CONTROL OF SALIVARY GLAND ENZYMES BY CATECHOLAMINES

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THESIS

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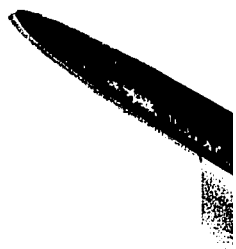
TABLE OF CONTENTS

T A B L E S



F I G U R E S

F I G U R E S

LIST OF ABBREVIATIONS

ATP	-	Adenosine triphosphate
UTP	-	Uridine triphosphate
Cyclic AMP	-	Cyclic-3',5'-adenosine monophosphate
NAD	-	Nicotinamide-adenine dinucleotide
DNA	-	Deoxyribonucleic acid
RNA	-	Ribonucleic acid
EDTA	-	Ethylenediaminetetracetic acid
TCA	-	Trichloracetic acid
DNSA	-	Dinitrosalicyclic acid
DDL	-	3,5-diacetyl-1,4-dihydrolutidine
DNase	-	Deoxyribonuclease
RNase	-	Ribonuclease
MAO	-	Monoamine oxidase
COMT	-	Catechol-O-methyl transferase
P-450	-	Cytochrome-P-450
LDH	-	L(+)-Lactate dehydrogenase

INTRODUCTION

It has been known for many years that salivary secretion in the mammalian organism is controlled by the autonomic nervous system. Adrenergic stimulation is particularly effective in causing the secretion of amylase from the parotid glands of many animals. Isoproterenol, a synthetic catecholamine with strong β-adrenergic action, elicits a very concentrated secretion of amylase. Recently, chronic administration of isoproterenol has been shown to cause hypertrophy of salivary glands, increased synthesis, changes in incorporation of amino acids into proteins, and changes in the concentration of some enzymes. After depletion of rat parotid amylase by isoproterenol, the concentration of the enzyme in the gland increased exponentially until the gland was fully repleted. This exponential increase did not correlate with observed changes in protein synthesis.

The present work started as an attempt to resolve these differences by studying the rate of amylase biosynthesis during repletion. This is described in the Experimental Section, Part I. De novo synthesis was measured by isolating amylase from the parotids of rats which had been injected with ^{14}C-amino acids at various times after isoproterenol. A moderate increase in apparent de novo synthesis of amylase was found, but this did not coincide with the rate of increase of amylase in the gland. Various possible mechanisms were explored, including; a changing rate of secretion superimposed on a constant rate of biosynthesis, activation of an inactive precursor, changes in degradation and turnover, mechanisms for stabilization of newly synthesized amylase, as well as such

possible experimental errors as contamination of the isolated amylase, and changes in the amino acid pool in the gland. A discussion of these mechanisms in the light of the present work and the work of others, is presented at the end of Part I.

Part II of the Experimental Section evolved out of recent reports that the activities of some non-exportable salivary enzymes were increased shortly after isoproterenol injection. The possibility that these changes might reflect a general change in enzymes associated with a particular subcellular structure was explored. Unfortunately, salivary mucins interfered in fractionation by differential and density gradient centrifugation. As an alternative, many enzymes associated with each subcellular fraction were measured during the repletion phase. These included monoamine oxidase, cytochrome oxidase, acid phosphatase, glucose-6-phosphatase, UDP-glucuronyl transferase, oxidative demethylation, cytochrome P-450, lactate dehydrogenase, and catechol-0-methyl transferase (COMT). None of the "microsomal markers" were found in the parotid or submaxillary. COMT increased two-fold, four hours after isoproterenol. No other changes in enzyme activity were found. These results are discussed in relation to the work of others at the end of the Experimental Section, Part II.

The section on experimental work is preceded by a review of literature which describes the concepts and evidence which prompted this work. The short introductions preceding several experiments include discussions of recent work of particular relevance to those experiments.

SECTION I

REVIEW OF LITERATURE

P A R T 1

AMYLASE

In a review of the literature, Fischer and Stein (1) described α-amylase as being among the earliest known enzymes. In 1811, Kirchhoff (5) described a factor in wheat extracts which could digest starch. The existence of amylase from many other sources were later described, eg. saliva in 1831 (6), malt in 1833 (7), blood in 1846 (8) and Aspergillus oryzae in 1881 (9).

Definition

The term amylase has been used to designate the enzymes catalyzing the hydrolysis of α-1 -> 4 glycosidic linkages of polysaccharides such as starch, glycogen, or their degradation products (1, 2, 4). α-amylase is an endoamylase (4) which means that it acts randomly on the α-1 -> 4 linkages. A rapid diminution of viscosity and average molecular weight of the substrate occurs. The primary products are oligosaccharides (dextrins) and as random cleavage continues a mixture of maltose, glucose, isomaltose and branch-chain products of low molecular weight are produced (1). The designation α-amylase originated from the finding that the reducing

hemiacetal group liberated by hydrolysis is in the α-optical configuration (10). (β-amylases are exclusively of vegetable or microbial origin, are exoamylases and produce maltose with the reducing sugar in the β configuration.)

Distribution

α-amylase has been found in a variety of organisms including plant seeds (11, 12), bacteria (13), fungi (14), paramoecia (15) and insects (16). This enzyme is widely distributed in mammals as a digestive enzyme secreted by salivary glands and pancreas (17, 18). The salivary enzyme content of mammalian species (17, 18, 19) shows great variation. The saliva of man, ape, rat, hog, mouse, rabbit, guinea pig, and squirrel contain substantial amounts of amylase. Other animals such as the cow, horse, dog, cat, fox and goat have negligible salivary amylase (17). Other tissues may have low concentrations of amylase (20) whose function cannot be the digestion of ingested food.

The source of serum and urine amylases is not clear. It has been suggested they originate in the pancreas, salivary glands or liver (18, 21, 22). The increased serum amylase in humans suffering from pancreatitis was immunologically similar to human pancreatic amylase, whereas normal serum amylase was immunologically distinct (18). Arnold et al (21) found that amylase was accumulated in the perfusate of rat liver and

concluded that the liver was a major source of serum amylase under normal physiological conditions. On the other hand, Mordoh et al (23) showed that liver microsome-bound amylase had a different Km than serum amylase. The function of liver amylase is unknown, but it may be involved in liver glycogen metabolism (23, 24).

A study of the enzyme and mucous secretions of salivary glands in many mammalian species pointed out the great differences between parotid and submaxillary glands in each species (19). The parotid was on the average more "serous" and the submaxillary more "mucous". At least five digestive enzymes (amylase, protease, lysozyme, DNase, and RNase) are secreted by salivary glands (19, 25). In rodents, the parotid gland secretes much higher levels of amylase and DNase than the submaxillary gland while the reverse is true of protease (19, 26). In the human, the parotid secretes more amylase than the submaxillary (27).

Isolation and Purification

Many α-amylases have been highly purified and crystallized by several methods. The earlier methods have involved solvent extraction, salt fractionation and crystallization (3). By this type of method, crystalline human salivary amylase (28, 29) human and hog pancreatic amylase (30, 31) and bacterial amylases (32) have been isolated. Bacterial amylases have also been purified after rivinol fractionation (33). Amylase is ideally suited to purification by adsorption on its substrate (34).

Starch was first used by Starkenstein (35) in 1910 to purify plant and bacterial amylases. Straub purified pancreatic amylase by adsorption on insoluble starch in a cold solution followed by centrifugation (36). An improvement of this method was proposed by Loyter and Schramm (37). Glycogen was used to adsorb amylase from a cold 40% ethanol extract of pancreas or parotid gland. By this method, highly purified, crystallized α-amylase was isolated from human saliva, porcine pancreas and rat parotid gland (37, 38).

Sephadex columns have been used successfully in the purification of amylase (39 - 42). A type of substrate adsorption is probably involved in this elution procedure since Sephadex is made from dextrans. The long retention time of amylase suggests substrate adsorption.

DEAE cellulose columns have also been used in the purification of amylase and in the separation of isoamylases (40, 43).

Multiplicity

The α-amylases from different species are similar in size and Ca^{++} requirement; however, they vary in amino acid composition, requirement for Cl^{-}, resistance to proteolysis, heat denaturation and other physical and chemical properties (1).

Structural differences among amylases have also been indicated by immunological studies. McGeachin (17, 18) formed antibodies to amylase from one source and measured their ability

to inhibit amylase from another source. Differences in degree of inhibition were found, not only between species, but also between organs from the same species. In all species studied, (rabbit, rooster, rat, goat, man, monkey) the amylase from the pancreas, serum and saliva were immunologically similar to one another but different from liver amylase.

In the rat liver, two types of amylases have been found. One is latent and microsomal-bound but can be activated by treatment with detergent, while the other is soluble and does not require detergent (44). The particulate and soluble amylases of liver displayed different kinetics (45) with the particulate amylase having a K_m of 2.5 mg/ml and soluble amylase having a K_m of 0.44 mg/ml (glycogen substrate).

Isoamylases have been detected in many species by electrophoresis on acrylamide or by column chromatography. In 1954, Doane (46) was able to demonstrate isoamylase in fruit flies by disc electrophoresis followed by staining for amylase activity. The six amylases separated were under genetic control (16, 47).

Muus and Vnenchak (48) in 1954 found four distinct zones after disc electrophoresis of crystalline human salivary amylase. By staining for amylase after disc electrophoresis of whole saliva or parotid saliva, Wolf and Taylor (49) and Boettcher and de la Lande (50) demonstrated that the pattern of isoamylases varied between individuals. They found from

5 to 8 isoamylases of varying activities. The isoamylase patterns of closely related subjects were more similar than those of unrelated subjects (20). Rat parotid saliva separated into five distinct amylase-staining bands, all with a greater mobility at pH 8.3 than the human salivary isoamylases (49). Robinovitch and Sreebny (25) separated rat parotid saliva by disc electrophoresis on both anionic and cationic acrylamide gels. They observed four distinct isoamylases separated in the anionic gel but only one in the cationic gel. They observed a slight change in the relative intensities of two of the bands if the saliva was not cooled immediately on collection and suggested this change may reflect recombination of subunits, or aggregation into dimers, trimers, etc., or that different molecular conformations may be involved.

Kauffman et al (45) were able to separate by disc electrophoresis six human parotid isoamylases which they designated z, 1, 2, 3, 4 and 5 in order of decreasing mobility. The relative concentrations of each isoenzyme varied among individuals, but for each individual were constant over a long period of time. Fractionation of parotid amylase on Bio-Gel filtration columns resulted in the separation of two peaks of amylase activity, designated A and B. Fraction A contained isoenzymes 1, 3 and 5. Fraction B contained isoenzymes z, 2 and 4. No differences were found in the amino acid compositions of Fractions A and B. A small amount of carbohydrate

was found covalently linked to the isoamylases in Fraction A. The carbohydrate moiety was composed of 8 moles of hexose and 4 of glucosamine. A long period of storage of amylase at 4° resulted in a change in the relative intensity of some bands. During 3 or 6 weeks storage, isoenzyme 4 was partly transformed to isoenzymes 2 and z.

A single amylase was found in rabbit parotid gland while three variants were isolated from rabbit pancreas (51).

In rat pancreas a single isoamylase was found (43) but two have been found in porcine pancreas (43, 52). The two porcine pancreatic amylases, separated by both disc electrophoresis and DEAE cellulose chromatography (43) were similar both in specific enzymatic activity, and in C- terminal residue (leucine). Cozzone et al (43b) separated three porcine pancreatic isoenzymes by DEAE cellulose chromatography, two of which could not be separated by disc electrophoresis. The two electrophoretically similar isoenzymes were identical in amino acid composition except for a moderate difference (5 moles) in aspartic acid and asparagine residues.

Two isoamylases of embryonic chick pancreas were detected by disc electrophoresis (53). Individual chicks revealed three phenotypes with respect to isoenzyme pattern which have respectively, only fast, only slow or slow plus fast bands. It was concluded that this was the result of two alleles of one gene. Changes in the relative concentrations of the two

isoenzymes during development were also observed (53).

Structure

The α-amylases from various sources are slightly acidic, (54, 55, 56) water soluble proteins of molecular weight around 50,000. Isoamylases probably do not differ in molecular weight but rather in charge since they are not separated by gel filtration on Sephadex, while they are separated by DEAE cellulose (25). We have confirmed this by separation of isoamylases using isoelectric focusing.

The amino acid compositions of several amylases have been determined (54, 55, 56). They present rather average amino acid compositions but display no striking similarities (1).

Fischer and Stein (1) reported that no non-protein organic material has been found in any α-amylase except that from A. oryzae which contained eight moles of mannose, one of xylose and two of hexosamine per mole of enzyme. The carbohydrate moiety was not necessary for enzyme activity. Recently, some human parotid isoamylases have been found with about 4% covalently-bound hexose and hexosamine (45).

End-group determinations have been made on A. oryzae (59, 60), B. subtilis (61), B. stearothermophilus (62), rat pancreatic (63) and porcine pancreatic (43, 58, 64) amylases. The results are confusing and somewhat contradictory.

A. oryzae amylase had an amino-terminal alanine (59) and C-terminal glycine, alanine and serine residues (60). In B. stearothermophilus (62) two moles of amino-terminal phenylalanine and two moles of C-terminal alanine were found. C-terminal lysine was found in rat pancreatic amylase (63). The end-group studies of porcine pancreatic amylase are in dispute. Caldwell (64) reported amino-terminal phenylalanine and C-terminal leucine. McGeachin and Brown (58), on the other hand, found three amino-terminal groups in crystalline porcine pancreatic amylase; phenylalanine, alanine and glycine. In each of two separated isoenzymes of porcine pancreatic amylase, Marchis-Mouren and Pasëro (43) found a C-terminal leucine residue. They could not, however, identify any free α-amino groups but suggested that the N-terminal residues were acetylated.

Several of these results suggest that α-amylase is made up of more than one chain of amino acids (58, 60, 62). This has been further explored with amylase from A. oryzea. Ca^{++} is required to maintain the tertiary structure of amylase (65). In the presence of EDTA, four disulfide bridges of taka-amylase can be broken by reduction (66, 67), resulting in the release of inactive subunits. Careful oxidation of the subunits in the presence of Ca^{++} restored enzyme activity and original structure (66). The reduced subunits could be alkylated (67, and reformed with complete recovery of amylase

activity. Tryptic digestion of the subunits resulted in peptides which could recombine in the presence of Ca^{++} to form an aggregate with enzyme activity and other properties similar to native taka-amylase (68). A similar type of inactivation and regeneration of B. subtilis α-amylase has been shown (69).

Properties

The properties of α-amylase have been well reviewed (1, 3, 27). Ca^{++} is an essential cofactor of all α-amylases. Dialysis does not completely remove Ca^{++} from the molecule; treatment with EDTA, however, does. Removal of Ca^{++} results in changes in molecular conformation (65) and a remarkable labilization of the enzyme. The pH ranges of stability of both mammalian and bacterial amylase are greatly reduced on removal of Ca^{++} (1). In contrast to native amylases, the Ca^{++}-free enzymes coagulate readily when heated. In the presence of Ca^{++}, α-amylases from several sources are remarkably resistant to proteolysis (70). On removal of Ca^{++}, the amylases can be easily hydrolyzed by a variety of proteolytic enzymes. Enzymatic activity of amylase can be abolished by treatment with EDTA, but restored by addition of an excess of Ca^{++}.

Mammalian amylases require chloride for activity but this requirement can be overcome by substituting other monovalent anions. The presence of chloride increases the isoelectric point and decreases the solubility of salivary

amylase (1). The temperature optimum and stability toward elevated temperatures and heavy metals are increased on addition of chloride.

The pH and temperature optima vary from one source to another. Mammalian amylases have pH optima between 6 and 7. The pH optima of amylases from other sources are: B. subtilis, pH 5.85 to 6.0; A. oryzae, pH 4.8 to 5.8; malt, pH 4.75 to 5.4. Other α-amylases can act at much higher temperatures (70a). Freezing can destroy amylase activity.

The various α-amylases have specific activities in the same order of magnitude (1). Schramm and Loyter (71) found the specific activities of human and rat salivary amylase were about twice as great as that of porcine pancreatic amylase. Activity depends on the substrate's degree of branching and branch length (27, 72). Long, straight chains are a better substrate than a highly branched substrate or very short chains. The apparent Michaelis constants of a variety of amylases are remarkably similar (1). Between 20° and 30°, values ranging from 1 to 5 x 10^{-3} mole of glucosidic bonds per liter have been obtained under a variety of experimental conditions with a variety of starches as substrates. The K_m is somewhat higher for B-dextrins and glycogen, and much higher for synthetic substrates such as α-phenylmaltoside. Energies of activation ranging from 10 to 16 kCal/mole have been reported (1).

Mechanism of Reaction

Surprisingly little is known about essential components and the active site of amylase, considering this enzyme has been available in a purified form for many years. This may be due to the complexity of the substrate and the reaction itself. Some possible reaction mechanisms have, however, been discussed (1, 73).

Early studies using chemical modification pointed out that free primary amino acids were indispensable for the catalytic action of pancreatic amylase. Free sulfhydryl groups were not essential (1). Both porcine pancreatic amylase and taka-amylase became inactive after acetylation of approximately half the free amino groups with acetic anhydride (74).

After dinitrophenylation of porcine pancreatic amylase, McGeachin and Brown (58) found three amino-terminal amino acids. No histidine or tyrosine had reacted. When only one mole of FDNP was bound to enzyme (presumably to the terminal phenylalanine residue) 70% of amylase activity was lost. Reaction of ε-amino groups with o-methyl isourea led to the loss of only 20% of the activity (due to 25% of the α-amino groups being guanidated). These studies suggested that the amino-terminal groups were important in catalytic activity of amylase.

Studies of the variation of reaction rates and Michaelis constants with pH or with chloride ions for several amylases

have been made (1, 73, 75). These studies indicated that the reaction rate is determined by the second phase of a Michaelis-Menten type reaction, i.e., that in which the glucosidic bond is actually hydrolyzed. The data for optimum pH of the reaction are consistent with the view that a carboxyl group of pK 4.0 to 5.5 and an amino or imino group of pK 6.0 to 8.5 are directly involved in the catalytic process. On this basis, several workers have tentatively postulated that a carboxyl group and a histidyl imino group are involved directly in the catalysis (75, 73).

Estimation

During incubation of α-amylase with starch or glycogen, the internal α-1, 4 links of highly cross-linked glycogen and amylopectin and the long straight chains of amylose are hydrolyzed, resulting in both a decrease in average molecular size and in production of reducing groups at the newly exposed 1 position of the broken carbohydrate-glucan link. These two properties are the basis for most methods of determining amylase activity. These methods are termed amyloclastic if they are based on the decreasing size of the substrate molecules, or saccharogenic if they are based on the measurement of sugars released from the substrate. The basic principles underlying the various methods of determining amylase activity are the following: 1. decrease in viscosity of substrate solution; 2. decrease in turbidity of a starch suspension; 3. diminution in the starch-iodine color; 4. increase in reducing power of

substrate solution; 5. release of soluble dyed-sugar residues from special dyed substrates.

Viscometric methods: The degree of viscosity occurring in a starch solution is a function of the interference between swollen granules. Thus when α-amylase randomly hydrolyzes α-1, 4-glycosidic linkages in both linear and branched fractions of the hydrated starch granule, the molecular architecture is disrupted with a resultant reduction in viscosity. Early methods (76, 77, 78) used an Ostwald viscometer to measure the change in flow rate. Chesley (78) demonstrated that the viscometric method was highly dependent on the nature of the starch substrate and Meyers and Reid (79) pointed out the unsuitability of this method in certain cases, particularly low levels of amylase in blood serum. The general lack of acceptance of viscometric methods is testimony to Gomori's (80) statement that viscometric methods of amylase assay are of historic interest only.

Turbidimetric methods: The reduction in turbidity of stable starch suspensions after incubation with amylase is due to a diminution in granule size due to starch hydrolysis. Methods have been described for saliva (81), pancreatic juice (82), urine (83), and serum (84). The turbidimetric methods are very simple and convenient. The main draw-back seems to be that the rate of reduction of turbidity is not

linear with time or with enzyme concentration. According to Guth (85), turbidimetric changes proceed in three distinct phases; the first minute after adding serum to the starch suspension hydrolysis progresses rapidly, then more slowly, before finally reaching a plateau.

Iodometric methods: The starch-iodine reaction has been used successfully for over a century to detect the presence of starch. The purple starch-iodine complex is due to the individual reactions of the components of starch (80% amylopectin, 20% amylose in potato starch) with iodine. According to Bates (86), amylose, the linear components, yields a blue color, and amylopectin, the branched fraction, forms a weak red color with iodine. Mould and Synge (87) related the color to the length of amylose chains with above 40 glucose residues giving red; 10 to 25, orange; and less than 10, colorless. Schoch (88) explained that the blue color can be attributed to the helical structure which linear chains assume with iodine. Each turn of the helix which contains 6 glucose units surrounds a molecule of iodine. The actual shade and intensity of color is a function of the number of helix turns and consequently on the length of the linear chains.

In 1908, the first quantitative iodometric amylase assay was described by Wohlgemuth (89). This, as well as the widely used adaptation of Somogyi (90), is based on incubating the enzyme with starch solution to the achromic end-point with

iodine. A shortcoming of this type of technique is that the achromic end-point is reached at a time when the substrate is no longer in excess. Spectrophotometric techniques (91, 92, 93) avoid this problem. The optical density of a blue staining incubation mixture produced by the action of amylase on starch is measured. Within certain limits there is a linear relationship between the amount of enzyme present and the decrease in optical density of the blue staining hydrolysate. Street and Close (93) argued that amylose, rather than starch is the only substrate suitable for use in conjunction with the starch-iodine reaction. However, Henry and Chiamori (72) demonstrated that human amylase acts faster on amylopectin than on amylose. Furthermore, the concentration of substrate called for in most of the iodometric methods is lower than that required for zero-order kinetics (94). Another problem was pointed out by Searcy et al (95) and Pimstone (96). Protein interferes with the starch iodine reaction either by binding of iodine or adsorption of starch. Therefore, solutions with high protein concentration could give a spuriously high amylase value.

Saccharogenic methods: Most amyloclastic methods suffer because suboptimum substrate concentrations must be employed since destruction of substrate, rather than formation of reaction products, is the measure of enzyme activity. A large excess of substrate concentration can be used

in saccharogenic methods without difficulty. Moreover, they are devoid of the shortcomings resulting from protein interaction and they can be applied to turbid or colored fluids. Most saccharogenic methods are based on the measurement of the reducing power of sugars released by hydrolysis of the substrate. A wide range of chemical reactions, with varying degrees of sensitivity have been utilized in the many methods used.

Copper reduction: Methods based on the reduction of cupric ions on heating with reducing sugars are among the earliest and most popular methods. The first was that of Moekel and Rost (97) in which the reduced copper was measured gravimetrically. Somogyi (90) stabilized the reduced copper with tartrate and measured it titrimetrically (KI was used as a redox indicator and the solution was titrated with thiosulfate to an achromic starch-iodine end-point). The Nelson method (98) and the method of Henry and Chiamori (72) are based upon formation of a colored complex of reduced copper with arsenomolybdate and phosphomolybdate respectively. In each of these methods proteins must be precipitated and removed. The supernatant containing the reducing sugars is then heated with an alkaline $CuSO_4$ solution.

Ferricyanide reduction: These methods are very similar to the copper reduction methods. The amylase-substrate

incubation mixture is treated with $ZnSO_4$ or tungstate to precipitate protein and the filtrate is heated with alkaline $K_3Fe(CN)_6$. The ferrocyanide produced is measured by titration with thiosulfate (99) or by reaction with phosphomolybdate (100).

Condensation of carbohydrate with anthrone (101) and aniline (102): These methods are based on formation of furfural by carbohydrate in hot acid. Furfural condenses with anthrone or aniline to form triphenylmethane dyes. Since the substrate, as well as products, can produce furfural, the enzymatic incubation must be followed by removal of substrate as well as protein by precipitation with TCA, and methanol or ethanol. Charcoal or Fuller's earth are also required to remove traces of dextrins.

A similar method proposed by Lorentz and Oltmans (103) is based on the reduction of triphenyltetrazolium chloride in a heated methanolic solution containing cyclohexylamine to form an intense red formazan. This method also requires the precipitation of protein with ethanol. The high sensitivity (measuring between 2 and 40 µg glucose) may be offset by the fact that the method is cumbersome.

Reduction of picric acid: Reduction of picric acid to the intensely colored picramic acid is the basis of the method first described by Myers and Killian (104) and later revised (105).

This is a sensitive method for measuring reducing sugars. A further advantage of this assay is that a single reagent, solid picric acid, stops the enzymatic hydrolysis, precipitates the protein, and forms the colored end product on heating. The procedure is therefore somewhat simpler than most other saccharogenic methods.

Dinitrosalicylic acid reduction: 3-5-Dinitrosalicylic Acid (DNSA) is very similar in structure and chemical properties to picric acid. A hot alkaline solution of DNSA can be reduced by glucose to 3-amino-5-nitrosalicylic acid, an intensely red compound (106). Sumner (107) adapted this reaction to measure small quantities of sugar in urine and later to assay saccharase activity in yeast (108). Its use to assay amylase has been described by Noelting and Bernfeld (3, 109). Amylase was incubated three minutes at 20° with soluble amylopectin, the action stopped by addition of alkaline DNSA reagent and color developed by heating at 100° for five minutes. Enzyme activity was estimated by comparing the absorbance at 540 mμ to a standard curve established with maltose (0.2 to 2 mg).

This method has several advantages. It is a simpler procedure than any of the other saccharogenic methods. Protein does not interfere in this method, therefore there is no need to precipitate and filter it (94). It has been reported (110) that the color produced is proportional to the number

of reducing groups and the average number of residues in the oligosaccharide products has no effect on the color. The sensitivity of this method is high. Searcy et al (111) point out that the chromogenic response to reducing sugars with DNSA reagent exceeds that yielded by alkaline solutions of copper (90), ferricyanide (100) or picric acid (105). The sensitivity of this method (3) has been increased by lengthening the enzymatic incubation to 60 minutes (111), increasing the incubation temperature to 37° (94), reducing the final volume to be read from 25 ml to 1.5 ml (111) and by reading the absorbance at the absorption maximum of 470 mμ (112).

Dyed substrates: In recent years attempts have been made to use dyed substrates in order to simplify the amylase assay. The basis for these methods is that enzymatic hydrolysis of dyed substrates releases soluble dyed oligosaccharides which remain after filtration or centrifugation and can be easily measured spectrophotometrically. In 1943 the first of these dyed amylase substrates, a mixture of α-p-nitrophenyl maltosides, was described (113, 114). This was a poor substrate for α-amylase because of its slight affinity for low molecular weight substrates. This problem has been more recently overcome by covalently linking various dyes to more natural substrates such as starch, amylose, amylopectin, glycogen, or higher dextrins. Some of these substrates for amylase assay

are: Remazolbrilliant Blue R bound to starch grains (115); Remazolbrilliant Blue R bound to dextrin (116); Reactone Red 2-B on soluble amylopectin (117); Cibachron Blue F-3 G.A. on highly cross-linked insoluble starch (118, 119); Cibachron Blue F-3 G.A. on insoluble amylose (120, 121); and Fluorescent Starch-Anthranilate (122).

Of these substrates, the Reactone Red 2-B on amylopectin has certain advantages. First, amylase acts much more readily on a soluble substrate. Amylopectin has been recommended as preferable to amylose as an α-amylase substrate (72). The fluorescent substrate (122) is of considerable interest because fluorometric assays are generally in the order of 1000 times more sensitive than spectrophotometric methods.

The use of dyed substrates for the determination of α-amylase will probably increase for several reasons. The procedures are generally simpler than most saccharogenic methods. Reducing substances in the sample do not interfere as they do in saccharogenic methods. Most of these dyed substrate methods are less sensitive, however, and they must be standardized by using an enzyme standard (standardized by a saccharogenic method).

Automated methods: Several workers have proposed automated methods for determining amylase, all using modules of the Technicon Auto Analyzer based on the work of Skeggs (123).

Each of these automated methods has certain shortcomings. Scheidt (124) in 1964 described a method based on the loss of iodine staining power of starch on incubation with amylase. This method is open to all the criticism of the amyloclastic-iodometric methods discussed earlier (ie. sub-optimal substrate concentration, and interference of protein). Other automated methods (116, 122, 125) are based on manual saccharogenic methods which require the removal of protein or unreacted substrate by precipitation. In automated methods, dialysis can often be substituted for precipitation in the removal of interfering substances. Unfortunately, the relatively large molecular size of the products of amylase action severely limits the amount of product passing across the dialysis membrane. The resulting low sensitivity restricts the usefulness of methods requiring dialysis.

The DNSA method is the only saccharogenic amylase assay which does not require the removal of protein or substrate. It is therefore, not only a much less complicated method manually, but it is also immensely suitable for use in an automated technique since dialysis is not required.

Biosynthesis

Most early reports on the biosynthesis of amylase were based on observed increases of amylase with time (1, 126). Such increases have been found *in vitro* after incubation of pancreatic (127), parotid (128) or liver (24) slices with amino

acids and ATP, and also in perfused liver (129). Amylase accumulation has been found *in vivo* after depletion of stored amylase by secretory stimulus in the pancreas (130, 131), submaxillary (26) and parotid (26, 132) glands. The most recent work on amylase biosynthesis is based on measuring the incorporation of radioactive amino acids into amylase.

Early studies of amylase biosynthesis in both mammalian and bacterial systems have been described by Fischer and Stein (1). The results of these experiments were consistent with a two-step formation of amylase outlined in the following scheme postulated by Nomura *et al* for the synthesis of *B. subtilis* amylase (134):

$$\text{Amino Acids} \xrightarrow[\text{Oxygen}]{\text{RNA}} \text{Precursor} \xrightarrow[\text{Factors}]{\text{Activating}} \text{Amylase}$$

They described the accumulation of amylase during incubation of both washed cell suspensions and cell-free lysates (1, 134, 135). Both systems were able to incorporate ^{14}C-glycine into protein and were inhibited by chloramphenicol and streptomycin. Incorporation of ^{35}S-methionine into amylase was observed only with the whole cell preparation. The increase in amylase was inhibited by p-fluorophenylalanine in the cell suspension but not in the cell-free lysate.

A number of early reports of pancreatic amylase synthesis by Straub *et al* also suggested the formation of an amylase precursor (130, 136). Increases in amylase activity were observed after incubation of ATP and amino acids with pigeon pancreas

homogenates, mitochondrial fraction, or aqueous extract of a dried acetone powder of pancreas. This "soluble system" increase in amylase was inhibited by streptomycin, chloroamphenicol, p-fluorophenylalanine, and RNase, but not by anaerobic conditions. Incorporation of ^{14}C-glycine into amylase took place in tissue slices but not in the "soluble systems".

The increase in amylase in the soluble systems described above may be simply a solubilization and activation of microsome-bound amylase. Laird and Barton (138) showed that a major portion of rat pancreatic amylase sedimented with the microsomal fraction. The microsome-bound amylase of pigeon pancreas was shown by Douglas and Munro (139) to be partially latent, and could be activated by a variety of treatments.

The work of Palade et al (140, 145) which combined biochemical and cytochemical techniques with electron microscopy in the study of pigeon and guinea pig pancreas has provided much information about the sequence of events occurring between enzyme synthesis and secretion. These experiments as well as similar experiments with rat parotid gland have been reviewed by Schramm (146).

Ribosomes isolated from guinea pig pancreas had secretory enzymes firmly bound. These enzymes (amylase, RNase, protease) could be released by chelation of Mg^{++} which is required for ribosomal stability (140a). By injecting ^{14}C-leucine into guinea pigs and isolating the secretory enzyme, α-chymotrypsinogen,

from each of the subcellular fractions at various times, the site of synthesis and sequence of transfer of secretory protein was deduced (140b). This study, supported by subsequent auto-radiographic experiments (141) led to the following hypothesis:

Secretory proteins are synthesized on the ribosomes attached to the membrane of the rough surfaced cisternae of the endoplasmic reticulum; they are subsequently transferred into the intracisternal space through which they travel to the Golgi region where they initially are found in small vacuoles. Then they are concentrated in larger condensing vacuoles which become zymogen granules. These move away from the Golgi zone and accumulate progressively in the apical region of the cell awaiting to be released in the glandular lumina following a secretory stimulus. Subsequent experiments in which pancreatic slices were pulse labeled and the crude microsomal preparation was further fractionated by sucrose gradient centrifugation (143, 144, 145) supported this hypothesis. Newly synthesized protein and amylase were shown to travel from the rough endoplasmic reticulum into small vesicles of the Golgi complex to condensing vacuoles.

A cell-free amylase synthesizing system was described by Redman et al (142). It consisted of pigeon pancreatic microsomes, a microsomal supernatant factor (pH 5 enzyme), amino acids, ATP and Mg^{++}. When ^{14}C-amino acids were used, labeled amylase could be isolated. Labeled amylase appeared

immediately on ribosomes. After 2 or 3 minutes, labeled amylase decreased on the ribosomes but increased in the smooth membranes. After 10 minutes, only 30% of the counts remained in ribosomal amylase while 50% were found in the smooth membranes.

An amylase synthesizing system from rabbit pancreas was described by Sreebny et al (147), in which ribosomes were isolated free from microsomal membranes and incubated with ATP, GTP, Mg^{++}, pH 5 enzyme, amino acids and other cofactors. Disc electrophoretically pure labeled amylase was recovered from the incubation mixture. This type of system was possible because the rabbit pancreas contains no RNase.

Some experiments similar to the studies of Palade et al (140) have been carried out in order to study amylase synthesis in the rat parotid gland (148, 149). Cell proteins were labeled in vivo during the secretion and repletion of amylase (148). Labeled amylase appeared first in the microsomal fraction and subsequently in the other fractions. Labeled total protein increased during repletion of amylase but did not coincide with the accumulation of amylase. In pulse-labeled rat parotid slices, the specific radioactivity of isolated amylase was initially highest in the microsomes and lowest in the zymogen granules. Two other fractions contained radioactivities higher than the zymogen granules. Subsequently, radioactively labeled amylase declined in the microsomes but

increased in all other fractions. After three hours the gland slice secreted 70% of the labeled amylase. It has been suggested on the basis of these studies that the sequence of events in parotid amylase synthesis (146) parallels that described by Palade et al (140 - 145) for pancreatic amylase synthesis.

A parotid gland slice system has been used by Gross et al (150, 151, 152) in studying epinephrine induced amylase synthesis. Incorporation of ^{14}C-amino acids into both amylase and total protein was increased after treatment with epinephrine (150, 151) but the increase of amylase incorporation was greater. This occurred concurrently with amylase secretion. Cycloheximide prevented amylase synthesis but did not inhibit secretion, thus indicating the independence of secretion and protein synthesis (150).

A cell-free system from rat parotid gland similar to that described for rabbit pancreas (147) has been able to incorporate amino acids into protein (153). Labeled amylase was not isolated, however, probably because of interference by RNase.

Tissue culture techniques have recently been used to study amylase biosynthesis. Both rat submandibular gland (154) and monkey parotid gland (155) tissue cultures secreted amylase. Labeled amylase could be isolated from human parotid tissue cultured with radioactively labeled amino acids (156).

The above experiments on pancreas and salivary glands all indicate de novo synthesis of amylase. No evidence of an amylase precursor, proposed by early workers (130, 134, 136) was found in these studies. A possible "precursor" is the

ribosomal-bound amylase described by Siekevitz and Palade (14). The rate of transfer of amylase from the ribosomes to the microsomes is so great that this cannot be described as a precursor in the usual sense (142).

The increase in rat submaxillary gland amylase after pilocarpine stimulation was assumed by Schneyer et al to be due to de novo synthesis (26). In vitro incorporation of amino acids into amylase was not increased, however, in proportion to the increased submaxillary amylase concentration (157). Furthermore, both puromycin and actinomycin D potentiated the pilocarpine effect. It was suggested by Chignell (132) that these effects were not consistent with an increase in de novo amylase synthesis but were probably the result of changes in degradation of amylase by catabolic enzymes.

An interesting test for de novo synthesis of barley amylase was described by Filner and Varner (158). Gibberellic acid induced the formation and secretion of α-amylase in barley seeds. By growing the gibberellic acid treated seeds in a medium composed of H_2O^{18}, newly synthesized amylase could be separated from pre-existing amylase (or amylase derived from a pre-existing precursor) by isopycnic equilibrium centrifugation. It was concluded on the basis of distribution of the enzyme on a CsCl gradient that essentially all of the α-amylase induced by gibberellic acid was newly synthesized from amino acids which arose primarily by hydrolysis of pre-existing seed proteins.

P A R T II

CONTROL OF SALIVARY FUNCTION

Introduction

The lubricating and cleansing actions of saliva were known from earliest times, but an understanding of the function of the salivary glands has come about slowly. One of the earliest studies was published in 1677 by Regner de Graff (160). He prepared dogs with chronic fistulae of both pancreatic and submaxillary ducts. He described the increase in salivary flow during mastication and swallowing as well as in response to the smell and taste of food. Other early studies on salivary function were by Johannes Müller (161), Claude Bernard (162) and Pavlov (163). These classical studies have been described by Burgen and Emmelin (159) in their excellent review of the physiological control of salivary gland function.

Anatomy

The anatomy of salivary glands is also described by Burgen and Emmelin (159) as well as other workers (164, 165). Comparisons of salivary glands of various species have also been made (164, 19). Vertebrate salivary glands have evolved into a fairly complex tissue (19), consisting of several cell types. Acinar cells are large cells involved in the synthesis and storage of secretory proteins. They are arranged in the form

of an alveolus. Acinar cells have been described as being either serous or mucous, depending upon whether enzymes or mucous are the main secretory products. Intercalated ducts are made up of small cells which form a narrow duct connecting the acini to the intralobular or "striated" ducts. These are generally granulated and in some animals convoluted. This duct is associated with water and ion transporting capacity (19).

The ultra structure of the glands has been studied by electron-microscopy (166, 167, 168, 169). The structure of the salivary acinar cells is very similar to that of the exocrine pancreas (140, 170). Changes in the ultra structure of these glands during secretion and repletion will be discussed below.

Salivary Secretion

Many types of cells may under certain conditions demonstrate specific transport of macromolecules from inside the cell into the extra-cellular medium. This process is most developed in glands which specialize in synthesis and export of digestive enzymes and hormones. The salivary glands are an example of this type of tissue.

The various salivary glands synthesize a number of proteins which are secreted during ingestion of food to aid in lubrication and digestion. The lubricating proteins, or mucins, are acid mucopolysaccharides or neutral glycoproteins which

contain a high proportion of carbohydrate (27, 159). At least five digestive enzymes (amylase, protease, lysozyme, DNase and RNase) are secreted by salivary glands (19, 25). These digestive enzymes, as well as the mucins, have been found concentrated in the acinar cell zymogen granules (146, 171-173).

The secretory cycle: The cyclic cellular changes underlying salivary activity which have been termed "the secretory cycle" can be defined in terms of secretory granules. It is generally accepted that secretory proteins are synthesized on the ribosomes and are transferred via smooth vesicles to the zymogen granules where they are stored in a concentrated form. When a specific hormone or nerve stimulus reaches the gland cell, a sequence of reactions occurs by which the exportable proteins are released from the membrane-bound granules to the exterior of the cell (146, 166, 169). The exact route of enzyme transport has been discussed previously in the section on biosynthesis of amylase.

Major changes in the subcellular arrangement of rat parotid acinar cells (the source of amylase, RNase, DNase) have been observed following secretion induced by starving - refeeding, pilocarpine administration, electrical stimulation (166) or isoproterenol stimulation (169). In a starved rat, a great number of electron dense zymogen granules were packed between the cell lumen and the nucleus at the base of the cell. Many of the zymogen granule membranes were in contact with one

another or with the lumen membrane. The endoplasmic reticulum at the base of the cell was well developed with flattened parallel cisternae. The Golgi apparatus and mitochondria were near the nucleus. Within minutes after induction of secretion, a spectacular change was observed. A great reduction in the number of zymogen granules was seen. As the granules fused with the cell membrane, the size of the lumen was greatly increased. One hour after secretion, no zymogen granules remained and involutions of the lumen were close to the nucleus (169). Scott and Pease (166) reported an increase in the endoplasmic reticulum which formed in whorls around zymogen granules during repletion (four hours after stimulation). Amsterdam et al (169) did not report such changes. They reported that small condensing vacuoles began to form near the Golgi membranes after two hours. By six hours, large condensing vacuoles were surrounded by Golgi membranes. The zymogen granules were beginning to form from the condensing vacuoles. Eleven hours after secretion, many zymogen granules had accumulated and 20 hours after secretion, they were densely packed in the cell. Scott and Pease (167) reported the formation of electron dense lamellar "crystalloids" near the Golgi membranes during the early stages of zymogen granule formation. They suggested the crystalloids might be lipoprotein associated with the formation of zymogen granule membranes and originating in the Golgi complex.

The work of Hokin et al (170, 174) in the field of phospholipid metabolism may explain some of the events taking place during the repletion phase of the secretion cycle. They observed that the induction of secretion in the pancreas (175), adenohypophysis (176), adrenal medulla (177), avian salt gland (178), stomach (179), submaxillary gland and parotid gland (179, 180) was associated with increased turnover of phospholipids and in particular phosphatidyl inositol. The increased turnover in pancreas slices occurred in both the smooth-surfaced Golgi membranes and in the rough-surfaced endoplasmic reticulum within a few minutes after stimulation of the pancreas (174, 181, 182). A large fraction of injected myoinositol-2-^{3}H was recovered in the pancreatic zymogen granules of guinea pigs stimulated with pilocarpine (183).

Hokin proposed a model (170, 174) in which phosphatidyl inositol links together lipoprotein subunits in the membranes surrounding secretion granules. This is represented diagrammatically in figure 1. When the granule membrane coalesces with the cytoplasmic membrane during the extrusion process, the granule membrane is released into the cytoplasm as subunits by the breakdown of the phosphatidyl inositol links between lipoprotein units in the granule membrane. Phosphatidyl inositol is re-synthesized in the intra-cellular membranes of the endoplasmic reticulum and Golgi apparatus. The membrane subunits may then be reassembled with phosphatidyl inositol. This new membrane becomes available for the formation of new zymogen granules.

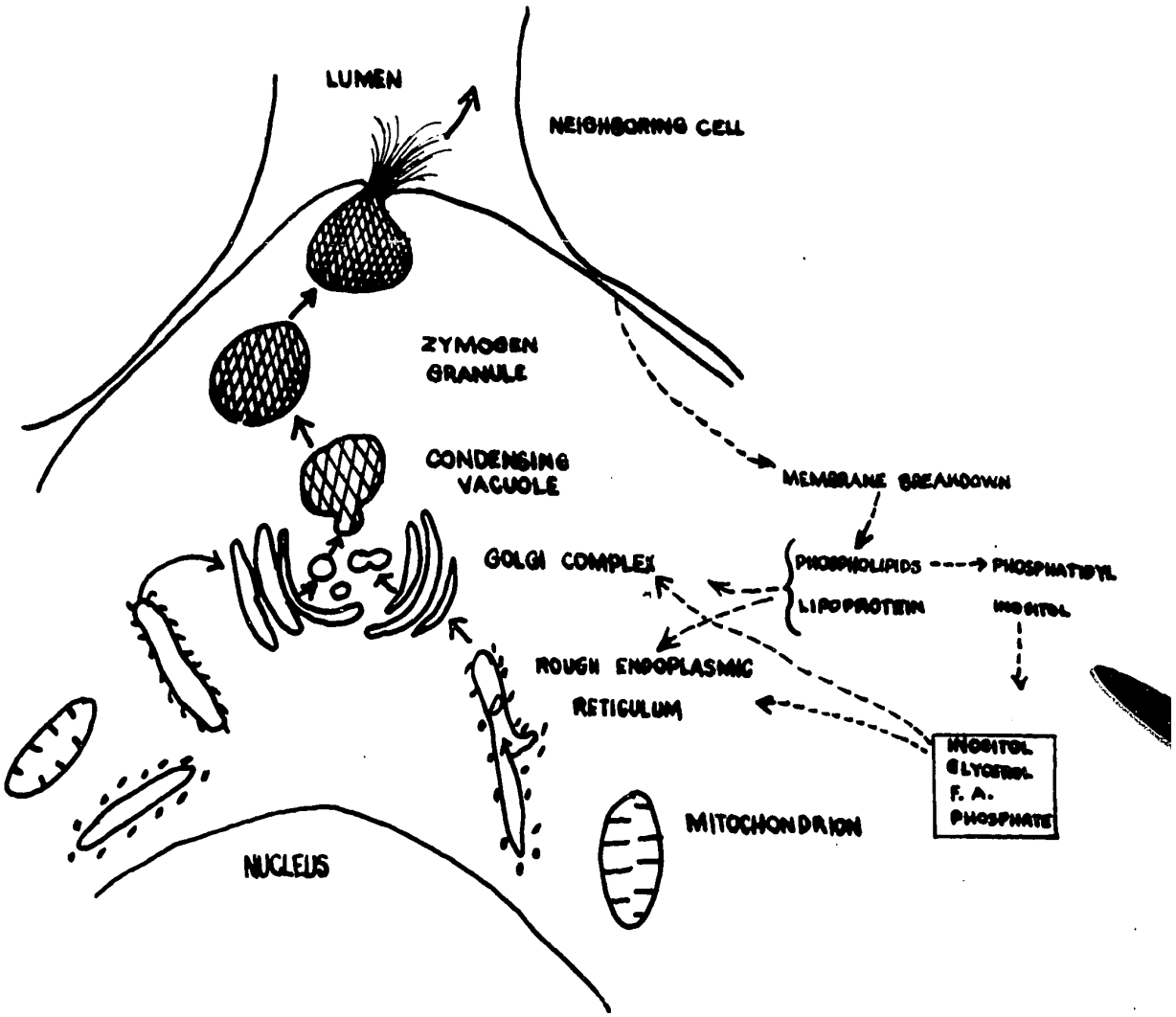


Figure 1 . Scheme of intracellular transport and extrusion of secretory protein in the parotid acinar cell. The solid arrows depict migration of secretory protein. The dashed arrows indicate the cycle of membrane breakdown and reassembly which follows extrusion of secretory protein.

It is possible that the new membrane formed may be stored as "crystalloids" before they are utilized for zymogen granule membrane.

These changes which have been described during induced secretion and repletion of the salivary glands are probably representative of the normal function of the glands. The secretion and repletion in salivary glands is controlled by food intake which follows a diurnal pattern. Sreebny and Johnson described the diurnal changes in the rat parotid gland (184). The gland levels of amylase, DNase and RNase progressively decreased during the eating cycle and then progressively increased until the start of a new eating cycle. Gland weight changed in a similar manner. There were corresponding changes in acinar diameter and granule content. It was concluded that the diurnal pattern of the rat parotid gland was due to storage and expulsion of secretory components located in the zymogen granules of the acinar cells. This view coincided with the conclusions of Amsterdam et al (169) and Pease et al (166) based on electron microscopic studies of parotid glands after artificial induction of secretion.

Induction of Secretion

Nervous control: Nervous control of salivary secretion was first described by Ludwig in 1851 after observing the flow from dog submaxillary gland which followed stimulation of the chorda-lingual nerve (185).

Salivary glands are unique in that their physiological functions are controlled solely and entirely by the central nervous system. Denervation renders these glands incapable of discharging saliva at any time some weeks after denervation (186). There is no evidence to suggest that secretion of saliva is normally induced by hormonal mechanisms similar to those activating, for example, the gastric or pancreatic glands (197). This by no means implies that the function of the salivary glands is not dependent on hormones. Thyroxine, corticosterone, somatotropin, and testosterone affect salivary gland growth and content (188, 189, 190).

Secretory activity of the salivary glands is known to be controlled by both branches of the autonomic nervous system. Although stimulation of either branch will result in elaboration of secretion, the composition of this secretion depends on the branch of the innervation stimulated (191, 192).

A detailed analysis of the action of the nervous system on salivary glands is given by Burgen and Emmelin (159). More recent work has been discussed in other reviews (19, 186, 187, 191).

The action of the nervous system occurs through chemical mediators in a process described as humoral transmission. These transmitter substances, or neurohumors, are found in parasympathetic and sympathetic nerve endings. Acetyl choline is found in parasympathetic nerve endings and norepinephrine is found in sympathetic nerve endings. Both acetyl choline and norepinephrine are present in substantial concentrations in salivary glands. This is consistent with the well-developed

sympathetic and parasympathetic innervation observed in salivary glands (133, 159, 193). In 1966, Nordenfelt (133) claimed that "all salivary glands studied so far have been able to synthesize acetyl choline", but with great species variation. In most species, the submaxillary synthesizes acetyl choline at a higher rate than the parotid. The acini of rat submaxillary and parotid glands are richly supplied by adrenergic nerves and contain noradrenaline. The sublingual gland has no adrenergic innervation and very little noradrenaline.

The actions of noradrenaline released at adrenergic synapses in tissues with sympathetic innervation have been discussed in detail by Iversen (194) and Burn (195).

The branched terminals of post-ganglionic sympathetic neurones have varicosities which contain a large number of membrane-bound vesicles. These vesicles, from which are derived the synaptosomes, are thought to represent specialized storage structures in which noradrenaline is sequestered in some way. The presynaptic nerve terminal contains the mechanisms for the synthesis and storage of noradrenaline. Part of the stored noradrenaline is released from the vesicles in response to nerve impulses reaching the nerve terminals according to the varying demand. The synaptic cleft is between the synaptic terminals, and the surface of the effector cells in direct apposition to them. Noradrenaline is released into the synaptic cleft and acts on specific receptor sites on the

post-synaptic cell surface, resulting in a specific response or series of responses within the effector cells. Noradrenaline is subsequently inactivated by: re-uptake into nerve terminals from which it was released; oxidative deamination catalyzed by monoamine oxidase (MAO) in the nerve cells; o-methylation catalyzed by catechol-o-methyl transferase (COMT) in the effector cells; or diffusion into circulation.

The integrated biochemical function of the adrenergic synapse can be experimentally altered at various levels by using several drugs. Drugs may depress the adrenergic response by: blocking of the nerve impulse in the post-ganglionic sympathetic nerve (bretylium); inhibiting noradrenaline biosynthesis (α-methyl-p-tyrosine); interfering with storage of noradrenaline (reserpine); or antagonizing the actions of released noradrenaline at post-synaptic receptor sites (phenoxybenzamine, propranolol). Drugs may enhance adrenergic response by: preventing re-uptake of noradrenaline (cocaine); preventing oxidative deamination (MAO inhibitors, eg., pheniprazine, iproniazid); preventing o-methylation (COMT inhibitors, eg., pyrogallol); imitating the action of noradrenaline (adrenaline, isoproterenol); or replacing noradrenaline from its normal storage sites (tyramine).

The activity at cholinergic synapses is much less complicated than at adrenergic synapses. Acetyl choline is released into the synaptic cleft and acts on effector sites of the target cells. It is immediately inactivated by cholinesterase. Cholinesterase inhibitors allow the accumulation of acetyl

choline in the synaptic cleft resulting in continuous stimulation of the effector cells.

Drugs, which can stimulate salivary secretion or interfere with normal salivary secretion, usually act in some way at adrenergic or cholinergic synapses. These have been discussed by Burgen and Emmelin (159). Parasympathomimetic drugs cause secretion similar to that caused by parasympathetic nerve stimulation. Acetyl choline, carbamyl choline, pilocarpine, and cholinesterase inhibitors all cause such secretion. Pilocarpine stimulation is not purely parasympathomimetic since it causes moderate sympathomimetic secretion (192).

Sympathomimetic agents produce secretion through stimulation of the effector sites in the adrenergic synaptic cleft. Noradrenaline, adrenaline, isoproterenol, synephrin, ephedrin, paredrinol, and amphetamine all act in this way.

Secretion of amylase from parotid glands is due to sympathetic stimulation. A large volume of salivary flow is induced by parasympathetic stimulation. The secretion of parotid amylase induced by parasympatholytic drugs is due to their partial action on sympathetic receptors (192, 196).

Adrenergic receptors: In 1948, Alquist (197) proposed the distinction between two types of adrenergic receptors. The α-receptors were defined as those with greater response to noradrenaline and adrenaline than to isoproterenol. The

α-receptors mediate such effects as vasoconstriction and inhibition of intestinal smooth muscle. The β-receptors showed a greater response to isoproterenol, mediating effects such as vasodilation, inotropic and chromotropic responses in the myocardium and relaxation of bronchial musculature. Other effects, such as decrease of cardiac glycogen, release of free fatty acids from adipose tissue (198) and amylase secretion from parotid glands (192) have also been attributed to β-receptors. Drugs which specifically block only one type of adrenergic receptor are used to study these receptors. Ergotamine, phentolamine, phenoxybenzamine, propranolol, and dichloro-isoproterenol are β-receptor blocking drugs. By use of these blockers, Kojima (199) showed that the increase in amylase secretion from rabbit parotid during stimulation of sympathetic nerves was due to β-adrenergic receptors. The initial promotion of flow rate was due to α-receptors. These findings agreed with the work of Photo (192) who measured the flow rate and amylase secretion from the parotid of anaesthetized rats. β-Adrenergic drugs evoked a concentrated amylase secretion with a low rate of flow.

Robison et al (200) have pointed out that the properties of α and β-receptors are not always clearly separated. They suggested that all β-adrenergic effects in all tissues may be mediated by adenyl cyclase. Some α-receptors may similarly act through adenyl cyclase, and in some tissues, both α and β-stimulation may activate adenyl cyclase. More recently,

(201) they indicated that β-stimulation resulted in an immediate increase in tissue cyclic AMP whereas α-stimulation resulted in a decrease in cyclic AMP.

Adenyl cyclase: A number of hormones and neurohumors have been found to exert their effect on their target tissues by altering their intracellular level of cyclic-3',5'-AMP (cyclic AMP). This is discussed in several excellent reviews on the control of cell functions by adenyl cyclase and cyclic AMP (200-204). Adenyl cyclase is part of the receptor site on a cell membrane. When a specific hormone or neurohumor interacts with the receptor, adenyl cyclase is stimulated to catalyze the synthesis of cyclic AMP from ATP. The cyclic AMP then interacts with another target system which results in a physiological response. This sequence has been described as a two-messenger system; the hormone is the first messenger and cyclic AMP is the second messenger in this system. Adenyl cyclase is involved in mediating the effects of many hormones on many tissues resulting in changes in the activity of a large number of enzymes (200).

The role of cyclic AMP in cellular function has been studied in many ways. Changes in the concentration of cyclic AMP or in the activity of adenyl cyclase can be measured (203, 205-208). Inhibitors of a specific phosphodiesterase, which catalyzes the breakdown of cyclic AMP to 5'-AMP, (caffeine, theophylline) can be used to mimic the activation of adenyl cyclase since it leads to the accumulation of cyclic

AMP (207, 209-211). Many derivatives of cyclic AMP, such as mono and dibutyryl cyclic AMP, have been used to mimic the effects of adenyl cyclase stimulation (206, 210-212). These derivatives are generally more active than cyclic AMP because they more easily cross the cell membrane.

Among the processes mediated by cyclic AMP is the secretion of macromolecules by various exocrine and endocrine glands. The secretion of amylase from pancreas, which can be stimulated by pancreozymin, carbamyl choline and catecholamines, is also stimulated by theophylline and derivatives of cyclic AMP (211).

Johnson et al (206) reported that the 3 to 9-fold increase in cyclic AMP found in cat pancreas within 30 to 60 seconds after injection of secretin, pancreozymin or acetyl choline accompanied the onset of secretion. The levels of cyclic AMP paralleled the subsequent decline and cessation of secretion. A second rise and fall in cyclic AMP was observed at 80 and 140 minutes respectively.

Inhibition of acetylcholine and pancreozymin induced enzyme secretion was abolished by atropine treatment, but not the changes in cyclic AMP levels. They suggested that the electrolyte secretion induced by secretin may be related to the rise in cyclic AMP whereas the enzyme secretion induced by acetyl choline and pancreozymin may not be linked to increased cyclic AMP levels. These workers pointed out the possibility that the large secondary rise in cyclic AMP after

stimulation might be associated with protein synthesis in the gland because the rise occurs at the beginning of the protein repletion phase of the secretory cycle.

In the rat parotid gland, the apparent role of cyclic AMP in electrolyte and enzyme secretion described by Batzri et al (213) was the opposite to that of Johnson et al. Amylase and K^+ secretion from rat parotid slices were induced by epinephrine. Mono-butyryl cyclic AMP, on the other hand, caused a rapid and intensive secretion of amylase without release of K^+. This suggested that cyclic AMP mediated the epinephrine induced secretion of enzymes but not of electrolytes in the rat parotid gland.

The early studies of the same group of workers (209, 210) established the involvement of the adenyl cyclase system in the secretion of amylase. These experiments demonstrated the release of amylase from parotid gland slices by treatment with phosphodiesterase inhibitors and cyclic AMP derivatives. Malamud (207) described an immediate three-fold increase in mouse parotid gland adenyl cyclase activity three minutes after injection of isoproterenol.

The results in our laboratory are similar. Injection of isoproterenol into rats caused 50% increase in cyclase activity at 2.5 minutes and a return to the basal level at two hours (214, 215). In rat parotid slices, a decrease to 60% of basal adenyl cyclase activity was observed 150 minutes after isoproterenol treatment (208).

Adenyl cyclase activity has been found in the isolated cell membranes of many tissues (203). In salivary glands, most adenyl cyclase activity was found by Schramm and Naim in the cell membrane (208). A substantial concentration was found in the microsomal fraction. The same group of workers (216) found that the microsomes were responsible for more than 50% of the rat parotid particulate fractions. It was mainly smooth microsomes which bound the cyclic AMP in rat parotid. This is derived from both the Golgi vesicles and the cell membrane, both of which are modified during salivary secretion (169, 213).

Protein kinase: The sequence of changes between the stimulation of adenyl cyclase activity and the physiological response in the salivary glands is unknown. In salivary glands, stimulation causes an immediate secretion (within seconds) of saliva, a repletion of salivary proteins over several hours, and in some cases, a stimulation of RNA synthesis, DNA synthesis and cell growth and division (217).

The intermediate steps are not clear. In several tissues, it appears that phosphorylation of enzymes is involved in some of these intermediate steps. For instance, in the liver, a recently discovered protein kinase (218) is involved in adrenaline stimulated glycogenolysis. This protein kinase which catalyzes the transfer of phosphate from ATP to serine residues of protein (218, 219) is activated by cyclic AMP (220) which is produced by the adrenaline stimulated adenyl cyclase.

Protein kinase catalyzes the ATP dependent phosphorylation of inactive phosphorylase kinase to an active form. In turn, phosphorylase kinase catalyzes the ATP dependent conversion of inactive phosphorylase b to active phosphorylase a, which initiates the breakdown of glycogen. Protein kinase is identical with glycogen synthesis kinase; thus, cyclic AMP, through its control of protein kinase activity, may regulate both the synthesis and breakdown of glycogen (221).

Protein kinase has been identified in many tissues including liver (222), skeletal muscle (220), adipose tissue (223), testes (224), and brain (225, 226). In all of these tissues, cyclic AMP causes a several fold increase in protein kinase activity.

In this laboratory, very high protein kinase activity was found in rat submaxillary and parotid glands (215). On stimulation of secretion by injection of isoproterenol, the activity of parotid protein kinase begins to increase. It reaches a maximum level about three-fold higher than the basal level between two and four hours and returns to slightly below basal level twelve hours after injection of isoproterenol (214). Thus, protein kinase reaches maximum activity after parotid secretion ceases, and before amylase biosynthesis reaches maximum. The sequence of events; induction of secretion and increased adenyl cyclase activity within minutes after injection of isoproterenol, increased protein kinase levels followed by increase in amylase biosynthesis, suggests that cyclic AMP

may cause immediate secretion. It may also be responsible for events leading to the increase in protein kinase activity which in turn affect the rate of enzyme biosynthesis or the accumulation of amylase within zymogen granules.

Protein kinase is found in most tissues, including salivary glands, in the soluble fraction (214, 226). Recently, Maeno et al (227) reported that rat brain protein kinase was found mainly in particulate fractions in a latent form. Triton X-100 activated and solubilized high specific activity protein kinase from the microsomes as well as synaptosomes. They concluded that cyclic AMP and cyclic AMP dependent protein kinase were closely associated with events underlying synaptic transmission in the brain. On the basis of these experiments, it would be interesting to look for a membrane-bound, latent protein kinase in salivary glands. Such an enzyme, if found, might be involved in the secretory process, or in events related to repletion of salivary proteins.

Calcium: Many reports indicate that calcium ions play a role in the induction of secretion. Thus, calcium is required for pancreatic secretion (amylase, proteins) stimulated by acetyl choline (228), and carbachol (229).

The rat parotid gland did not, in early experiments, display any requirement for Ca^{++} in catecholamine-induced secretion (230) . This was probably because the salivary glands contain such high levels of endogenous Ca^{++} under normal conditions (231, 232). Recently, Selinger and Naim (233), by

use of EGTA to chelate Ca^{++} and propranolol to block the activation of endogenous catecholamine, showed that both epinephrine and butyryl cyclic AMP induced secretion of amylase from parotid gland slices only when Ca^{++} was added to the medium.

It has been suggested that Ca^{++} is involved in the events which take place between the activation of adenyl cyclase and the secretion of exportable protein (146, 233, 234, 235). Following the isoproterenol induced secretion of amylase, the uptake of Ca^{++} in the acinar cells of parotid gland was considerably reduced (231). Selinger et al (234) have shown that the binding of Ca^{++} in parotid gland homogenates was dependent upon ATP. Bound Ca^{++} was located mainly in the microsomal fraction, and particularly in the smooth membranes derived from the Golgi complex. The binding of cyclic AMP was also in the smooth microsomal membranes (216), and high adenyl cyclase activity was located in the parotid microsomes (208). The fact that ATP is required for the secretion of parotid amylase (232) further supports a possible secretory mechanism involving adenyl cyclase, cyclic AMP, ATP and Ca^{++} in the smooth membranes of the Golgi complex.

Recent work on rat pancreas is consistent with the involvement of Ca^{++} in the secretory process. Heisler and Tenenhouse (229) have shown that the carbochol induced protein secretion from pancreatic slices was inhibited in the absence of Ca^{++}. Addition of dibutyryl cyclic AMP eliminated this

inhibition. They suggested that cyclic AMP may be utilized during secretion. This supports the theory of Rasmussen and Tenenhouse (235) that Ca^{++} and ATP play a role in maintaining membrane structure. Conversions of membrane from one state to another were thought to involve a transition from a Ca^{++}-associated to a Ca^{++}-dissociated state. A possible model was proposed. When membrane-bound ATP was converted to cyclic AMP, Ca^{++}, which is strongly chelated by ATP, would be released because of its weak chelation by cyclic AMP. The Ca^{++} may be freed into intracellular fluid or it may be complexed by other groups such as phospholipids in membranes or with exportable proteins.

The involvement of Ca^{++} in secretory tissues may be related to events in the cell following secretion of proteins. This has been suggested by several workers (146, 228, 234) but with little supporting evidence. The fact that ATP dependent binding of Ca^{++} in parotid glands is localized mainly in the Golgi complex suggested a role of "packing the proteins to be secreted within the zymogen granules by forming a concentrated calcium-protein complex" (234). The reduction of Ca^{++} uptake two hours after isoproterenol (231) and the well-known requirement of Ca^{++} for amylase stabilization (70) might explain the very low rate of accumulation of amylase at this time (132). Some of these possibilities are further examined in the discussion at the end of the Experimental Section, Part I.

Secondary Changes Following Stimulation

Growth: In 1961, Selye et al (236) showed that repeated injections of isoproterenol over a period of several months produced an enlargement of salivary glands in rats. This was attributed to both increase in the number (236-239) and size (236, 238-240) of cells. These changes are described in a review by Seifert (167). The mean diameter of acinar cells doubles as does the nuclear diameter. The increased number of acinar cells was preceded by an increase in mitotic activity between one and five days after initiation of daily isoproterenol injections (241).

These changes have been related to sympathetic and parasympathetic stimulation in the salivary glands (239). The results of Hall and Schneyer (242) that rats maintained on a liquid diet exhibited a decrease in gland size, suggested the role of functional stimulation in regulating growth of salivary glands. The same workers (243) found that parasympathectomy resulted in lower amylase activity and acinar cell size, but sympathectomy had no effect. Wells (239) found that both branches of the autonomic nervous system were responsible for initiating the changes in gland size. The isoproterenol induced growth of salivary glands was inhibited by propranolol (244), a β-receptor blocking agent. Theophylline treatment induced salivary growth (245) and it appears to mediate both the sympathetic and parasympathetic induction of salivary growth (239).

Biosynthesis of RNA, DNA and protein: In salivary glands enlarged by repeated isoproterenol treatments, not only was increased chromosome division observed in the acinar cells (241), but there was increased acinar ergastoplasm which was matched by an increase in cellular RNA content (167). Several authors have noted other changes which occur during the early stages of isoproterenol induced salivary gland growth. These changes have been mainly in the glandular concentrations and rates of biosynthesis of DNA, RNA and protein (167).

Using autoradiographic techniques, Seifert (167) found a 46% increase in ^{3}H-cytidine incorporation into mouse parotid and submaxillary RNA, one hour after isoproterenol treatment. Barka (246, 247) and Baserga et al (248, 249, 250) have studied the incorporation of ^{3}H-uridine into rat and mouse salivary glands. The rate of incorporation decreased about 40% between two and four hours then increased to about 200% of the basal level between 12 and 24 hours after isoproterenol stimulation. It returned to the basal level after 48 hours (251). These changes occurred to the same extent in microsomal, nuclear and t-RNA (252). The concentration of RNA in rat submaxillary increased steadily during the first four days of chronic isoproterenol treatment and subsequently remained 50% higher than the basal level. The changes in the activities of several enzymes involved in the biosynthesis of UMP paralleled the changes in submaxillary RNA content (251).

The apparent peak in RNA biosynthesis during the pre-replicative stage was thought to be related to a subsequent increase in DNA biosynthesis (246, 247). This theory was seriously weakened when Malamud and Baserga (249) showed that the increase in ^{3}H-uridine incorporation into submaxillary RNA resulted from corresponding changes in specific activity of the immediate precursor, UTP. An estimation of RNA biosynthesis was made after taking into account these changes in precursor specific activity. On that basis, no significant increase in RNA biosynthesis was found after isoproterenol stimulation. The only significant change was a 55% decrease one hour after stimulation.

Several groups have reported that large increases in DNA synthesis (measured by ^{3}H-thymidine incorporation into DNA) in rat and mouse salivary glands occur after injection of isoproterenol (217, 247, 248, 253-256). As a rule, DNA synthesis does not start to increase until about 20 hours after isoproterenol. It reaches a peak at about 28 hours, then subsides and by 48 hours, it has returned to the control level. The parotid gland responds to isoproterenol with maximal increase in DNA synthesis, the sublingual and minimal. The submaxillary gave an intermediate response (217). Both acinar cells and cells of intercallated ducts were stimulated while cells of the striated ducts were not (217, 256). In mouse parotids, a large single dose induced a 250-fold increase in the number of DNA synthesizing cells (217). The peak of DNA synthesizing activity was about 20-fold greater than the

control in rat submaxillary gland (253). These changes in activity were dependent upon the dose of isoproterenol (217, 253, 257). Doses greater than 3 mμmole/g body weight were required because this dose did not stimulate DNA synthesis in mouse salivary gland (253, 257). This might be considered a "physiological dose" because a dose of 2.5 mμmole/g body weight (0.1 mg in a 150 g rat) resulted in only a 17% depletion of parotid amylase (132). A dose of 5 mg isoproterenol/150 g rat (120 mμmole/g body weight) was used in most of the experiments described in the Experimental Section. This dose was sufficient to induce an approximately ten-fold increase in DNA synthesis in rat and mouse salivary glands (217, 253).

The changes in DNA synthesis were closely paralleled by changes in enzymes, such as deoxythymidine kinase, deoxythymidylate kinase, deoxythymidylate synthetase or DNA polymerase, which are involved in the replication of DNA (253, 258, 259). Glycogen also appeared to be involved. It decreased immediately after isoproterenol stimulation, then rose to a level six-fold greater than the basal level immediately preceding the increase in DNA synthesis (260).

Protein synthesis may be involved in the control of DNA synthesis. An increase in ^{14}C-amino acid incorporation into proteins has been reported in rat submaxillary four hours after isoproterenol (247). Incorporation into rat parotid proteins initially decreased at one hour, then increased to a maximum, 18 hours after stimulation by pilocarpine-refeeding (148).

In parotid slices, incorporation into total protein was doubled one hour after epinephrine or dibutyryl cyclic AMP induced secretion (150). In mouse salivary glands, a peak of amino acid incorporation into ribosomal and polysomal protein was reported by Baserga et al (217, 261). Inhibition of protein synthesis immediately after isoproterenol stimulation with cycloheximide, puromycin or sodium pentabarbital inhibited the increase in DNA synthesis which occurred 20 hours later (217, 255, 261). At two hours, an increase in incorporation of amino acids into an acidic, non-histone, nuclear protein was observed (262). In rat submaxillary gland, isoproterenol injection first depressed, then stimulated amino acid incorporation into total protein. One specific group of proteins showed a peak of synthesis during the first three hours when overall protein synthesis was depressed (263). Actinomycin D, which inhibited the increase in DNA synthesis without affecting protein synthesis (248, 253), inhibited the increased synthesis of that specific group of proteins. It has therefore been suggested that certain specific proteins may be related to the control of DNA synthesis and growth of cells (217, 261, 262).

The ability to stimulate DNA synthesis in the salivary glands is usually correlated with capacity to stimulate secretion of saliva. Several studies have pointed to the independence of these two effects, and suggest that the action of isoproterenol on DNA synthesis is direct, rather than related to secretion or repletion of salivary components. D-actinomycin and actinomycin D inhibited the synthesis of DNA without inhibiting the secretion

and repletion of mouse salivary amylase and DNase (217, 258, 259). Kirby et al (264) studied the effect of structural modifications of the isoproterenol molecule on the relative ability of the new compounds to induce secretion and promote DNA synthesis. The molecular changes, in most cases, resulted in fairly consistent changes in secretion of amylase, salivary DNA synthesis and liver glycogenolysis. When both phenolic OH groups were absent, however, DNA synthesizing activity was abolished without affecting secretion of amylase. Furthermore, the d- and l-isomers of isoproterenol are equally effective in stimulating amylase secretion and DNA synthesis, but d-isoproterenol does not produce glycogenolysis in the liver.

Baserga et al (217) found that pyrogallol, which inhibited the metabolism of isoproterenol by COMT, enhanced the stimulation of DNA synthesis in mouse salivary glands. This indicated that isoproterenol itself may be the active compound. A conflicting report was published by Barka (254) in which an inhibition of isoproterenol-induced DNA synthesis in rat submaxillary was observed after pyrogallol administration. Injection of o-methyl isoproterenol did not result in increased DNA synthesis. Barka suggested that methylation of isoproterenol is a step in the complex metabolic processes that lead to the triggering of cell multiplication.

Changes in enzymes: The concentrations of many enzymes in salivary glands change following nervous or drug induced secretion. Some of these changes can be related to the cycle of secretion and repletion, to the stimulation of DNA synthesis, general growth of acinar cells, atrophy of tubular tissue and other changes in microanatomy which have been reported. Some of these mechanisms are discussed below.

The increase in gland size and enzyme activity may be the result of general stimulation of protein biosynthesis and level of metabolism by secretory stimuli. This type of change could be similar to changes induced by thyroid and pituitary hormones (189). For example, thyroxine increase the level of secretory enzymes in the parotid, submaxillary, and pancreas (189), as well as increasing the salivary gland weight (188). The changes have been related to cytological changes such as the increased number and size of acinar cells with a simultaneous regression of tubule cells (188-190). Catecholamines, which induce salivary secretion, however, do not appear to be similar to thyroxine in their effects on general protein synthesis (265, 266).

Changes in the enzyme activity of salivary glands after isoproterenol treatment may simply reflect the general increase in glandular mass in response to repeated secretory stimulation. Cytochemical studies have shown an increase in the activity of the enzymes monoamine oxidase, succinic dehydrogenase and acid phosphatase in salivary acinar cells which paralleled the increase in gland size (167).

In contrast, the secretory proteins such as amylase (132) and salivary mucins (167), which are stored in the acinar zymogen granules, were not increased during the induced gland growth. This is consistent with electron microscopic studies which have indicated that in the enlarged acinar cells, zymogen granules are replaced by less dense secretory vacuoles (167). Similarly, the decrease in salivary protease following isoproterenol-induced growth (267) can be correlated to atrophy of tubule cells, the site of protease storage (167).

Changes in enzyme activity in salivary glands may be due to changes in the number and size of certain subcellular particles induced by the secretory stimuli (268).

An increase in endoplasmic reticulum has been described in salivary glands shortly after secretory stimulus (167, 269, 270). Long term stimulation,which resulted in gland growth, also resulted in an increase in the size of acinar nuclei, and mitochondria, increased endoplasmic reticulum and RNA content as well as enlarged Golgi complex (167).

An increase in salivary MAO and succinic dehydrogenase, along with an enlargement of mitochondria, indicated that in this case, enzyme changes were merely a reflection of increased mitochondrial mass (271). It has been reported that chronic administration of isoproterenol increases total succinic dehydrogenase without affecting the activities of cytochrome oxidase or fumerase (272). A short term increase in MAO without a simultaneous increase in succinic dehydrogenase or cytochrome

oxidase was found in the heart (273, 274) and salivary glands (271) of isoproterenol stimulated rats. This suggested either that isoproterenol affects mitochondrial enzymes unequally, or else that the isoproterenol stimulated MAO is not mitochrondrial in origin. This latter suggestion was supported by the finding that a large portion of heart and salivary MAO is microsomal (267, 275). It has thus been suggested that early increases in salivary MAO activity (3 hours after injection of isoproterenol) are the result of the increased endoplasmic reticulum (169, 271).

Overall changes in salivary gland enzyme activities may result from a response of specific enzymes to salivary stimulation rather than to changes in general protein syntheis or growth of subcellular particles. Some of the enzyme changes which do not seem to be directly related to any general long-term glandular changes, may be related to secretion and repletion of salivary enzymes; e.g., amylase (132, 258), DNase (259) and protease (267). Other changes are the decrease in RNA biosynthesis 1 hour following isoproterenol stimulation (249) and the increased peroxidase activity 36 hours after stimulation (276).

Some changes in enzyme activities may be simply a response of the gland to metabolize the drug or hormone. This type of response may, for example, be similar to the increase of microsomal drug hydroxylating enzymes in response to many drugs.

Metabolism of Catecholamines

The metabolism of catecholamines has been the subject of several doctoral research programs in this laboratory. The various aspects of catecholamine metabolism, are thoroughly reviewed in several theses resulting from this work particularly in reference to MAO and COMT (279, 280). Other authors have also reviewed the role of COMT and MAO in catecholamine metabolism (281, 282, 283). It is generally accepted that MAO plays a role in inactivation of endogenous catecholamines (mainly noradrenaline in adrenergically innervated tissues). COMT inactivates exogenous catecholamines such as circulating adrenaline or other extracellular amines. Noradrenaline is present mainly in the intracellular vesicles of sympathetic nervous tissues. There is an equilibrium between noradrenaline in the vesicles and the unbound noradrenaline in cellular fluids which is available for destruction by MAO. Drugs like reserpine, which cause a release of norepinephrine from storage granules, expose it to MAO. Nerve stimulation causes a release of norepinephrine into the synaptic cleft, where, after acting on adrenergic receptors, it may be inactivated by COMT.

The above general pattern seems to be true of salivary glands. Most salivary MAO is contained in parenchymal cells but approximately 30% is found in sympathetic synaptic terminals (271, 284, 285). The synaptic MAO metabolizes virtually all

endogenous noradrenaline whereas exogenous noradrenaline is o-methylated in the parenchymal cells (286, 287). In the liver, MAO is mainly mitochondrial, but in rat salivary glands, it is almost equally divided between microsomal and mitochondrial fractions (268). Sympathetic denervation,which causes atrophy of the adrenergic nerve endings, resulted in a decrease of both mitochondrial and microsomal MAO.

The salivary glands have a comparatively high concentration of COMT, being exceeded only by the liver and pancreas in the monkey (288). It has been estimated that approximately 70% of salivary COMT activity is in the parenchymal cells (289, 290).

Anderson and D'Iorio (291) demonstrated multiple forms of COMT in rat liver. Subsequently, multiple forms have been shown within many tissues of several species (292) and these isoenzymes displayed different kinetics. The concept that the origin of the two isoenzymes may be neuronal and parenchymal was supported by the work of Jarrott (289) who discovered that the enzyme kinetics of neuronally located COMT (in several tissues including rabbit submaxillary) was different from total COMT. The neuronal COMT may play a role in metabolism of exogenous noradrenaline under certain conditions. Jonason (293) reported that salivary noradrenaline released by reserpine is rapidly metabolized by MAO. Inhibition of MAO retards the removal of noradrenaline, inhibition of COMT does not. Inhibition of both MAO and COMT retards the reduction more than MAO inhibition alone. This suggested that free endogenous noradrenaline may be metabolized by COMT only after it reaches a high concentration.

Induced changes in salivary gland COMT have been reported. The reductions in rat submaxillary COMT after duct ligation or sympathectomy simply reflected the atrophy of parenchymal and neuronal cells respectively (289, 290). The dramatic decrease in rat submaxillary COMT (to 10% of basal level), 24 hours after a large dose of isoproterenol (254) is very difficult to explain in terms of other changes induced by isoproterenol or in terms of catecholamine metabolism. The same paper (254) also reported on the inhibition of salivary DNA synthesis after COMT inhibition. This was disputed by the results of other workers who reported the opposite effect of COMT inhibition (217).

Isoproterenol, an artificial catecholamine, is not deaminated by MAO but is an excellent substrate for COMT (294). Its major pathway of metabolism in the human appears to be O-sulfate conjugation. COMT plays an important role, however, since substantial amounts of urinary 3-O-methyl isoproterenol were also found (295). In the rat, the isoproterenol metabolites were 3-O-methyl isoproterenol and the glucuronides of isoproterenol and 3-O-methyl isoproterenol (294). Eight hours after a small administered dose of labeled ispproterenol, 92% of the label had been recovered in the bile and urine of rats.

In several tissues, ten minutes after injection of ^{3}H isoproterenol, most radioactivity was found as O-methyl isoproterenol. Two hours later, the radioactivity (except in

kidneys and liver) decreased 20-fold (294). Baserga et al (217) studied the metabolism of isoproterenol within the mouse salivary glands and substantiated the role of COMT and glucuronyl transferase in its metabolism. They found that even with large doses of isoproterenol (0.8 μM/g body weight) most isoprpterenol was catabolized within six hours. At this dose of isoproterenol, a maximum concentration of free isoproterenol appeared in the salivary gland at thirty minutes, decreased to one-sixth at one hour and decreased at a slower rate between one and six hours when it was virtually absent. At six hours, 0-methyl isoproterenol was still present in moderate amounts.

The relative concentrations of isoproterenol and its metabolites in the salivary glands depended upon the dose administered. Thirty minutes after injection of 0.08 μM/g body weight of ^{3}H-isoproterenol, approximately 5% of total salivary radioactivity was due to unmetabolized isoproterenol. When the dose was 0.8 μM/g, over half the isoproterenol was unmetabolized.

SECTION II

ANALYTICAL*

Amylase: Manual Method of Bernfeld (3)

Reagents

DNSA reagent: 1% 3,5-dinitrosalicylic acid and 30% sodium potassium tartrate in 0.4 N NaOH

Substrate solution: 1% amylopectin, 0.02 M phosphate, 0.007 M NaCl, pH 6.9

The amylopectin was prepared: a, by isolation from corn starch as described by Schoch (296); or b, by treating a commercial soluble starch with sodium borohydride as suggested by Strumeyer (297).

a. A 3% suspension of corn starch was gelatinized on a steambath and autoclaved three hours at 20 lb. pressure. n-Amyl alcohol, 10% by volume, was added with stirring to the hot solution and the mixture was allowed to cool to room temperature. Amylose, which precipitated on cooling, was removed by centrifugation. The supernatant was treated with excess methanol to flocculate the amylopectin. The amylopectin was removed by centrifugation, resuspended in methanol, filtered and dried. To prepare the substrate, the amylopectin was autoclaved with the buffer solution, and a slight amount of undissolved amylopectin was removed by centrifugation.

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* All chemicals described in this section were purchased from Fisher Scientific Company unless otherwise specified.

b. A commercial preparation of amylopectin, (Sigma Chemical Co.) resulted in a high blank with the DNSA reagent. Sodium borohydride pretreatment was used to attack the free reducing groups in the amylopectin, thus lowering the blank value. A suspension of 10 g amylopectin in 180 ml water was dissolved by boiling. The solution was cooled on ice and a previously cooled solution of 300 mg of sodium borohydride in 20 ml water was gradually added while magnetically mixing with a Teflon-coated stirring bar. The mixture was allowed to stand at room temperature for at least one hour. This 5% stock solution could be stored in the refrigerator for several weeks without retrogradation or microbial growth. (There was no microbial growth after four months but some retrogradation of the amylopectin had occurred. The solution could be used, however, by heating it to boiling in order to redissolve the amylopectin.) For daily use, 25 ml of the 5% stock solution was removed, and 0.05 ml acetone was added with shaking to destroy excess sodium borohydride. After standing twenty minutes at room temperature, the solution was brought to pH 7.0 with 1 N acetic acid. The volume was adjusted to 125 ml with the 0.02 M phosphate buffer pH 6.9. The solution was centrifuged ten minutes at 5000 X g to remove a small amount of undissolved starch. The supernatant was a clear colorless, slightly opalescent, solution.

Procedure

One ml of properly diluted enzyme was incubated three minutes at 37^{o} with 1 ml of substrate solution. The tube was

heated for five minutes in boiling water and cooled. After dilution with 20 ml water, absorbance was measured at 540 mμ. A blank was prepared in the same way without enzyme. A calibration curve established with maltose monohydrate (0.2 to 2 mg in 2 ml H_2O) was used to convert spectrophotometric readings to maltose concentration. Amylase activity was expressed in terms of milligrams of maltose liberated in three minutes at 37° by 1 ml of enzyme solution.

Amylase: Automated Method

Preliminary Experiments

In order to automate the method of Bernfeld for the determination of amylase, some information on the color reaction between reducing sugars and the dinitrosalicylic acid reagent was required. Experiments were performed to determine optimum wave length, required heating time and other factors which affect the sensitivity or reproducibility in methods adapted to the Technicon Auto Analyzer.

Optimum wave length: Spectra of maltose standards which were read against the reagent blank by the Unicam SP 200 spectrophotometer are shown in figure 2. The absorbance peak was between 490 and 500 mμ. The readings at this wave length were erratic due to the high absorption of the reagent blank shown in figure 3. The absorbance peak of the reagent blank was at 370 mμ (figure 4) but absorbance at 500 mμ was high enough to

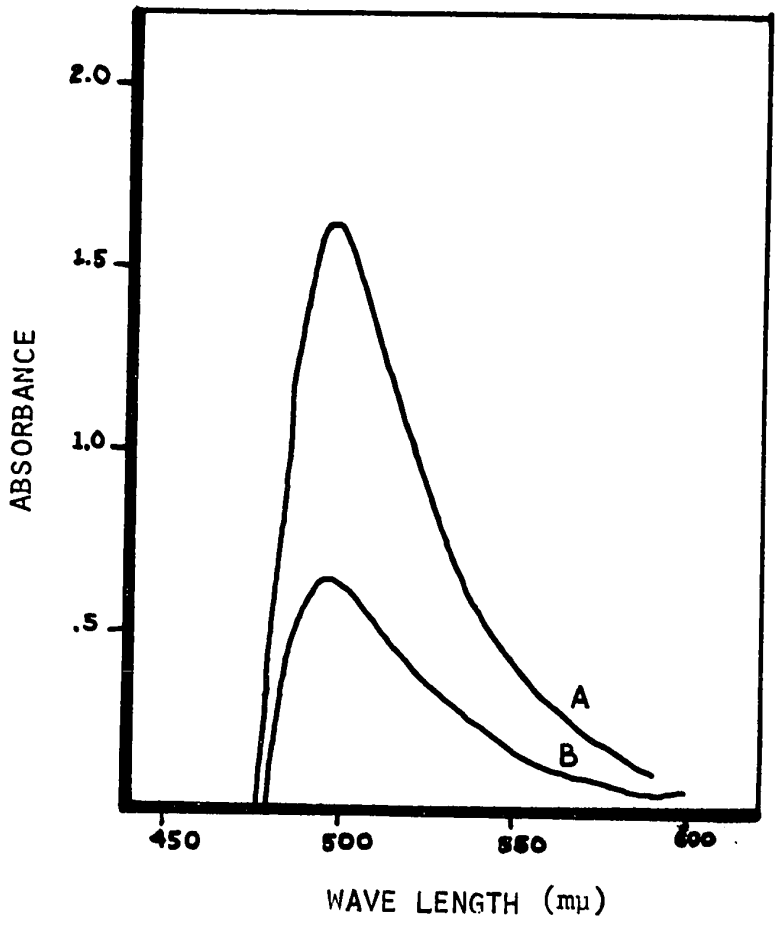


Figure 2 . Absorption spectra of DNSA reagent heated with maltose : A - 1.2 mg maltose ; B - 0.4 mg maltose. Absorbance was measured against a reagent blank.

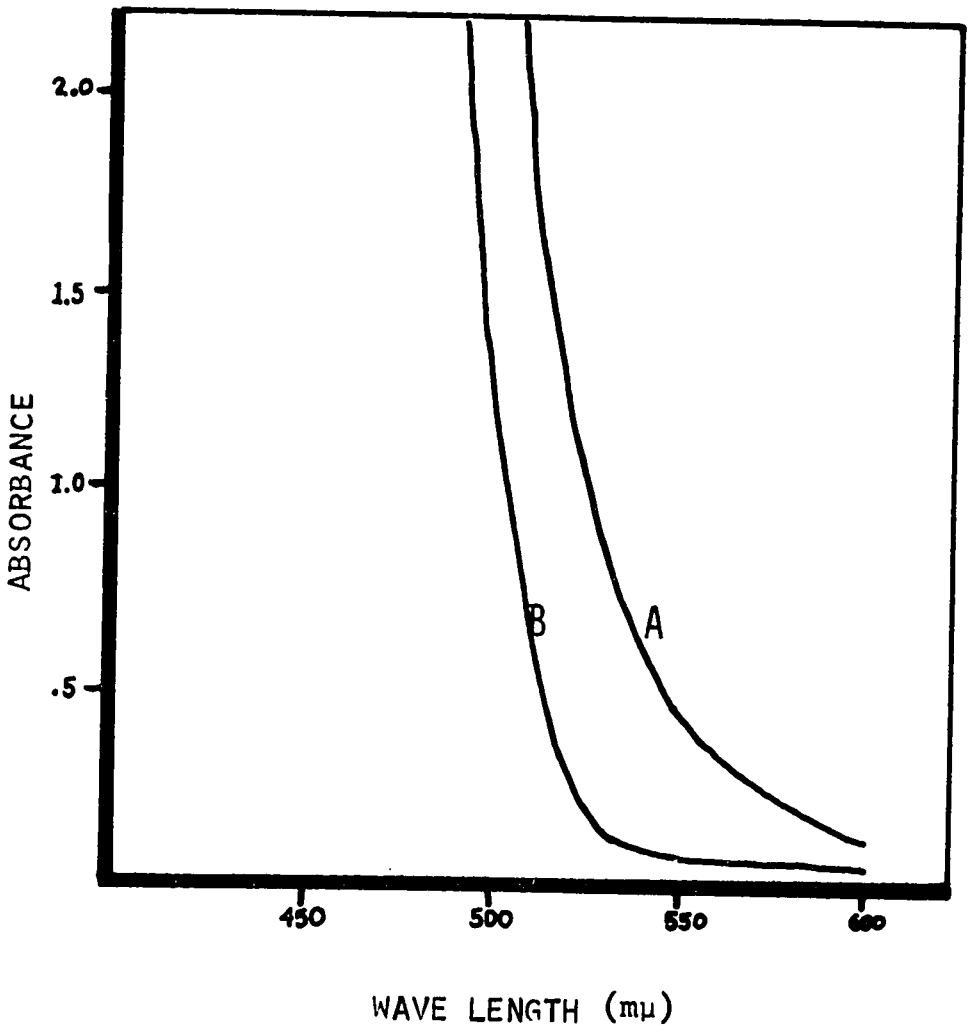


Figure 3 . Absorption spectra of the DNSA - maltose reaction products : A - 1.2 mg maltose ; B - reagent blank. Absorbance was read against water.

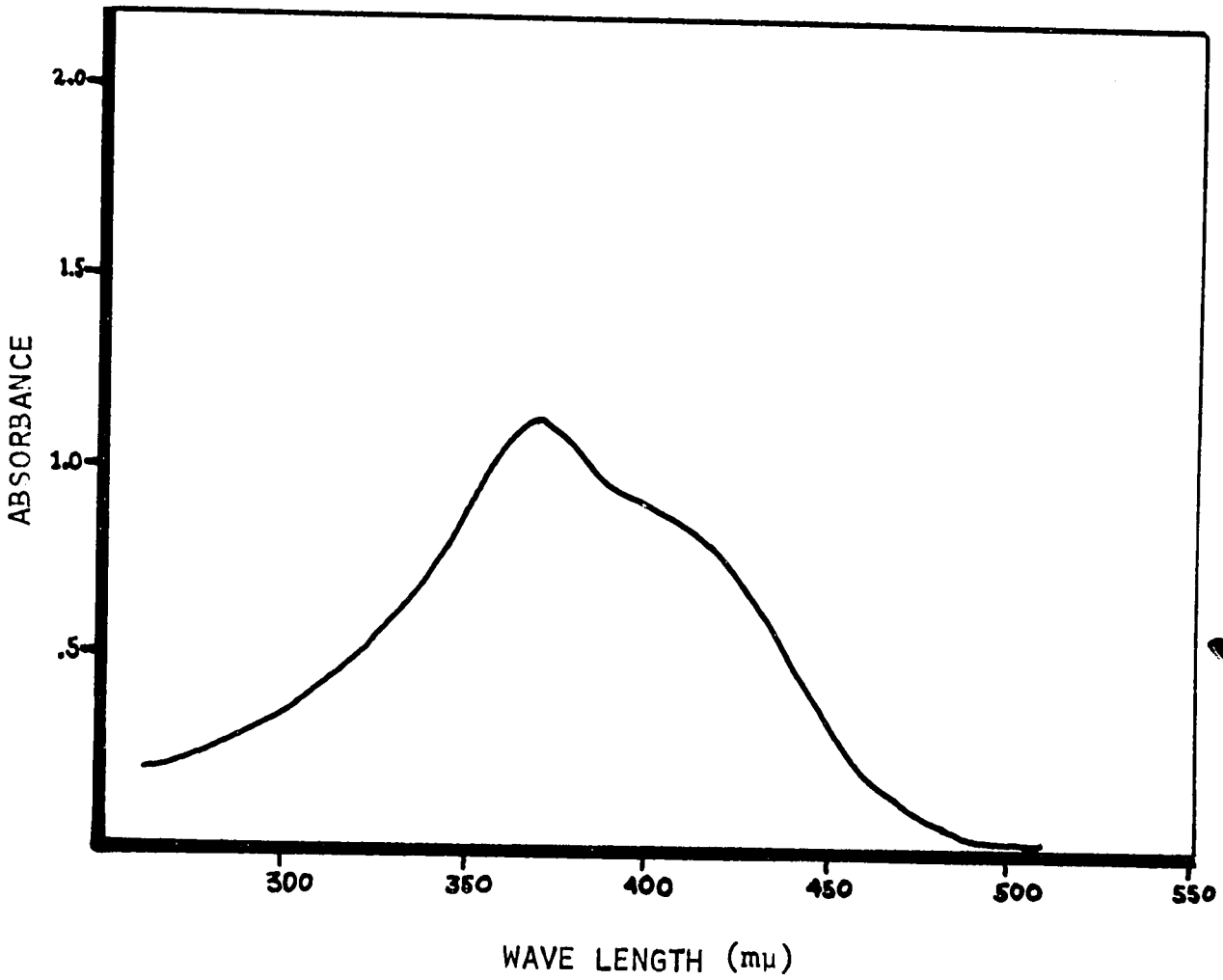


Figure 4 . Absorption spectrum of the DNSA reagent. The DNSA reagent was diluted and its absorbance was measured against water.

interfere with the amylase determination at this wave length. In figure 5, the absorbance of standard solutions (0.2 to 2.0 mg maltose) was plotted against maltose concentration for wave lengths between 500 and 570 mμ. At 500 mμ, where greatest sensitivity was achieved, the standard curve was not linear. The standard curve at 550 mμ, although less steep, was linear. Because of this linearity and in order to keep the reagent blank at a low value, a wave length of 550 mμ was chosen as an acceptable compromise between sensitivity and reliability.

Sensitivity of the AutoAnalyzer colorimeter: The absorbance of maltose standards was read at 540 mμ on a Beckman D.U. spectrophotometer. The standards were also passed through the 1.5 cm flow cell of the AutoAnalyzer colorimeter and absorbance measured using 550 mμ filters. In figure 6, these standard curves are compared. It is obvious that any loss of sensitivity caused by increasing the wave length from 540 to 550 mμ was more than compensated by the increased light path of the flow cell.

Temperature and heating time: Bernfeld (3) recommended five minutes in a boiling water bath for maximum color development. Since the heating bath of the AutoAnalyzer was set at 95°, it was necessary to determine the heating time required for maximum color development below 100°. Maltose was heated with DNSA reagent at 95° or 93° for various time periods.

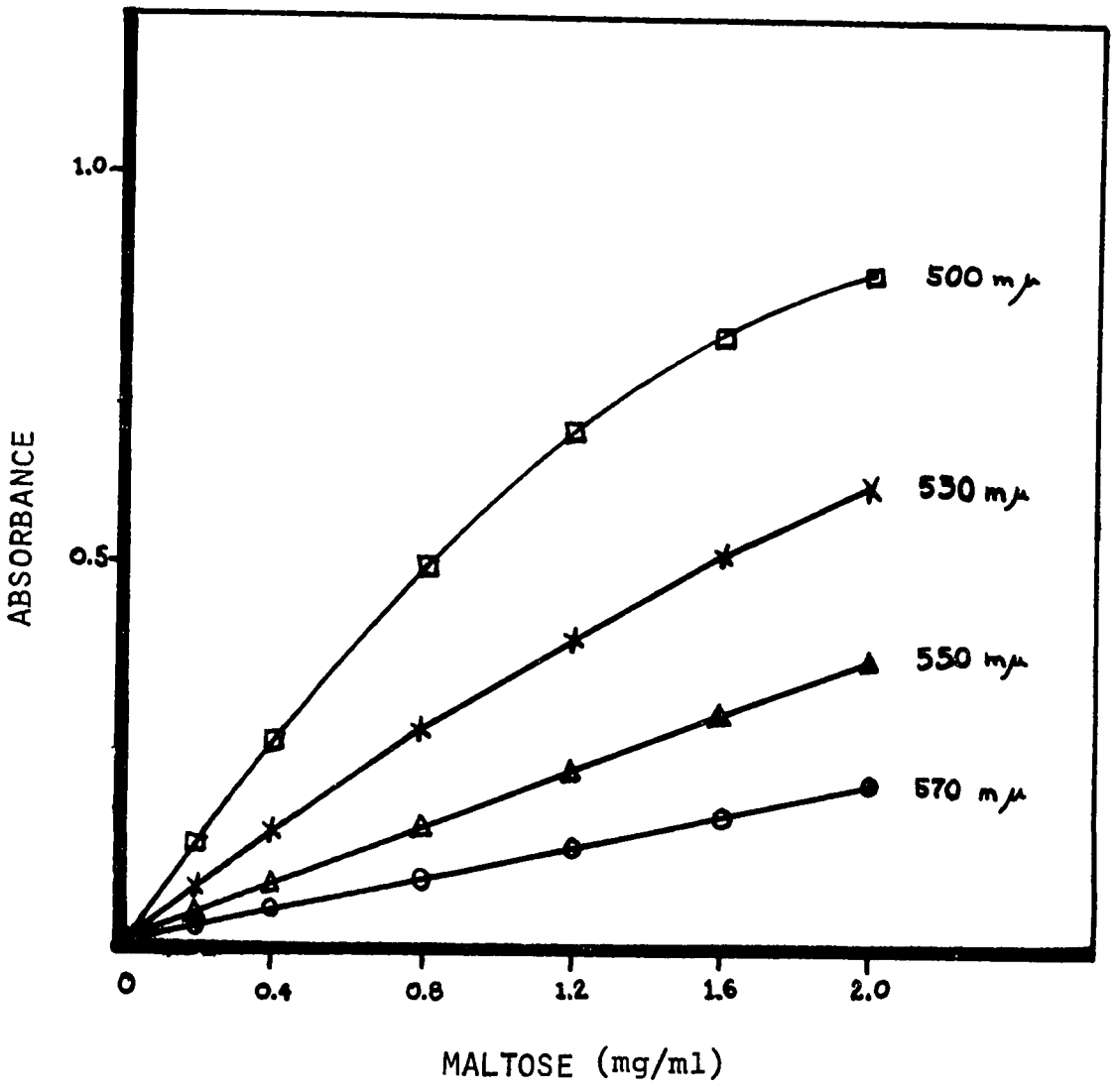


Figure 5 . Effect of wave length on maltose calibration curves, for the Bernfeld amylase assay.

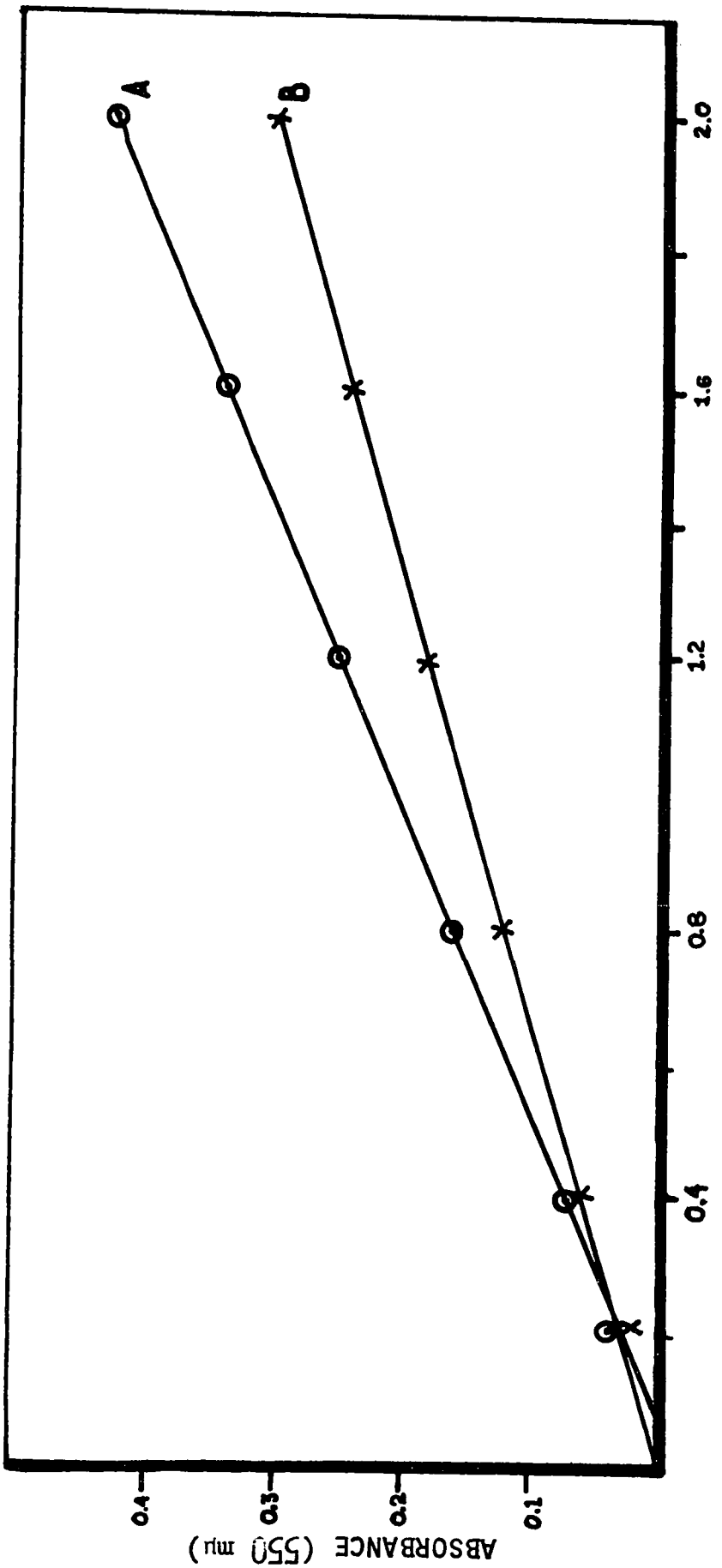


Figure 6 . Calibration curves for the Bernfeld amylase assay : A - standard maltose solutions were measured, using a Technicon AutoAnalyzer colorimeter equipped with a 1.5 cm flow cell ; B - standard maltose solutions were measured, using a Beckman DU spectrophotometer and a 1.0 cm cuvette.

In figure 7, it can be seen that color development had reached maximum by five minutes at 95° and by seven minutes at 93°.

Interference from air: It has long been known that reducing sugars and reduced DNSA can be oxidized by atmospheric oxygen, thus affecting the amylase assay (108, 94). This interference was particularly strong in our first attempts to automate the DNSA method. In the AutoAnalyzer flow technique, solutions are segmented with air bubbles in order to prevent streamlining and back-mixing due to friction and turbulence in the tubing. The resulting intimate contact of air and reaction mixture produced a standard curve which does not pass through the origin (figure 8). Low concentrations of maltose standard exhibited no more absorbance at 550 mμ than the reagent blank. By segmenting the solutions with pure oxygen (figure 8), the interference was greatly amplified. It has been suggested that simply by including a small amount of glucose in the DNSA reagent in order to increase the blank absorbance, this interference can be overcome (298). In the automated technique, however, it was very easy to remove the interference by segmenting the solutions with pure nitrogen instead of air. This resulted in a standard curve (figure 8) which passed through the origin, allowing very low concentrations to be determined.

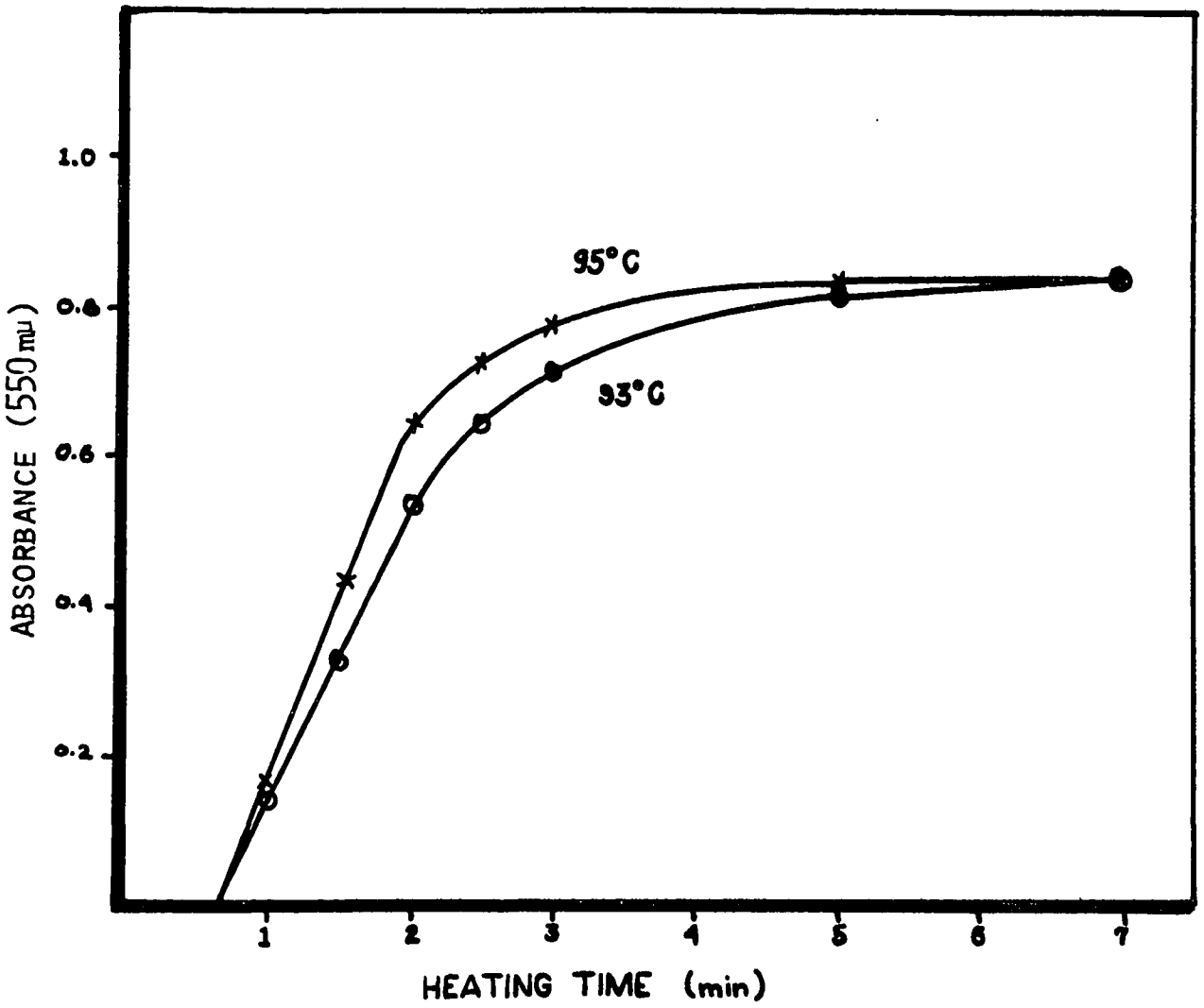


Figure 7 . Effect of temperature and heating time on color development in the Bernfeld amylase assay.

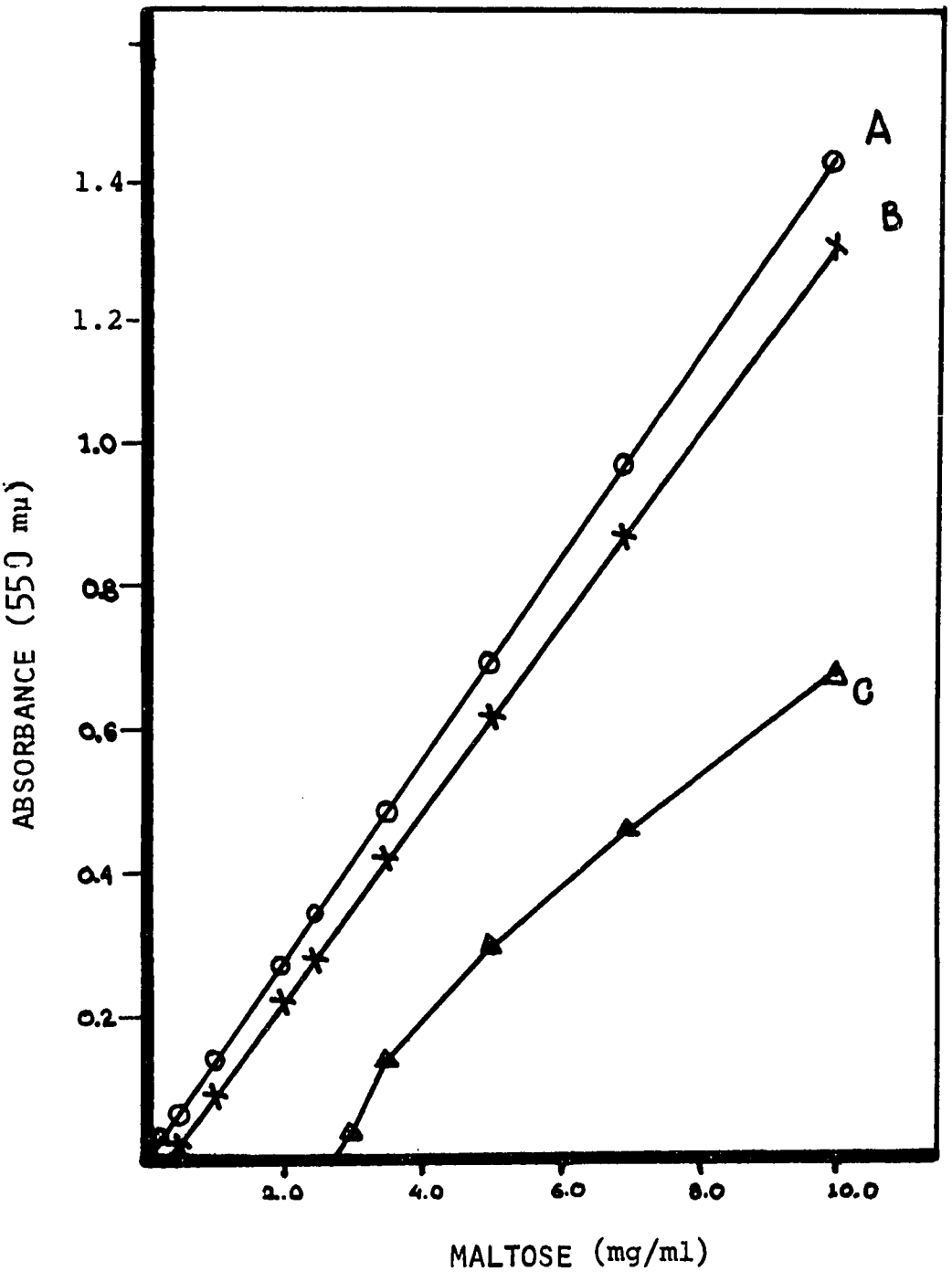


Figure 8 . Effect of air, oxygen and nitrogen on the automated amylase assay : The flow stream was segmented with ; A - nitrogen ; B - air ; or C - oxygen.

Automated Procedure

All reagents were prepared as described in the manual amylase procedure but with the non-ionic detergent, triton X-100 added to each solution (0.02%) for improved mixing and flow pattern. Standard maltose solutions were stored frozen in 2 ml conical sample cups. Amylase samples were determined automatically using the ordinary modules of the Technicon Auto-Analyzer, including sampler, proportioning pump, mixing coils, 37^{o} and 95^{o} baths, colorimeter with 550 mμ filters, 15 mm flow cell and recorder. Samples and reagents were mixed in the proportions indicated in the schematic diagram (figure 9).

The following describes all the automatic manipulations occurring between the sampler and the colorimeter. Amylase samples or maltose standard solutions were aspirated at a rate of 0.23 ml/min with a total sampling time of two minutes. The sample and substrate solution (0.8 ml/min) were segmented with nitrogen gas and mixed in a 37^{o} bath for three minutes. The DNSA reagent (0.60 ml/min) was immediately mixed to stop the enzymatic reaction. This mixture is passed through the pump at 0.6 ml/min after removal of excess solution and nitrogen in a debubbler. It was forced through a glass coil in a 95^{o} oil bath for six minutes to develop the color. A portion, 0.42 ml/min was diluted with water (2 ml/min) and passed into the flow cell of the colorimeter, where absorbance at 550 mμ was read.

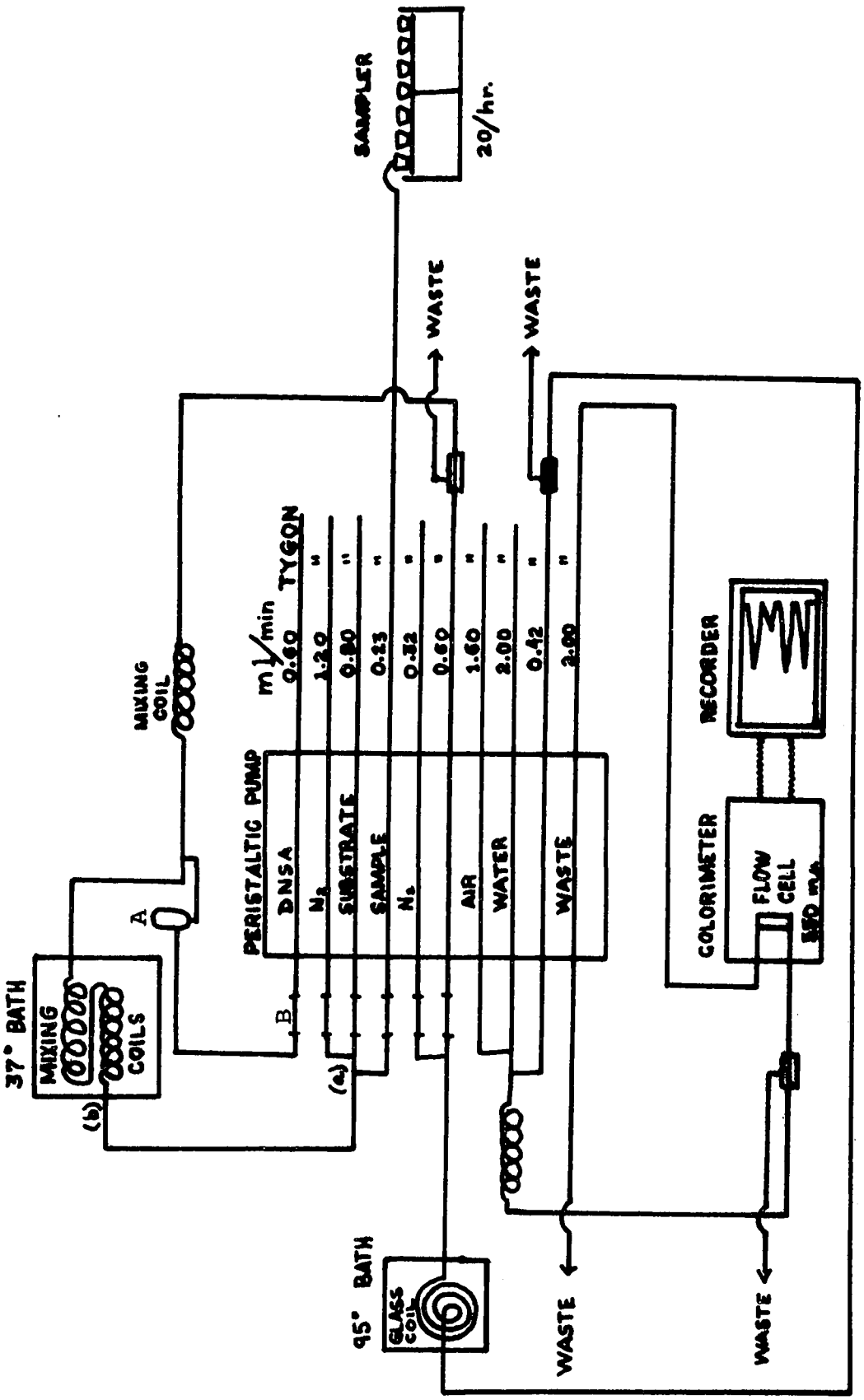


Figure 9 . Schematic flow diagram for the automated amylase assay :
A - pulse supression chamber ; B - pulse supression tubes.

All pulse suppression tubes and chambers indicated were required to ensure smooth flow, a constant baseline and reproducible standards. The tubing is tygon transmission tubing except between a and b where glass tubing (2mm i.d.) was used. This tubing, as well as the glass mixing coils in the 37° bath, required occasional cleaning with chromic acid solution. The nitrogen gas was stored in a 1 liter collapsible polyethylene bag connected to the Technicon manifold by tygon transmission tubing.

Discussion

As with the manual method (3), the standard curve of absorbance vs maltose concentration for the automated method (figure 10) was linear over the complete range of 1 to 10 mg/ml. Although the DNSA reagent could be stored many months in a sealed dark bottle, with day-to-day use, the standard curve was gradually lowered and it was therefore necessary to prepare a standard curve for each day that amylase was assayed.

Amylase units were expressed as mg of maltose monohydrate liberated in 3 minutes at 37°. Amylase units/ml were thus read directly from the standard curve in terms of concentration of maltose. Amylase units described here have been compared to those proposed by other workers in table 1.

When several dilutions of amylase isolated from rat parotid gland were assayed, the results,shown in figure 11, were obtained. Amylase activity (mg/ml maltose) was proportional to the enzyme

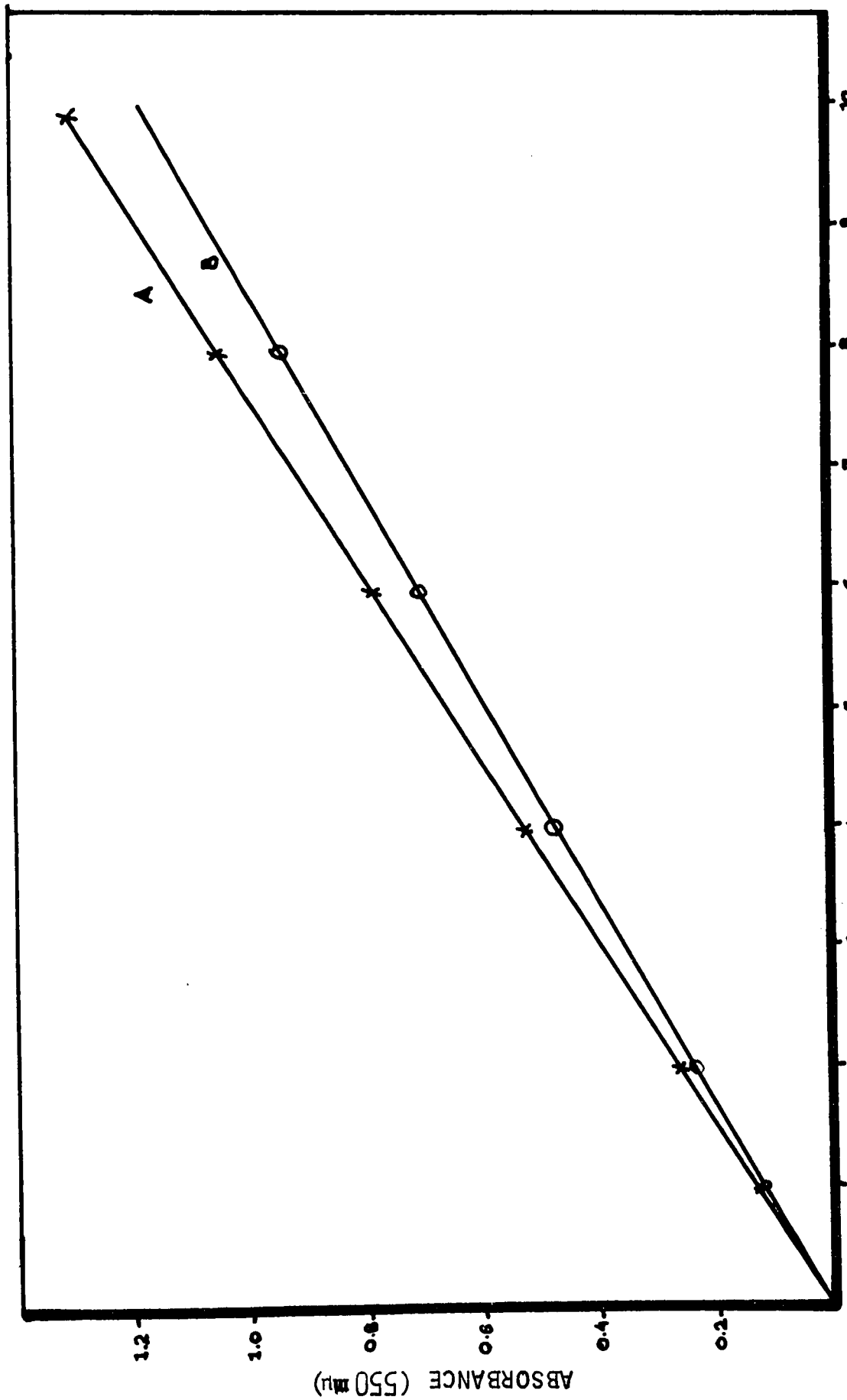


Figure 10 . Standard curve for the automated amylase method : A - freshly prepared DNSA reagent ; B - DNSA reagent, stored 5 days.

Table 1

Comparison of amylase units proposed by several authors.

Definition of Unit	Incubation Temperature	Relative size of units *	Reference
Reducing equivalent of 1 mg maltose monohydrate released in 3 minutes	37° 40° 30° 25° 20°	1.00 1.08 0.81 0.66 0.52	This Thesis 71 3
Moles of reducing groups released in 1 minute	37° 25°	0.925 0.61	IUPAC † 94
Reducing equivalent of 1 mg glucose released in 30 minutes	37°	5.0	90

* The size of units are expressed relative to the amylase unit defined in this thesis (mg maltose/3 min at 37°). Calculations were made on the basis of reported temperature-amylase activity relationships (3, 94).

† International Union of Pure and Applied Chemistry

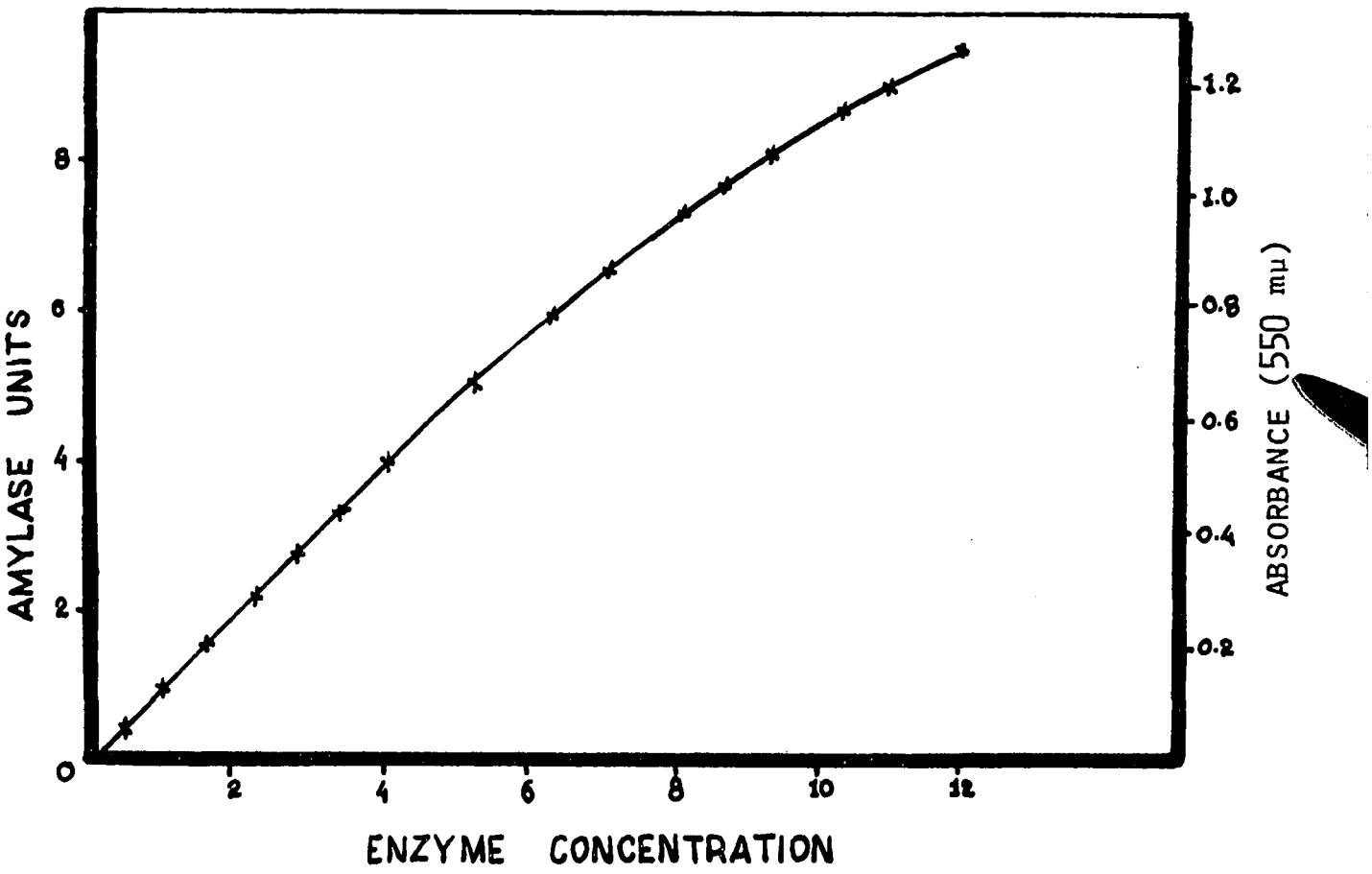


Figure 11 . Linearity of amylase activity with concentration. The curve was prepared by assaying a series of dilutions of several parotid homogenates. Enzyme concentration is in arbitrary units. Amylase units are mg maltose released in 3 minutes at 37°.

concentration up to about 5 units. The activity fell off slightly at higher concentrations. Whenever possible, sample dilutions were prepared to bring the enzyme concentration within the linear part of the curve. However, with concentrations slightly above this, the curve could be used to find a correction factor.

In order to define the reliability of this method, some of the kinetic parameters of continuous flow, described by Thiers et al are given. Delay time, the time between the aspiration of a sample and the response of the recorder pen to that sample, was 12 minutes. This included three minutes for enzyme incubation in the 37° bath and six minutes for color development in the 95° bath. The half-wash time, the time required for the absorbance to be reduced 50% following transfer of the sample intake tube from sample to water, was 18 seconds. Consequently, with a sampling rate of 20 samples per hour (2 minutes aspiration of sample with one minute of air between samples), the absorbance peak was virtually the same as the sample steady state (absorbance reached during continuous aspiration of sample). The interaction between samples was less than 1%. The minimum volume of sample required was 0.5 ml.

Continuous Monitoring of Amylase Secretion

Introduction

The release of products from tissues has been monitored with the AutoAnalyzer as, for example, in the production of

corticosteroids by adrenal slices (299). This type of continuous flow analysis was considered to be suitable for measuring the secretion of amylase from the parotid glands of anaesthetized rats. Collection of saliva directly from the parotid duct of anaesthetized rats has been described by Schneyer et al (300). Our attempts to canulate the ducts were unsuccessful. Instead, parotid saliva was collected in a simple flow tube, diluted, and analyzed for amylase. The saliva collector, the diluter and the amylase assay were all fully automated with the AutoAnalyzer as described below.

Procedure

The collection of parotid saliva for the continuous monitoring of amylase is shown in figure 12. Rats were anaesthetized with Nembutal (50 mg/kg i.p.) or urethane (500 mg/kg i.p.). An incision was made in the left cheek to expose the parotid duct. The parotid duct, formed by the union of three principal branches, crosses the masseter muscle parallel with the buccal and mandibular branches of the facial nerve and opens opposite the molar teeth. This was severed close to the opening into the mouth cavity and carefully pulled away from the muscle. The nerve was left undisturbed as much as possible since handling of the nerve caused contractions of the facial muscles which pulled the parotid duct out of the collector. The rat was placed on a board and immobilized on its side with the aid of strings tied to the incisor teeth, and legs, and with adhesive tape behind the head. The board was tilted so that the rat's head was slightly lower than

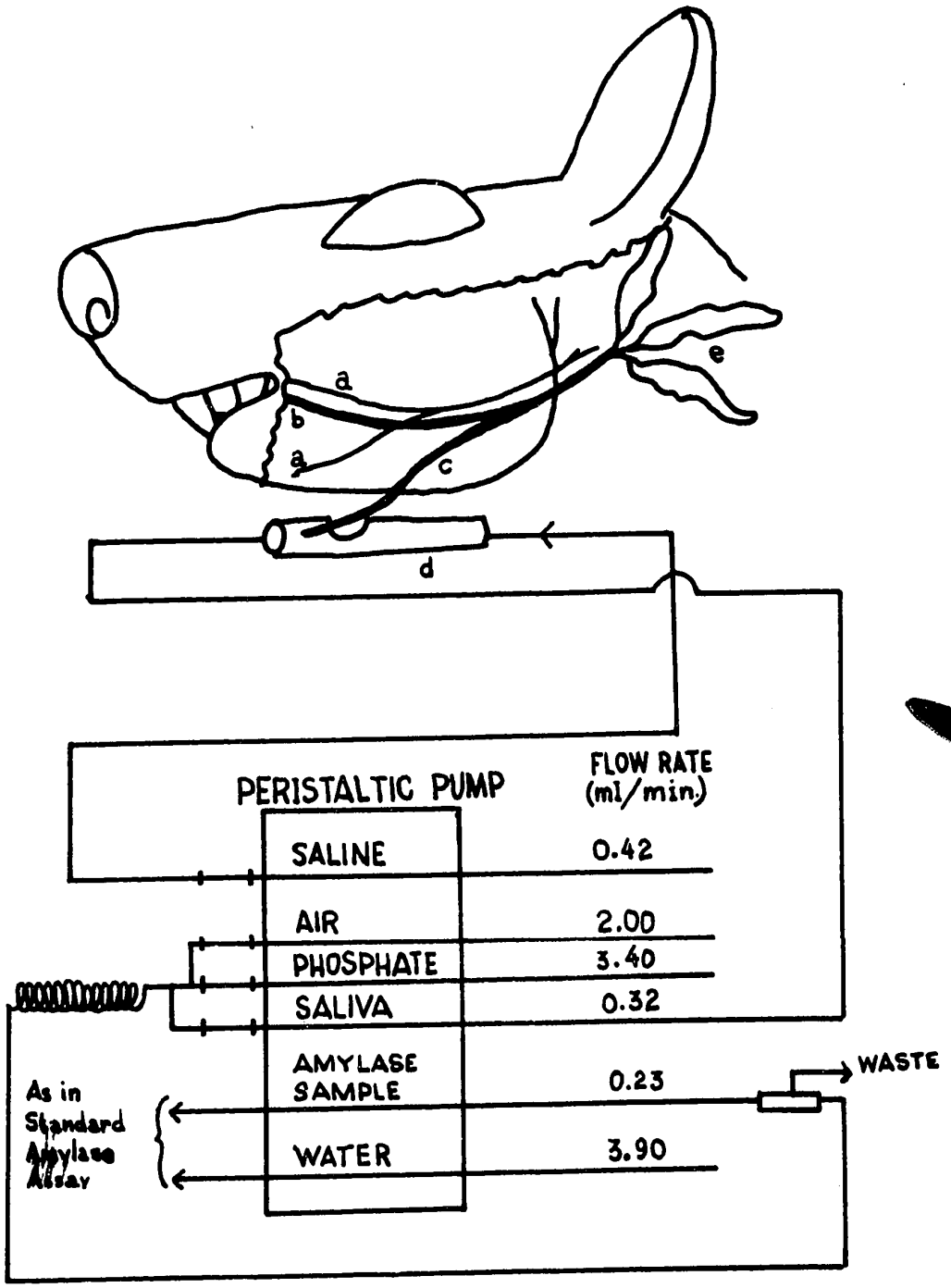


Figure 12 . Schematic diagram of the continuous monitoring of amylase secreted from rat parotid gland. This utilizes essentially the same AutoAnalyzer manifold as the automated amylase assay (figure 9) but modified as indicated: a - facial nerve ; b - parotid duct <u>in situ</u> ; c - parotid duct severed and placed in d -- saliva flow collector ; e - parotid gland.

its body to prevent saliva from flowing back into the lungs.

The parotid duct was carefully cleaned of surrounding tissue and placed in the collector as shown in the diagram (figure 12). The collector was held in place with adhesive tape and attached to the amylase manifold of the AutoAnalyzer.

In order to measure amylase in the range applicable to the previously described automated amylase technique (figure 9), a few changes were made in the manifold. The saliva which was collected by a flow of isotonic saline past the end of the parotid duct was diluted automatically as shown in figure 12. The sample inlet of the amylase manifold continuously pumped a portion of the diluted saliva, and the excess was removed from the debubbler as waste. Sensitivity was further reduced by changing the water tube of the amylase manifold from a flow rate of 2.0 ml/min to 3.9 ml/min.

By continuous sampling of maltose solutions with concentrations between 2 and 40 mg/ml directly into the sample tube (i.e. without automatic dilution), a calibration curve relating optical density to maltose concentration was prepared, similar to that in figure 10. A series of dilutions of saliva were measured and a curve of this type shown in figure 11, (an enzyme activity-concentration curve) was plotted. By continuous sampling of some dyes from both the collector tube and the sampler tube, the dilution factor for the automated diluter was found to be 1:12. On the basis of 1:12 dilution of saliva, sampling rate of 0.23 ml/min and the enzyme concentration-activity

curve, the flow rate of amylase was calculated and plotted against optical density. This calibration curve is shown in figure 13. The flow rate was expressed as units of amylase per 100 seconds because the AutoAnalyzer chart paper is in 100 second divisions. Total amylase secreted was calculated by adding the flow rates for each 100 second interval.

Dissection and Treatment of Salivary Glands

The rats used in all experiments were Sprague-Dawley males. Each rat was stunned by a sharp blow on the head, then bled by puncturing the heart with a scalpel. The salivary glands were rapidly removed and placed in ice-cold isotonic NaCl, or 0.25 M sucrose.

The removal of the entire parotid gland free from associated lymph nodes and adipose tissue was difficult, particularly in animals treated with isoproterenol shortly before killing. Familiarity with the exact shape and position of the parotid gland was necessary because of the difficulty in distinguishing it from surrounding structures.

Removal of the skin from the neck and cheek of the rat reveals many closely associated structures including not only the salivary glands but lachrimal glands, many lymph nodes and adipose tissue (300, 301). The exorbital lachrimal gland,which has occasionally been mistaken for the parotid gland (146), is slightly below and in front of the ear. The parotid gland is less obvious. It consists of three lobes joined together by

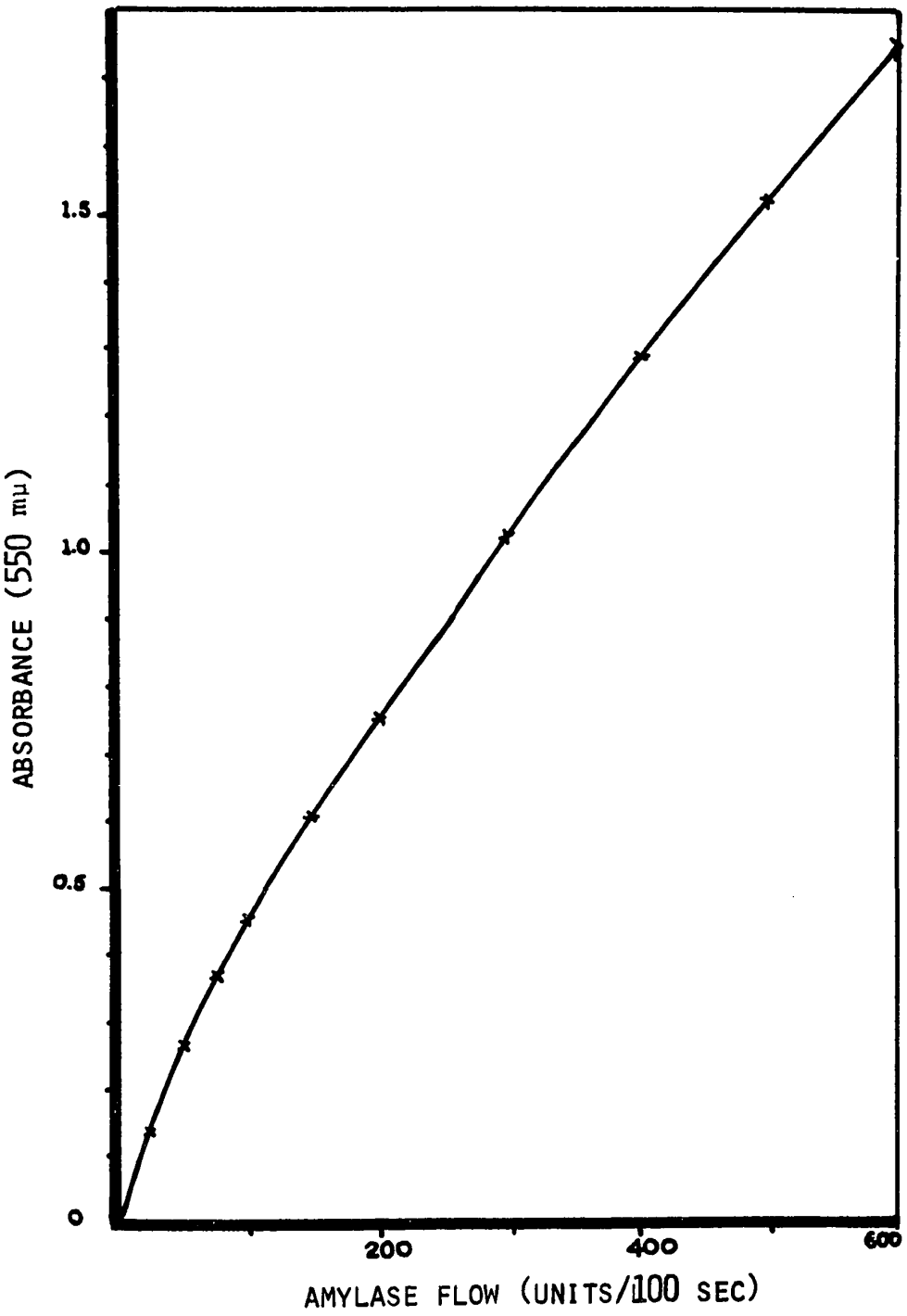


Figure 13 . Calibration curve for the continuous monitoring of amylase secretion.

ducts to a common parotid duct (Stenson's duct) which crosses the cheek to the mouth. The upper lobe is attached to the base of the ear and is partly obscured by the lachrimal gland. It runs along to the posterior edge of the mandible. The central lobe runs from the shoulder to the edge of the cheek. The lower lobe is closely associated with the submaxillary gland and the lymph nodes, and is partly obscured by these structures. The two large submaxillary glands are the most prominent structures. The pair lie together along the midventral line of the neck.

Removal of the salivary glands was accomplished with the aid of blunt forceps, scissors and a bright lamp. The glands were exposed by removal of the skin from the neck and removal of the lachrimal glands. The upper lobe of the parotid gland was teased away from the base of the ear. Removal of some adipose tissue at the base of the neck exposed the central lobe. It was carefully pulled away from the neck towards the cheek. The lower lobe was parted from the edge of the submaxillary gland. Careful lifting of the lower and central lobes enabled the lymph nodes to be pulled away. The three lobes were then lifted and removed by cutting the common parotid duct. Parotid tissue is very weak and is easy to tear in pulling it away from the neck. With young rats, however, it was usually possible to remove the glands without damage. Rats larger than 200 g were much more difficult because connective tissue surrounding the glands was quite tough.

Preparation of Parotid Gland Homogenates

Methods of treating tissues for release of amylase activity have generally involved pulverizing the tissue in isotonic sucrose (149), isotonic NaCl (132), or hypotonic solutions (139). It has been suggested that membrane-bound amylase must be released by treatment with detergent or with 0.2 M tris, pH 8.0 (302). Virtually all the amylase of zymogen granules was released by suspension in hypotonic solution or treatment with triton X-100 (172). Repeated freezing and thawing has also been suggested to release bound amylase (303).

In order to determine the conditions of homogenization optimal to the determination of amylase in rat parotid glands, three experiments were done. In each experiment, a group of rats were killed, the parotid glands excised and finely minced. Aliquots of the mince were weighed and homogenized in one of the following solutions:

1. Isotonic sucrose - 0.25 M sucrose
2. Isotonic saline - 0.9% NaCl
3. Distilled water
4. Phosphate buffer - 0.02 M phosphate, 0.007 M NaCl pH 6.9
5. Detergent - 0.025% Triton X-100 in phosphate buffer
6. Tris buffer - 0.2 M tris pH 8.0

In Experiment I, glands from two rats were used and a homogenate was made with each of the six solutions. In Experiment II, the minced glands from three rats were divided into eight portions and two homogenates were prepared with each of solutions

2, 3, 5 and 6. Two rats were used in Experiment III and three homogenates each were prepared with solutions 5 and 6.

After thorough homogenization with a manual tissue grinder, each sample was centrifuged at 2000 X g for ten minutes. The supernatant was then assayed for protein and amylase.

The results of the three experiments are summarized in table 2. Triton X-100 and tris buffer were more effective in releasing amylase than the other solutions used for homogenization. Experiments II and III indicated that there was very little difference in the total amylase released by these two media but there was much less variation with Triton X-100. There was also much less protein released with Triton X-100 than the tris buffer. This is probably an advantage when amylase must be isolated since it has a higher specific activity to start with.

Stability of Amylase in Parotid Glands and Homogenates

Effect of Freezing

Three rats were starved overnight and killed in the morning. The parotid glands were removed. One gland from each rat was immediately frozen and the other was placed in ice-cold isotonic saline. Both were stored 24 hours (frozen or refrigerated). Amylase activity in each gland was determined after homogenization in a buffer solution containing 0.02 M phosphate, 0.007 M NaCl, 0.05% Triton X-100 at pH 6.9. The results are shown in

TABLE 2

Effect of homogenizing medium on amylase and protein in parotid glands.

		HOMOGENIZING MEDIUM					
		1	2	3	4	5	6
	Experiment	Isotonic Sucrose	Isotonic Saline	Water	Phosphate Buffer	Triton X-100	Tris Buffer
AMYLASE	I	16	20	20	20	25	26
	II	-	18.6±0.2	22.4±0.1	-	21.8±0.1	22.4±2.1
	III	-	-	-	-	20.1±1.0	20.2±2.5
PROTEIN	I	10.5	6.5	9.5	10.5	11.5	33
	II	-	10.5±0.9	15.3±0.9	-	12.3±0.6	28.3±1.2
	III	-	-	-	-	16.0±1.2	32.2±1.5

Amylase is expressed in units/mg tissue. Protein is expressed as mg/100 g tissue.

table 3. In three cases, the amylase activity of frozen glands was 24, 35 and 53% lower than glands which were refrigerated for 24 hours.

Storage of Glands and Homogenates

A series of experiments were done to determine the stability of parotid amylase activity to storage of the glands or homogenates in the refrigerator. Parotid glands from four rats were homogenized in 0.02 M phosphate pH 6.9 containing 0.05% Triton X-100. After centrifugation (2000 X g for 10 minutes) an aliquot of the supernatant was taken for amylase determination. The homogenates were refrigerated for three days, after which amylase was again determined. The results in table 4 indicated that the homogenates could be stored several days without much change in amylase activity.

The effect of storage of the whole parotid gland was determined in the following way: Parotid glands were removed from rats and immediately placed in 1 ml ice-cold isotonic saline. The glands were stored refrigerated for various times after which they were homogenized and amylase determined. The results, shown in table 5, show the effects of storage for one hour, four hours, 18 hours and three days. In most cases, each of the pair of glands in a rat was stored for a different time. The isotonic saline in which the glands had been stored were also assayed for amylase. The two glands of rat number 1 were both homogenized and assayed within one hour. In that time, very little amylase

TABLE 3

Effect of freezing on the amylase activity of parotid glands.

Rat Number	AMYLASE ACTIVITY* Stored † Refrigerated	Stored† Frozen	% Difference
1	9910	4650	53
2	7500	4850	35
3	12880	9760	24

* Total amylase units (mg/maltose/3 min at 37°) in the whole parotid gland.

† Parotid glands were stored 24 hours either frozen or at 4°.

TABLE 4

Effect of storage on amylase activity of parotid gland homogenates

Rat Number	AMYLASE ACTIVITY* Control	Stored 3 days	% Change
1	17750	16875	-5
2	16750	16250	-3
3	11500	11875	+3
4	16500	16875	+3

* Total amylase units (mg/maltose/3 min at 37°) in the whole parotid gland.

TABLE 5

Effect of storage time on amylase activity in parotid glands. Activity is expressed as total units in each gland or in the isotonic saline in which the gland was stored.

Rat Number	AMYLASE ACTIVITY*						
	In Parotid				In Saline	Total	% difference between pairs of glands
	Storage Time:						
	1 hr	4 hr	18 hr	3 days			
1	17750	-	-	-	200	17950	6
	16750	-	-	-	220	16970	
2	11500	-	-	-	165	11665	2
	-	11750	-	-	180	11930	
3	13250	-	-	-	180	13430	4
	-	-	13500	-	550	14050	
4	490	-	-	-	86	576	5
	-	-	310	-	234	544	
5	570	-	-	-	142	712	6
	-	-	-	460	296	756	
6	-	16000	-	-	130	16130	14
	-	-	-	10750	3120	13870	

* Total units per gland or in the saline solution, mg/maltose/ 3 min at 37°.

had escaped into the isotonic saline. The total amylase of the two glands was fairly close (6% difference). For rats 2, 3 and 4, storage times of 4 hours and 18 hours resulted in very little difference in total amylase (less than 6%). There is an indication, however, that with storage for 18 hours, there is increased amylase in the isotonic saline. For longer storage time, there may be a decrease in total amylase. The 14% difference in storing three days for rat number 6 may be due to either loss of amylase activity or it may be due to normal differences found between pairs of glands. Rat number 5, which was depleted of amylase by isoproterenol, showed no decrease in amylase on storage for three days.

The results shown in tables 3, 4 and 5 indicated that parotid glands should not be stored frozen, but could be stored refrigerated several hours without loss in amylase activity. There may be some loss in activity when stored for longer than 18 hours. When homogenates are prepared immediately, they can be stored for three days without loss of activity.

Isolation of Amylase

Amylase was isolated from parotid homogenates by precipitation of amylase-glycogen complexes as described by Schramm and Loyter (71, 304).

Reagents

Glycogen reagent: A 2% solution of shellfish glycogen (Mann Research Chemicals) was centrifuged at 10,000 x g

for ten minutes. The supernatant was autoclaved and could be kept in the refrigerator for up to four months.

Procedure

All operations were below 4°. Parotid gland homogenates were centrifuged at 10,000x g for ten minutes. Ethanol was added to the clear supernatant to give a final concentration of 40%. The mixture was centrifuged at 10,000 x g for 20 minutes. The precipitate was discarded and an aliquot of the supernatant was transferred to a test tube. The reagents were added with mixing in the following order: 0.05 ml 0.2 M phosphate buffer pH 8.0, for each ml of supernatant, 0.1 ml glycogen reagent (2 mg glycogen) for each 1000 units of amylase activity and 95% ethanol to readjust the concentration to 40%. The mixture was shaken five minutes and then centrifuged at 2000 g for six minutes. The precipitate containing amylase was resuspended with the help of a Teflon-coated homogenizer pestle in 40% ethanol containing 0.01 M phosphate buffer pH 8.0 (a volume equal to that of the 40% ethanol supernatant). The centrifugation was repeated, followed by another washing with 40% ethanol and another centrifugation.

The final precipitate, a fine white solid, contained amylase and glycogen. The precipitate was suspended in aqueous buffer and on standing at room temperature one hour, completely dissolved. (The glycogen was hydrolyzed to maltose, oligoglucosides and dextrans). Aliquots were taken for amylase, and protein

assays as well as for determination of radioactive incorporation in some experiments.

In most cases, where amylase was isolated from homogenates of parotid gland, the recovery of amylase activity in the purified enzyme was 90-95%. Specific activity was between 2700 and 3400 units/mg protein.

Protein: Automated Biuret Method

For the determination of protein in parotid gland homogenates, the automated biuret method of Lane and Mavrides (305) was slightly modified to increase sensitivity.

Reagents

Biuret reagent: Three g of $CuSO_4$ and 12 g of sodium potassium tartrate were dissolved in 500 ml, 200 ml of NaOH were added, the volume was made up to 1 liter, and a few drops of triton X-100 were added.

Protein standards: Fraction V bovine serum albumin (Nutritional Biochemicals) was dissolved in water to a final concentration, between 2 to 20 mg/ml, and stored frozen in sample cups until needed.

Procedure

A schematic diagram of the system is shown in figure 14. The difference from the method of Lane and Mavrides is in the sample flow rate and the number of samples per hour. Since the homogenates to be analyzed had protein concentrations

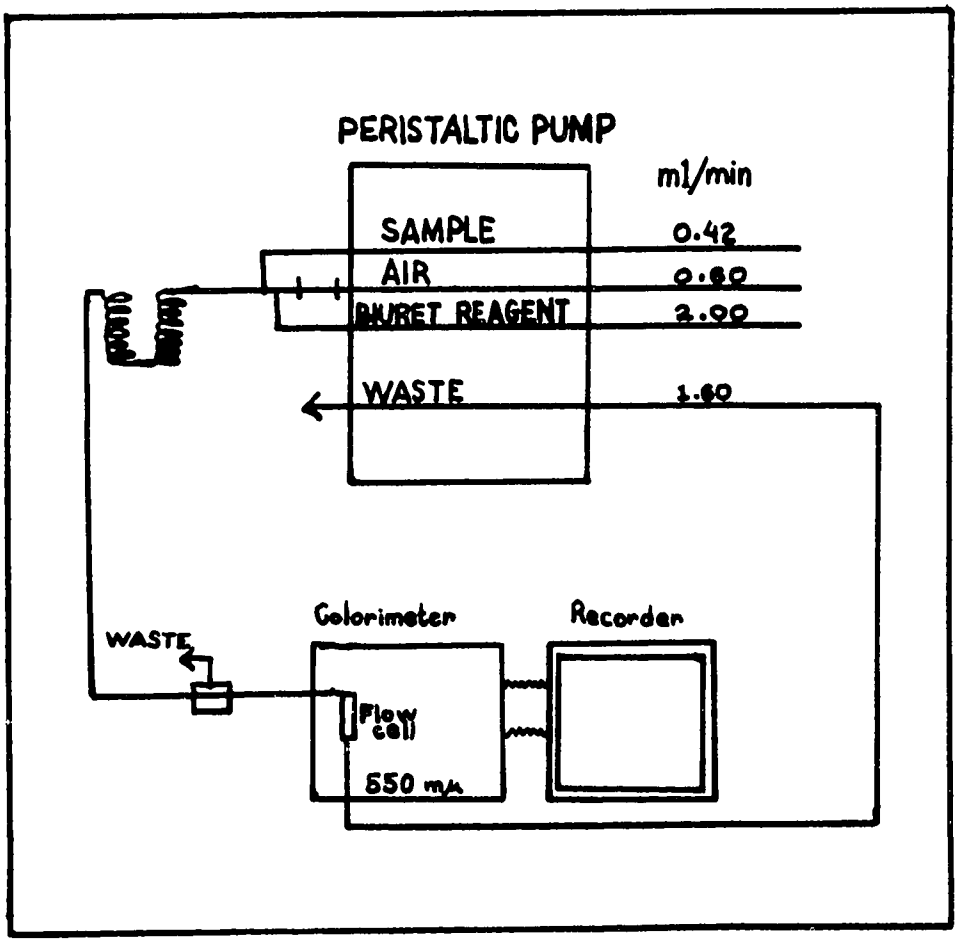


Figure 14 . Schematic diagram of the automated biuret method.

below 2 mg/ml in many cases the sensitivity had to be increased. The sample flow was therefore increased from 0.32 ml/min to 0.42 ml/min and the sampler was set at 40 samples per hour rather than 60 per hour. This resulted in an increased slope of the standard curve in figure 15. The increase in sensitivity was obtained at the expense of the volume of sample required. This was of no consequence in our case since the original homogenate was 10 ml and 1 ml was sufficient for this modified biuret method.

Sources of Interference

Some expected contaminants in the protein assay were tested for interference in the biuret method. The concentrations of protein, ethanol, homogenizing buffer and maltose shown in table 6 were prepared and assayed with the automated biuret method. In each case, a slight interference was found. In cases in which protein was assayed in the crude parotid homogenate only, the phosphate buffer would interfere (an increase of 4% in the apparent protein level). In each case where protein was determined in the crude homogenate, the buffer was the same and the slight error would be constant from sample to sample. Triton X-100 did not interfere in this method.

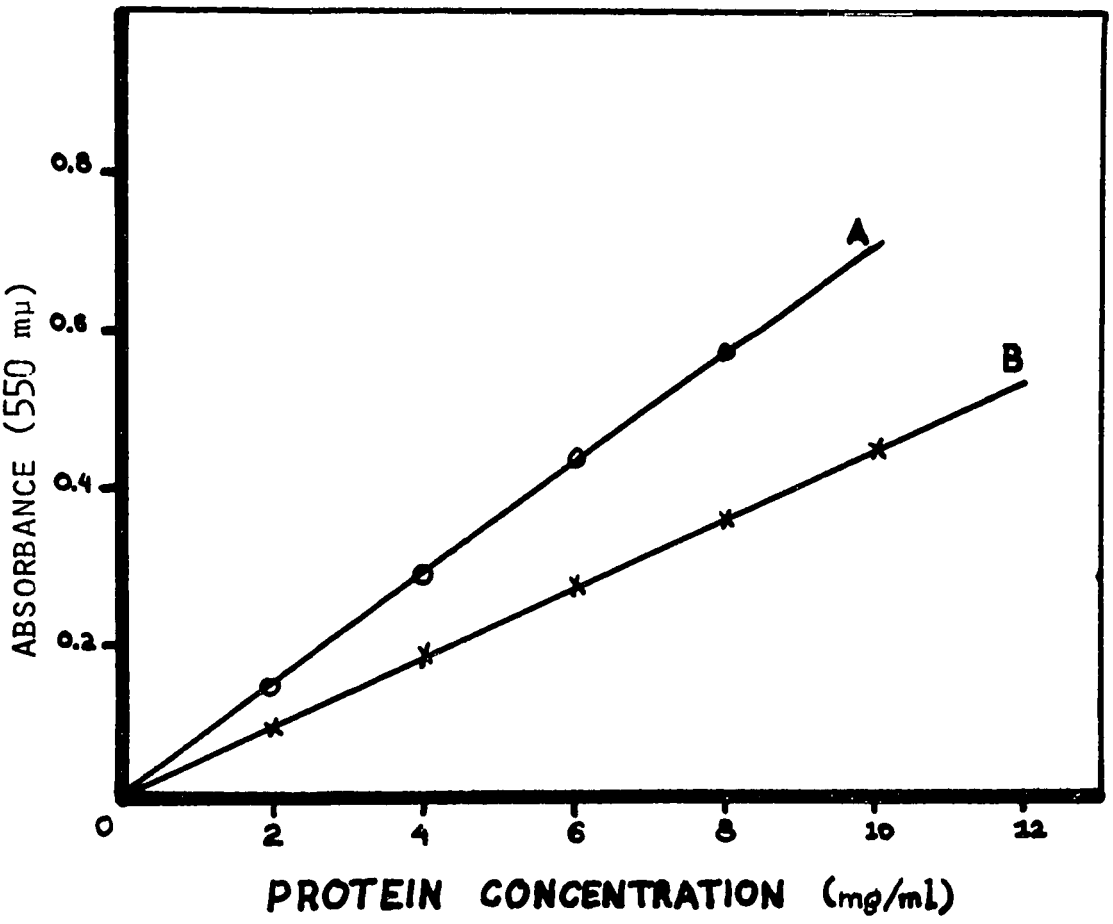


Figure 15. Calibration curves for the automated biuret method. The curves represent two different sample rates : A - 0.42 ml/min ; B - 0.32 ml/min.

TABLE 6

Effect of various substances on the determination of protein by the automated biuret method.

Sample Composition		Biuret Protein (Mg/ml)	Increase due to interference
Protein (mg/ml)	Contaminant		
5.0	20% Ethanol	5.1	2 %
5.0	0.01 M PO_4, pH 6.9	5.2	4 %
5.0	5 mg/ml Maltose	5.3	6 %

Protein: Automated Lowry Method

In the experiments where amylase was isolated from crude homogenates, the concentration of protein in the crude homogenate was just sufficient for determination with the automated biuret method. The isolated amylase was at a very much lower protein concentration. Since the method described by Lowry et al (306) is much more sensitive than the Biuret method, we decided to automate the Lowry method in order to combine great sensitivity with rapidity and convenience. Schuel et al (307) attempted to automate the Lowry method. To avoid interference from sucrose, they did not use the $CuSO_4$ reagent and their method was essentially a measurement of tyrosine and tryptophan residues. Other methods suffered from interaction between samples, or lower sensitivity (308, 309).

We have devised an automated Lowry method which is fifty times more sensitive than the automated biuret (305) and ten times more sensitive than Schuel's method (307).

Reagents

All reagents were essentially those described by Lowry (306) but slightly modified. They were all stored at room temperature. Standard protein solutions were stored frozen in 2 ml sample cups.

Alkaline carbonate solution: 2% Na_2CO_3 in 0.1 N NaOH (or 2% Na_2CO_3 in water for samples dissolved in 0.5 N NaOH).

Copper sulfate solution: 0.5% $CuSO_4.5\ H_2O$ in 1% sodium potassium tartrate, stabilized at pH 6.0.

Folin reagent: Commercially prepared 2 N Folin Phenol reagent was diluted to 0.5 N.

Protein standards: Standards were made with fraction V bovine serum albumin (Sigma Chemical Co.) in concentrations of 20 µg/ml to 500 µg/ml in distilled water or in 0.5 N NaOH.

Procedure

Protein samples were determined automatically using the ordinary modules of the Technicon AutoAnalyzer including sampler, proportioning pump, mixing coils, colorimeter with 660 mµ filters, in the proportions indicated in figure 16. The mixing jets were 8 cm lengths of glass transmission tubing with 4 constrictions formed by softening the glass in a flame. The turbulence caused by the liquid being forced through the constrictions resulted in very rapid mixing of reagents.

Discussion

As with the manual method (306), the automated standard curve in figure 17 was nonlinear but reproducible. Lowry pointed out that poorly soluble samples could be dissolved in 0.5 N NaOH and assayed by using Na_2CO_3 reagent without NaOH. This is possible with the automated method since the standard curve was the same when the protein standards in 0.5 N NaOH were measured using 2% Na_2CO_3 in water.

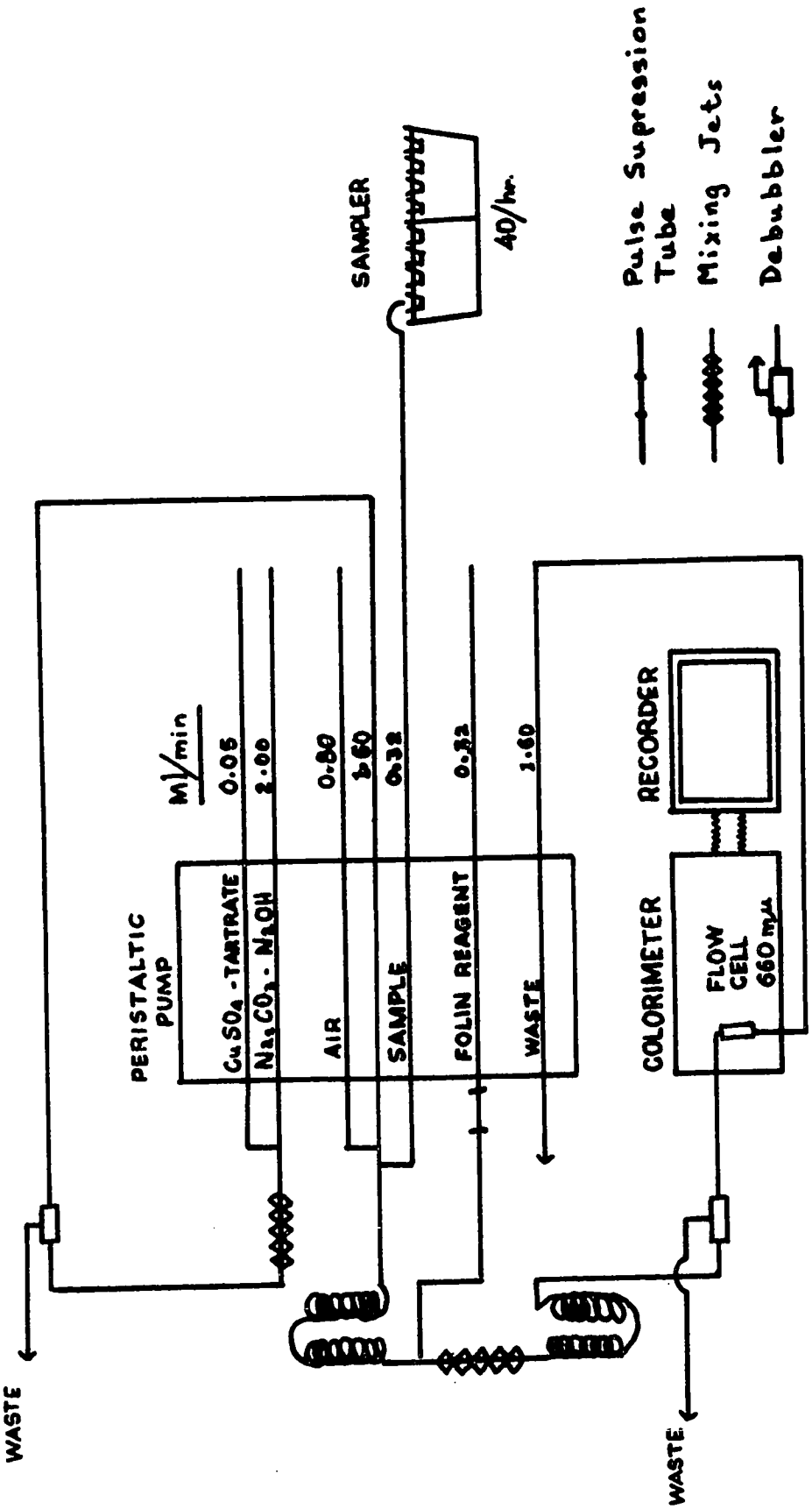


Figure 16 . Schematic flow diagram for the automated Lowry protein assay.

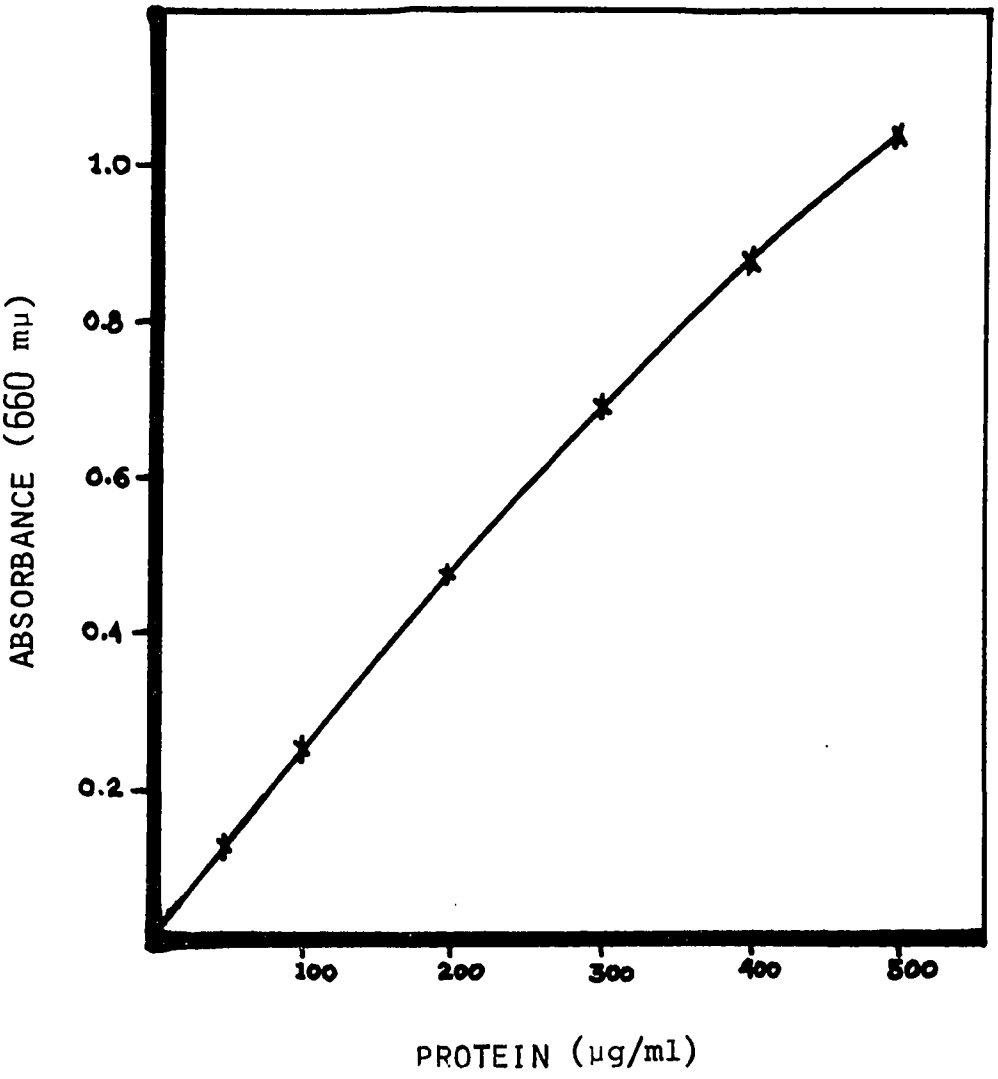


Figure 17 . Calibration curve for protein by the automated Lowry method. Protein concentration is in μg/ml of bovine serum albumin.

The automatic mixing of carbonate and $CuSO_4$ solutions was convenient. The separate solutions were stable for many weeks but the mixture was stable only for hours. When a large number of samples were determined, a premixed $CuSO_4$ carbonate solution changed with time, thus affecting the baseline. With automatic mixing, the baseline was steady for four hours.

In order to define the reliability (310) of this method, some of the kinetic parameters of continuous flow described by Thiers et al (311) are given.

By making the delay time (311) for color development fairly short, interaction between samples due to laminar flow was minimized. The total delay time, i.e., the time for sample to reach the colorimeter after entering the reagent stream, was 5.5 minutes. This included 2.8 minutes during which protein reacted with the alkaline copper reagent and 2.7 minutes for development of color with the Folin reagent. Little sensitivity was lost by using a short delay time since 75% of the color development took place within one minute of addition of the Folin reagent (306). The short delay time resulted in a half-wash time (311) of ten seconds. Consequently, with the sampler set at 40 samples per hour (60 second aspiration of sample with 30 seconds of air between samples), there was only 1.1% interaction between samples. For accurate estimate of protein, 1.1% of the previous sample value must be subtracted

from the uncorrected estimate. During the sampling time, 99% of the sample steady state (311) was reached. Consequently, the slight variation of sampling time due to different sample volumes did not affect the protein estimates and allowed sample volumes as low as 0.4 ml.

Precision, as defined by Anastassiadis and Common (310), was measured by determining the standard deviation of ten determinations at each of several protein concentrations. The standard deviation was 0.4% at 500 µg/ml, 0.5% at 100 µg/ml, 1% at 50 µg/ml, 2% at 25 µg/ml and 15% at 5 µg/ml. Differences of 1 µg/ml could be distinguished, which means that sensitivity (310) was 1 µg/ml.

Interference

Several substances have been reported to interfere in the determination of protein by the Lowry method (313 - 315). Therefore, some expected contaminants were tested for interference in the automated Lowry method. The contaminants, listed in table 7, are normally present in the protein assay after isolation of amylase by the method of Schramm and Loyter (71). Of these, the buffer, glycogen and maltose had little or no effect on the assay by the automated Lowry method. In almost all routine analyses, the concentration of buffer, glycogen and reducing sugar would have been lower than in table 7 because most samples were diluted to bring protein concentration into the range of this analysis. A definite

TABLE 7

The effect of expected contaminants on the determination of protein by the automated Lowry method.

Composition of Assay Mixture		Lowry	
Protein µg/ml	Contaminant	Protein µg/ml	% Decrease Due to Interference
150	Ethanol, 20%	139	7.5
150	Buffer*	148	1.5
150	Maltose, 0.5 mg/ml	149	1.0
150	Glycogen, 1 mg/ml	147	2.0
250	Ethanol, 10%	242	3.2
250	" 20%	239	4.4
250	" 30%	233	6.8
200	" 20%	192	4.5
200	" 40%	184	7.6
500	" 20%	475	4.9
500	" 40%	450	9.8

* 0.01 M phosphate buffer, pH 6.9

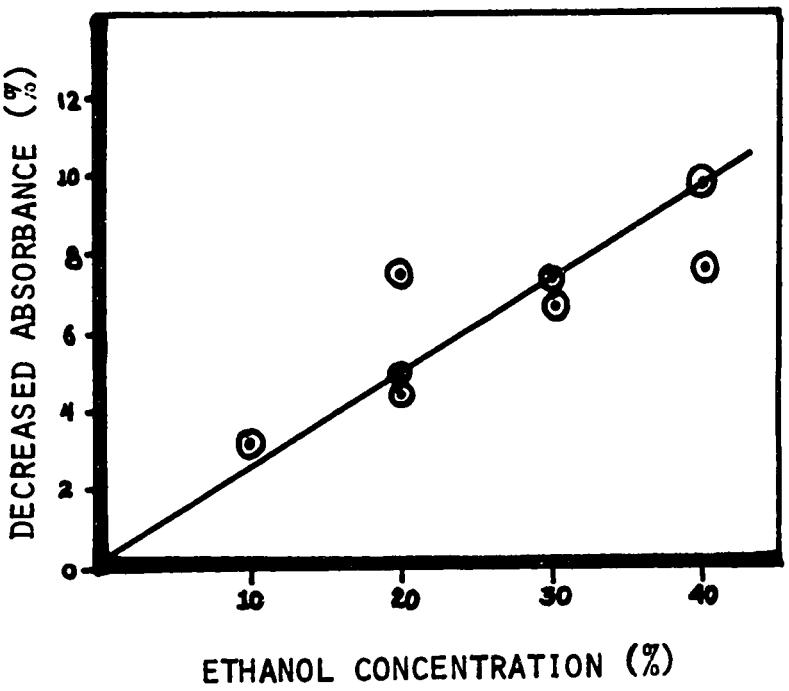


Figure 18 . Interference of ethanol in the automated Lowry method. Interference is expressed as % decrease in absorbance at 660 mμ of bovine serum albumin dissolved in varying concentrations of ethanol.

interference by ethanol was found. It was approximately a 5% reduction with 20% ethanol and 10% reduction with 40% ethanol (figure 18). In the few cases where samples contained significant concentrations of alcohol, the appropriate adjustment was used to correct the protein values.

Triton X-100, commonly used to improve the flow in automated methods, caused precipitation of the alkaline copper reagent. A good flow pattern was ensured by rinsing all lines with a Triton X-100 solution followed by distilled water before each run.

Monoamine Oxidase (MAO)

Simple spectrophotometric methods such as those based on oxidation of benzylamine (316) and kynuramine (317) were not sensitive enough for the determination of MAO in salivary glands. A sensitive radioisotopic assay of MAO, described by Otsuka and Kobayashi (318) was used in our preliminary experiments. Because of the possibility of interference of aldehyde dehydrogenase (319) in this method, we subsequently used the fluorometric method of Kraml (320). This method, based on the oxidation of kynuramine and immediate cyclization to the fluorescent 4-hydroxyquinoline (317), has been modified by Drujan and Diaz-Borges (321) and Century and Rupp (322).

Method A: Radioassay of MAO

The method of Otsuka and Kobayashi (318) was adapted as follows to smaller volumes.

Reagents

Substrate: 1-^{14}C-tyramine hydrobromide (New England Nuclear) and unlabeled tyramine hydrochloride, 1 μM; 10^6 DPM/ml. This solution was stored frozen between assays.

Buffer: 0.3 M phosphate, pH 7.5.

Procedure

The incubation mixture was prepared by mixing 0.1 ml each of substrate, sample solution, and buffer in the bottom of 5 ml centrifuge tubes. The tubes were incubated for 30 minutes in a shaking water bath at 37°. The reaction was stopped by addition of 0.1 ml of a 2 M citric acid solution. This acidification allows the selective extraction of the products into anisole. Anisole, (2.5 ml) was shaken with the acidified reaction mixture, centrifuged, frozen, and the anisole poured off into scintillation vials. This extraction procedure was repeated once more and 10 ml of standard scintillation fluid, 0.4% Omnifluor (New England Nuclear) in toluene, was added to the anisole extracts. Two drops of NCS solubilizer (New England Nuclear) were added to clear the solution. Counting was done in a Mark 1 liquid scintillation counter (Nuclear Chicago) operated at the balance point for C^{14}. Two channels were monitored in order to detect quenching effects. Quenching was low, and did not vary from sample to sample.

Units of MAO activity were expressed as CPM released after 30 minutes at 37°. On the basis of extraction efficiency

of about 80% (318), counting efficiency of 72%, and substrate specific activity of 1000 DPM/mμmole, 1 unit as described here is approximately equivalent to 3.5 mμM/hr at 37°.

Under the conditions described above, enzyme activity was proportional to enzyme concentration up to 30,000 CPM/ 30 min/sample (figure 19).

Method B: Fluorometric Assay of MAO

Kraml's method (320) was modified as suggested by Drujan and Diaz-Borges (321) and Century and Rupp (322) and adapted to small volumes as described below.

Reagents

All reagents were prepared by using glass redistilled water.

Substrate: kynuramine dihydrobromide, (Regis Chemical Company) 400 μg/ml.

Buffer: 0.2 M phosphate, pH 7.4

PCA solution: 0.6 M perchloric acid

Standards: 4-hydroxyquinoline trihydrate (Koch-Light Laboratories) in concentrations of 0.2 to 20 mμmole/ml These were stable when refrigerated.

Procedure

The incubation mixture was prepared by mixing 0.5 ml of sample, 1 ml of buffer and 0.5 ml of substrate in 5 ml centrifuge tubes on ice. Sample blanks were prepared by substituting

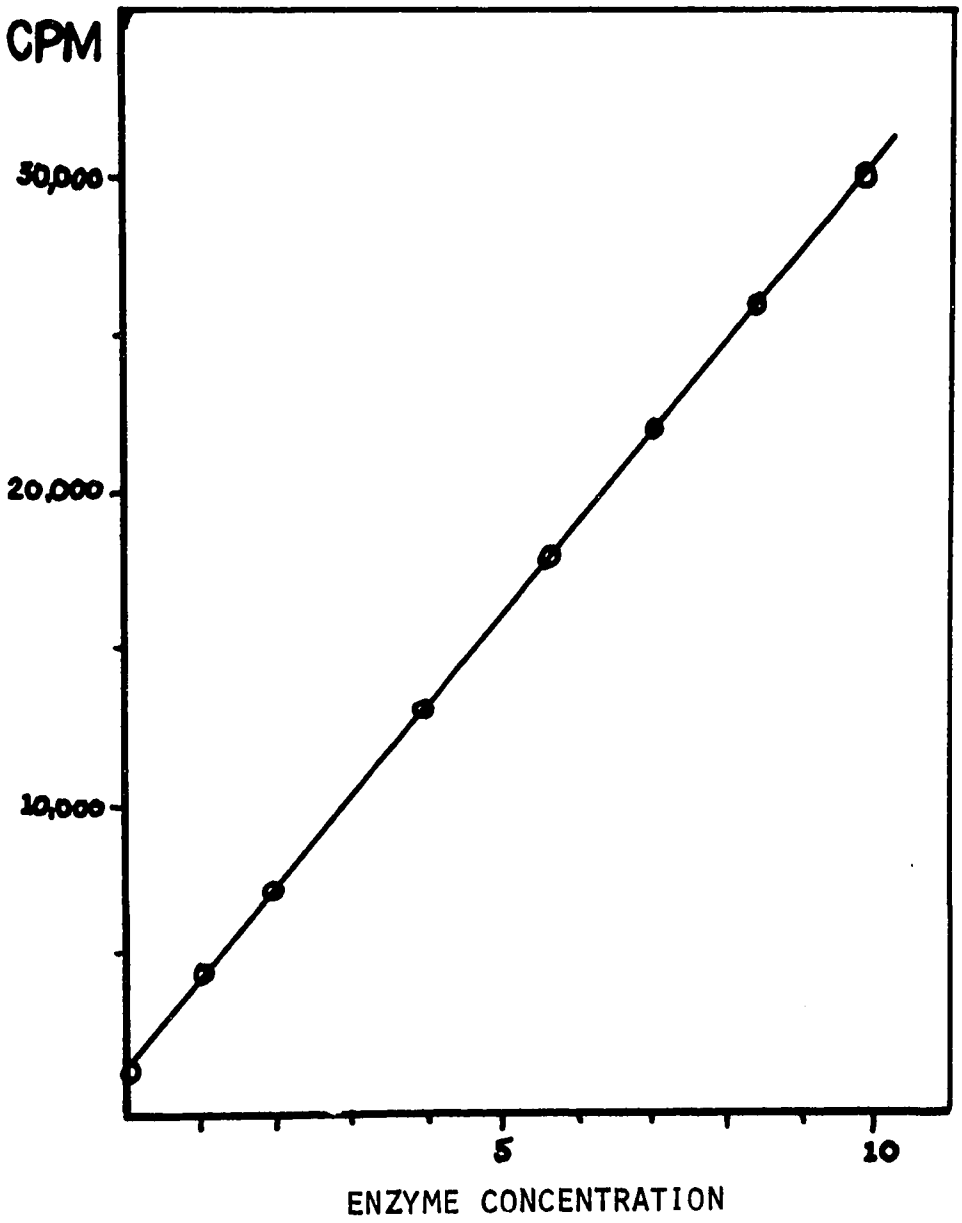


Figure 19 . Calibration curve for the radio-assay of MAO with ^{14}C-tyramine as substrate. Activity is expressed as CPM released in 30 minutes at 37^{o}. No blank was subtracted. The various concentrations of enzyme were prepared by dilution of a liver mitochondrial fraction. Enzyme concentration is expressed in arbitrary units.

water for substrate solution. The tubes were incubated for 30 minutes in a shaking water bath at 37°. The reaction was stopped by transferring the tubes to ice. Two ml of perchloric acid solution was added to precipitate protein and the tubes were centrifuged. One ml of supernatant was mixed with 2 ml of 1 N sodium hydroxide. Fluorescence of the basic solutions was measured in an Aminco-Bowman Spectrofluorophotometer at 388 mμ. A standard curve was prepared by treating 1.0 ml each of a series of solutions of the product, 4-hydroxyquinoline, with 2 ml of 1 N sodium hydroxide and measuring the fluorescence as described above. A typical standard curve is shown in figure 20. The sample blank was subtracted from each sample reading and from the standard curve, the enzyme activity was read in mμM 4-hydroxyquinoline/ml incubation mixture.

Under the conditions described above, enzyme activity was proportional to enzyme concentration over a wide range (figure 21).

Cytochrome Oxidase

Cytochrome oxidase activity was measured in early experiments by an adaption of the method of Cooperstein and Lazarow (323), as described in Method A. Several changes were required for the assay of cytochrome oxidase of salivary glands. Method B describes the modified procedure.

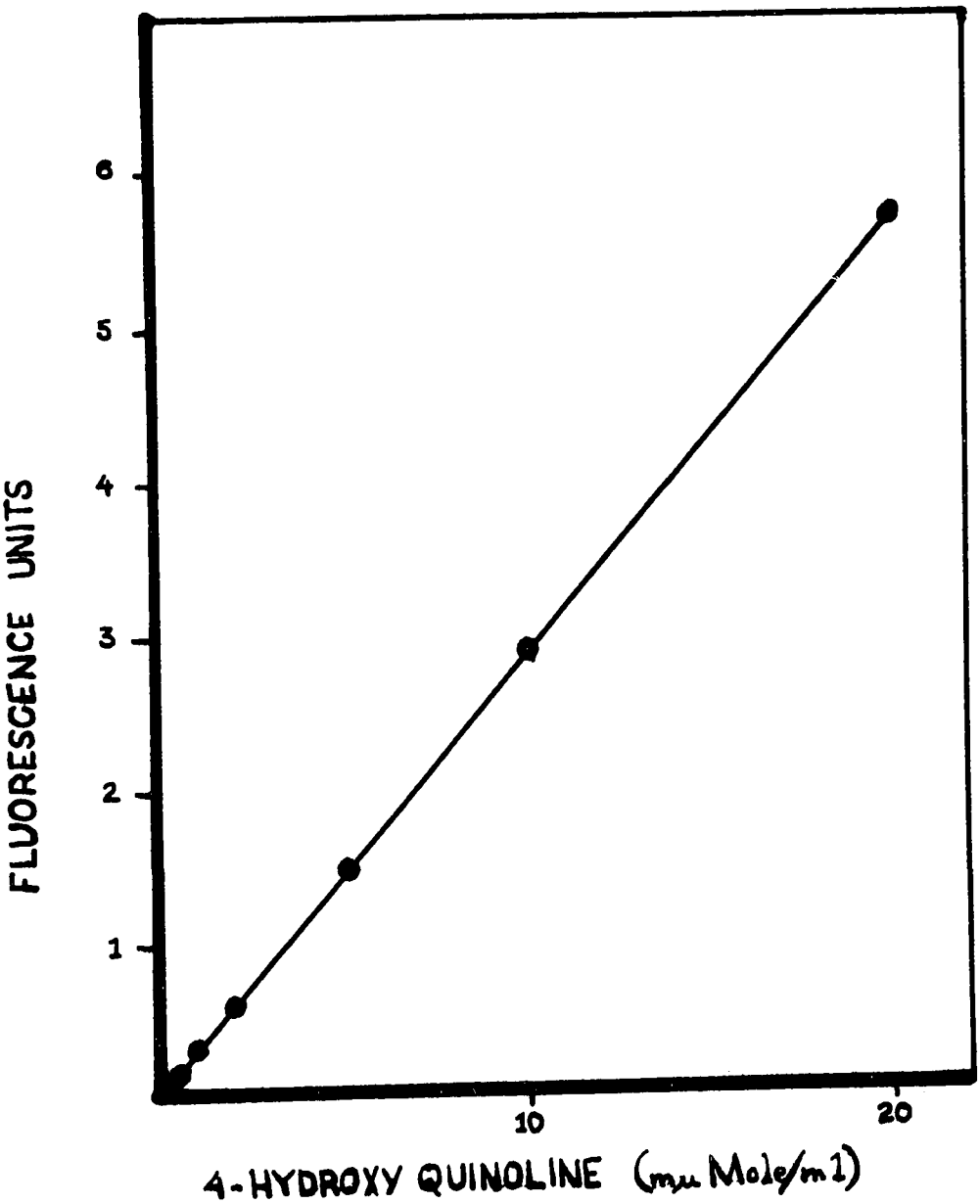


Figure 20 . Calibration curve for the fluorimetric detection of MAO. Enzyme activity is expressed in terms of mμMole/ml of 4-hydroxyquinoline released in 30 minutes at 37°. No blank was subtracted.

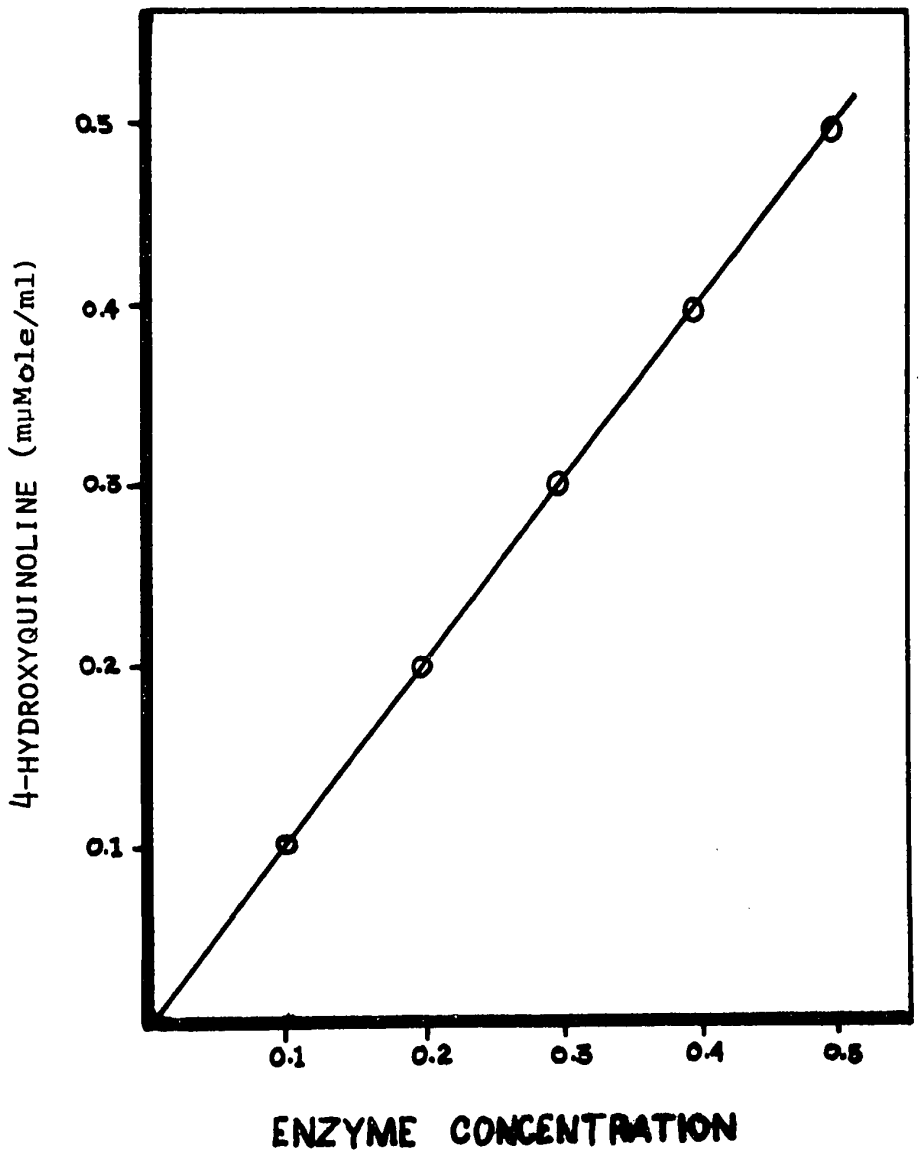


Figure 21 . Fluorimetric detection of MAO: linearity of MAO activity with enzyme concentration. Activity was expressed as mμMole/ml of 4-hydroxyquinoline released in 30 minutes at 37°. Enzyme concentration is expressed as ml of a 10% rat liver homogenate.

Method A: Cytochrome Oxidase, Cooperstein and Lazarow (323)

Reagents

Stock substrate solution: This was prepared by dissolving 35 mg horse heart cytochrome c (Type III, Sigma Chemical Co.) in 100 ml 0.03 M phosphate buffer pH 7.4. It could be stored in a dark bottle under refrigeration for several weeks.

Procedure

Reduced cytochrome c solution was prepared at the start of each day by treating 30 ml stock substrate with 25 µl of fresh 1.2 M sodium hydrosulfite solution. The mixture was shaken to oxidize the excess sodium hydrosulfite. To test for complete oxidation of hydrosulfite, an aliquot was used to assay a sample of cytochrome oxidase. Shaking the substrate solution in air was repeated until further shaking produced no change in the rate of oxidation of cytochrome c by the sample.

Incubation took place in 1.5 ml water-jacketed cuvettes in the automatic sample changer of the Bausch and Lomb Spectronic 505 spectrophotometer. The incubation temperature was maintained at 25° by circulating water from a constant temperature bath (Heto: Berkerod Denmark). After addition of 1.3 ml of reduced substrate solution to each of the sample cuvettes, 5 to 100 µl of sample solution was added to the first cuvette and the absorbance at 550 mµ was recorded 1 to 5 minutes on a constant

speed recorder (linear/Log Varicord #43, Photovolt Corp. N.Y.). The next cuvette was placed in the light path, sample added, and the absorbance recorded. After four samples had been assayed in this way, a few crystals of $K_3Fe(CN)_6$ were added to each sample cuvette to completely oxidize the cytochrome c. The absorbance of each was the sample blank.

To calculate cytochrome oxidase activity, the sample blank was subtracted from the sample absorbance at any given reaction time, and the logarithm of this difference, log D, was found. Cytochrome oxidase activity was calculated from the following formula:

$$\text{Enzyme Activity} = \frac{\log D_1 - \log D_2}{t_1 - t_2}$$

where t is time after initiation of the reaction in minutes.

Discussion

Under these conditions, the reaction was first order. Therefore, the logarithm of cytochrome c absorbance decreased linearly with time (figure 22). The rate of oxidation of cytochrome c was proportional to enzyme concentration over a fairly wide range (figure 23). This was true, however, only during continuous measurement of absorbance. When absorbance was measured intermittantly, (with the solution in the dark between measurements) the oxidation of cytochrome c was very much slower (figure 22).

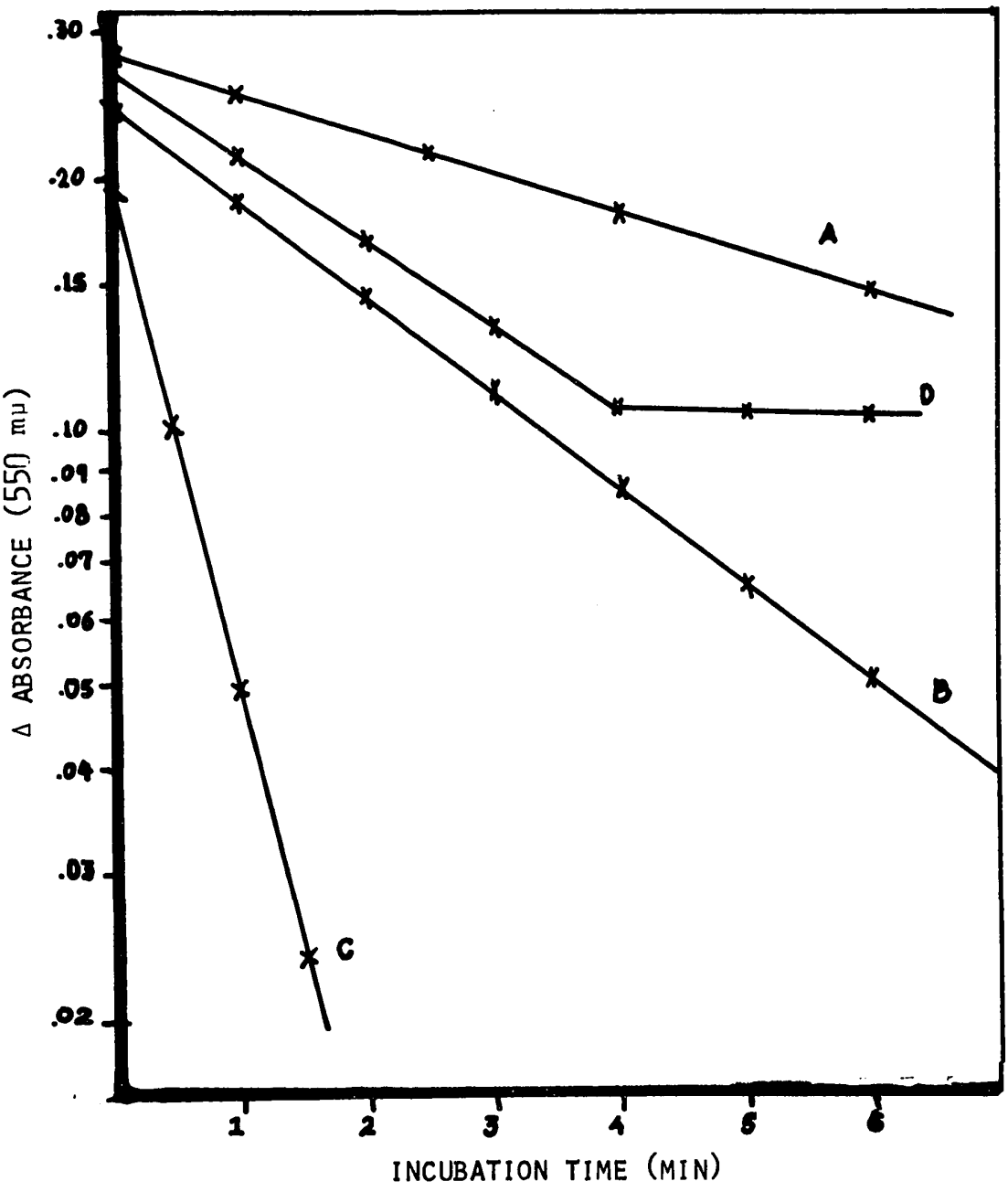


Figure 22 . Cytochrome oxidase activity : Change in cytochrome c absorbance with time. Samples A, B and C contained 10, 25 and 100 μl of a submaxillary gland homogenate, respectively. Sample D (25 μl) was monitored for 4 minutes then between 4 and 6 minutes it was kept in the dark except briefly to measure absorbance.

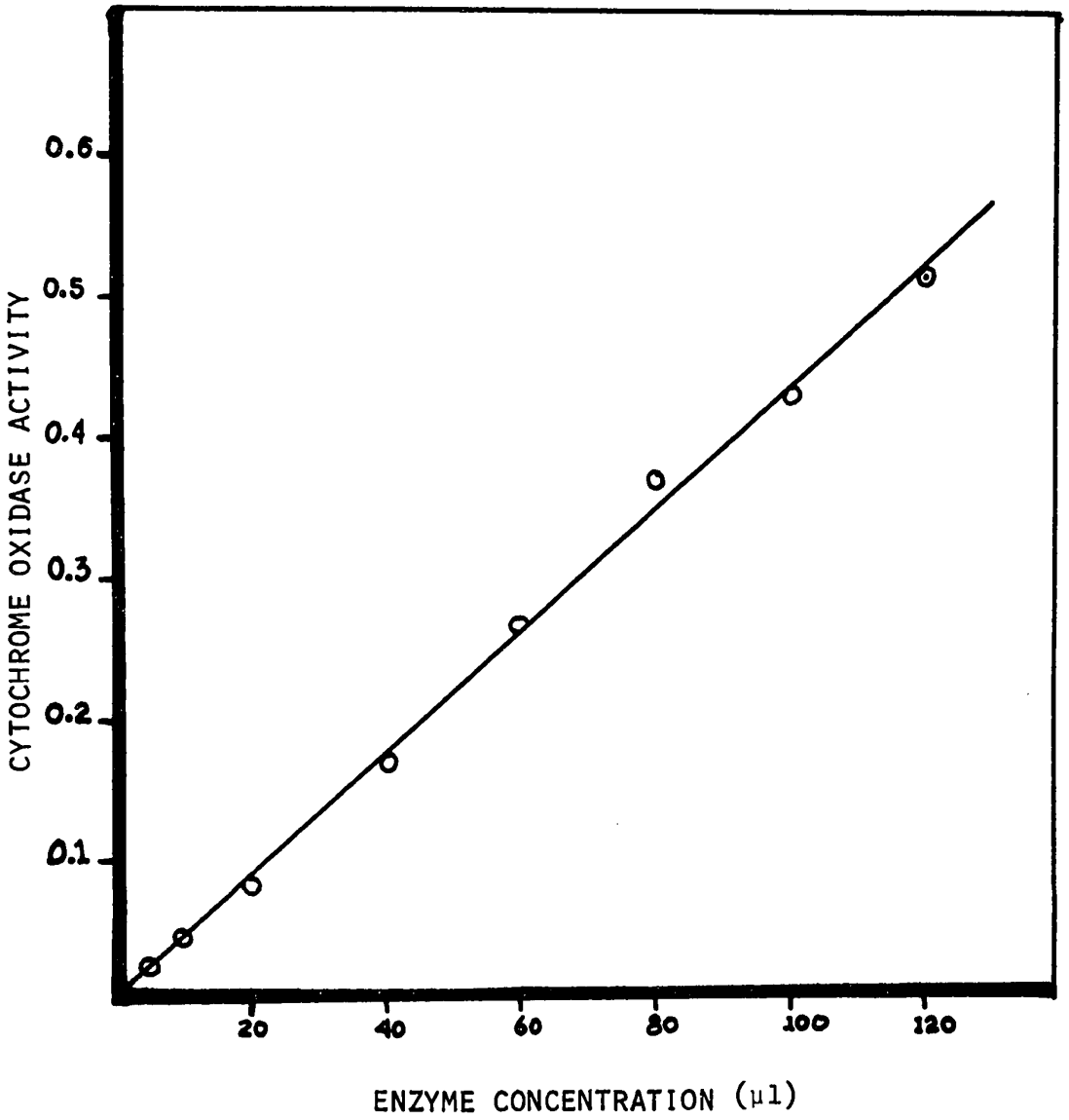


Figure 23 . Linearity of cytochrome oxidase activity with enzyme concentration. Enzyme concentration is expressed as µl/assay of a parotid gland mitochondrial preparation. Enzyme activity is expressed in terms of change in absorbance of a cytochrome c solution as explained in the text.

During routine use of the method of Cooperstein and Lazarow (323), several drawbacks became apparent, particularly in the assay of crude homogenates. The reduction of cytochrome c with sodium hydrosulfite was time consuming because of the necessity of destroying excess sodium hydrosulfite. Successive assays were required each time the solution was shaken followed by the immediate calculation of cytochrome oxidase activity in order to determine when the excess hydrosulfite was oxidized. Excessive shaking oxidized some of the cytochrome c. The reduced cytochrome c was slowly oxidized at room temperature so that after two hours, it was only about 50% reduced. The cytochrome oxidase activity may also be inhibited by the breakdown products of sodium hydrosulfite (324). For these reasons, other methods were tried. For us, the most satisfactory method was that described by Smith (325), the catalytic hydrogenation of cytochrome c with palladium on asbestos as described below (Method B).

When parotid and submaxillary glands were homogenized and the subcellular components fractionated by centrifugation, the sum of the cytochrome oxidase activities of the fractions was greater than the cytochrome oxidase activity of the whole homogenate (table 8). Smith and Conrad (326) described the inhibition of cytochrome oxidase activity by substances in the nuclear and the soluble fractions of rat liver. They showed that high concentrations of cytochrome c were inhibitory (327) and suggested that other basic proteins such as salmine

TABLE 8

A typical distribution of cytochrome oxidase activities among subcellular fractions of salivary glands.

Fraction	CYTOCHROME OXIDASE ACTIVITY* Parotid	Submaxillary
Nuclear	56	170
Mitochondrial	271	264
Microsomal	49	38
Soluble	0	0
Sum of all fractions	376	472
Whole homogenate	236	205

* Cytochrome oxidase activity expressed as total units per whole fraction or homogenate. A cytochrome oxidase unit is defined in the text.

TABLE 9

The effect of aging and additions of DNA, RNA and detergent upon cytochrome oxidase activity in parotid, submaxillary, heart and liver.

	CYTOCHROME OXIDASE ACTIVITY				
			Additions†		
	Control	Aged*	DNA	RNA	Triton X-100
Parotid	0.419 0.385	0.414	0.352	0.419	0
Submaxillary	0.173	0.180	0.174	0.182	0
Heart	1.62	1.61	2.117	2.236	
Liver	0.543	0.540	0.503	0.640	

* The enzyme, a $_0$ tissue homogenate in isotonic KCl, was stored 24 hours at 4^0.

† The amount of DNA added was 20 µg/assay, the amount of RNA added was 50 µg/assay, and triton X-100 was added to a final concentration of 0.2%.

could inhibit by binding to cytochrome oxidase (326). This was supported by Person and Fine (328) who demonstrated the inhibition of cytochrome oxidase by polycations (protamine, lysozyme, histone, ribonuclease). They were able to block and reverse this inhibition with polyanions (polyglucose sulfate, polyethylene sulfonate, carageenins, sulfochitosans, DNA, chondroitin sulfate and heparin). Decrease of activity of aged enzyme preparations could also be partly reversed by polyanions. Activation of various preparations of cytochrome oxidase with detergent (329, 330),by sonication (330) and by incubation with dilute buffer (326) have been proposed. The optimum conditions for the assay of purified cytochrome oxidase has been variously reported to be pH 5.6 in 0.04 M phosphate (329), pH 5.95 in 0.075 M phosphate buffer (329), and pH 6.0 in 0.1 M phosphate buffer (331). These optima would not necessarily apply to crude preparations since inhibition by polycations and reversal of inhibition by polyanions are sensitive to pH and buffer concentration (328, 332).

The optimal conditions for the assay of cytochrome oxidase in crude homogenates, particularly with regard to reversal of inhibition were therefore explored. In the following experiments, rat heart, liver, submaxillary glands and parotid glands were homogenized in isotonic KCl and stored on ice until assayed. The conditions for the cytochrome oxidase assay were those described in Method A except that the pH and molarity of the buffer was varied.

Additions of RNA (Sigma, yeast, type II-S) and DNA (Sigma, calf thymus, type V) were also tried. Table 9 indicates that the cytochrome oxidase activity of samples stored at 4° was stable at least 24 hours. Further storage, however, was accompanied by a loss of enzyme activity. The effect of DNA and RNA is also shown in table 9. At pH 7.0, 0.075 M phosphate, addition of RNA to the reaction medium increased the oxidation of cytochrome c whereas DNA increased cytochrome oxidase activity only with heart muscle homogenate, and may decrease it in other organs. Triton X-100, a non-ionic detergent, completely inhibited cytochrome oxidase activity at a concentration of 0.2%.

Figure 24 indicates that the pH optimum depends very much on the buffer concentration. Optimum conditions for a parotid homogenate were 0.1 M phosphate, pH 6.6. (With these conditions, activity was increased almost threefold from thoseof Method A (.03 M, pH 7.4). The amount of RNA added for maximum increase of activity under those conditions was 5 µl where a 40% increase was observed (figure 25).

Method B: Cytochrome Oxidase, Modified

Reagents

Reduced cytochrome c: To 10 ml of 0.01 M phosphate buffer, pH 7.0, 52 mg of cytochrome c (Sigma, type III) was added, and nitrogen was bubbled through this solution. About 200 mg of 5% Pd on asbestos was added and the mixture was stirred in an atmosphere of hydrogen for two hours. Nitrogen was again

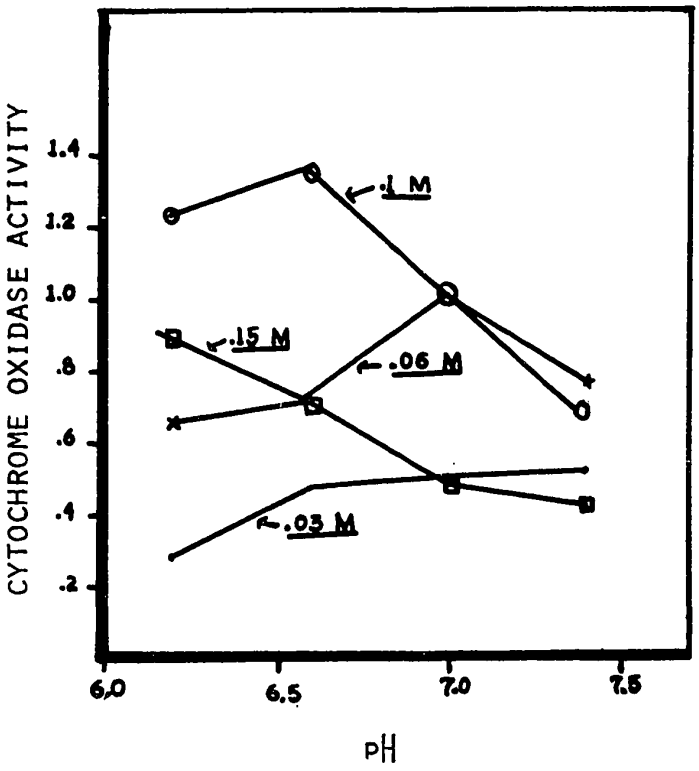


Figure 24 . Optimum conditions for parotid cytochrome oxidase activity. Effect of molarity and pH of phosphate buffer in the incubation medium. Enzyme activity is defined in the text.

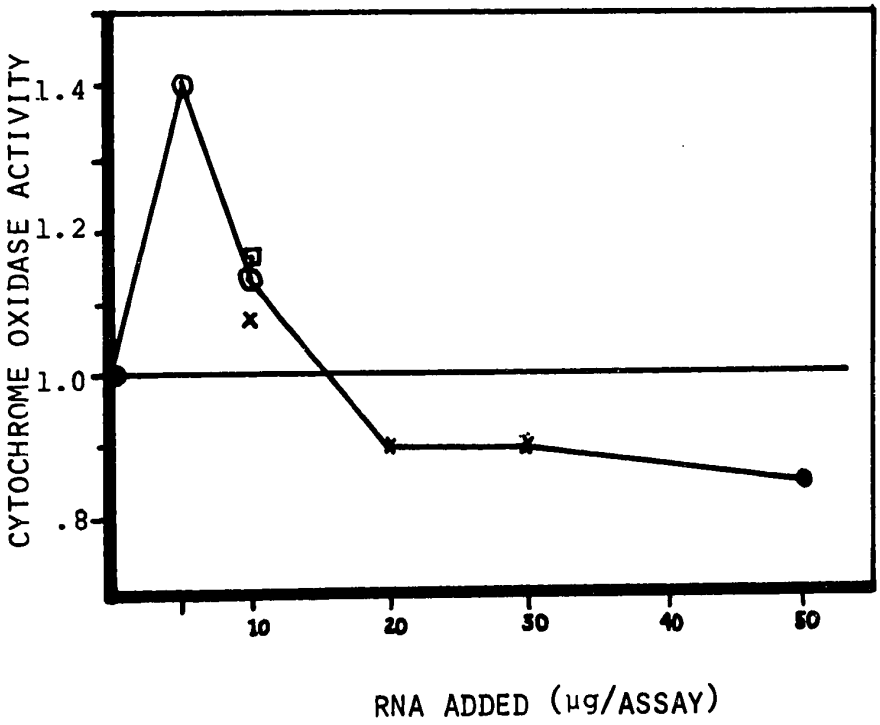


Figure 25 . Effect of RNA on parotid cytochrome oxidase activity. Enzyme activity is defined in the text.

bubbled through, the solution was filtered and stored in a dark bottle under nitrogen. The reduced cytochrome c solution could be stored refrigerated under nitrogen for three days with little auto-oxidation.

Procedure

The equipment described in Method A was used. Into each of the cuvettes, maintained at 25°, was pipetted 1.2 ml of 0.1 M phosphate buffer pH 6.6. Sample (5 to 100 µl) was added with mixing to each cuvette. At least three minutes was allowed for each sample to equilibrate before addition of substrate. To the first cuvette was added with mixing 0.2 ml reduced cytochrome c solution, and the reading of absorbance at 550 mµ was immediately begun. (The amount of cytochrome c added resulted in a final oxidation absorbance of 0.33, i.e., the difference in absorbance at 550 mµ between fully oxidized and fully reduced solutions was 0.33. This was equivalent to a concentration of 25 µM.) Absorbance was measured until a change of 0.1 to 0.2 units had been recorded. The second cuvette was placed in position, reduced cytochrome c was added, and absorbance again followed. This was repeated for the third and fourth samples, after which one drop of saturated $K_3Fe(CN)_6$ solution was added to oxidize the cytochrome c and the absorbance of each solution was recorded.

The enzyme activity was calculated exactly as described in Method A.

Acid Phosphatase

Acid phosphatase activity was determined by estimating the p-nitrophenol released from p-nitrophenyl phosphate.

Reagents

Substrate: 4 mg/ml p-nitrophenyl phosphate (Sigma)
Buffer: 0.2 M citrate, pH 4.9

Procedure

In 0.4 ml plastic centrifuge tubes, 0.05 ml of enzyme (tissue homogenate) was mixed with 0.3 ml of citrate buffer, and stored cold until analyzed. From the diluted solution, 0.1 ml aliquots were transferred to each of two sets of tubes labeled sample and blank respectively. To the sample tubes, 0.5 ml of substrate and 0.5 ml of buffer were added. In the blank tubes, water was substituted for substrate. The tubes were incubated at 37^{o} for thirty minutes followed by the addition of 2 ml of 0.2 N NaOH to each. The samples were read at 410 mμ against a reagent blank (prepared by using water in place of sample). The sample blanks were read against water at 410 mμ and each was subtracted from the equivalent sample reading.

A standard curve was prepared by adding 3 ml NaOH to 0.1 ml of a series of concentrations of p-nitrophenol solutions, and measuring absorbance at 410 mμ. Activity was expressed as absorbance units/30 minutes.

L(+)-Lactate Dehydrogenase (LDH)

Introduction

Most methods for the determination of LDH activity in mammalian tissues are based on the measurement of NADH at 340 mμ. In the following equation:

$$\text{L(+)-Lactate} + \text{NAD}^+ \rightleftharpoons \text{Pyruvate} + \text{NADH} + \text{H}^+$$

it can be seen that with the "forward reaction" (formation of pyruvate from lactate), NADH is formed and thus, the increase in absorbance at 340 mμ can be used to measure the reduction of lactate. Conversely, in the "backward reaction", the decrease of absorbance at 340 mμ indicates the oxidation of pyruvate by NADH.

Under physiological conditions, the equilibrium greatly favors the backward reaction (333, 334) and for this reason has been proposed for measurement of LDH in serum (334, 335), in skeletal muscle (333) and heart muscle (336). In general, the initial conditions for the backward reaction described by these authors were 0.3 to 0.8 mM pyruvate, and 0.07 to 0.17 mM NADH in phosphate buffer pH 7.0 or pH 7.4. The oxidation of NADH at 25° or 37° was determined by measuring absorbance at 340 mμ.

The forward reaction has also been proposed for the measurement of LDH in serum (337) and heart muscle (338). Amador et al (337) claim that the forward reaction produced

a more linear activity-concentration curve with blood sera. Both methods utilize a higher concentration of substrate and a higher pH than the methods based on the backward reaction.

In order to obtain the most favorable conditions for the determination of LDH in salivary gland tissue, several methods were tried. Included were the methods of Kornberg (334), Nielands (338), Amador et al (337) and various modifications of these.

In figure 26, curve A represents the change in absorbance due to LDH in parotid homogenate as determined by the backward reaction of Kornberg. In curve B, exactly the same conditions were used except that L(+)-Lactate and NAD^+ were substituted for pyruvate and $NADH^+$ respectively and the change in absorbance was due to the forward reaction. It appeared that the forward reaction (curve B) was more sensitive but that the absorbance curve fell off very quickly. The increased pH and L(+)-Lactate concentration used in the Nielands method (curve C) and the Amador method (curve D) greatly increased sensitivity. In an attempt to further improve the conditions, the concentration of substrate was further increased. In figure 27, by using the conditions of Nielands, but varying lactate concentration, it was shown that increased lactate concentration increased the sensitivity of LDH measurements. At a concentration of 250 mM L(+)-Lactate, absorbance was linear with time up to 0.2 absorbance units. At 450 mM or

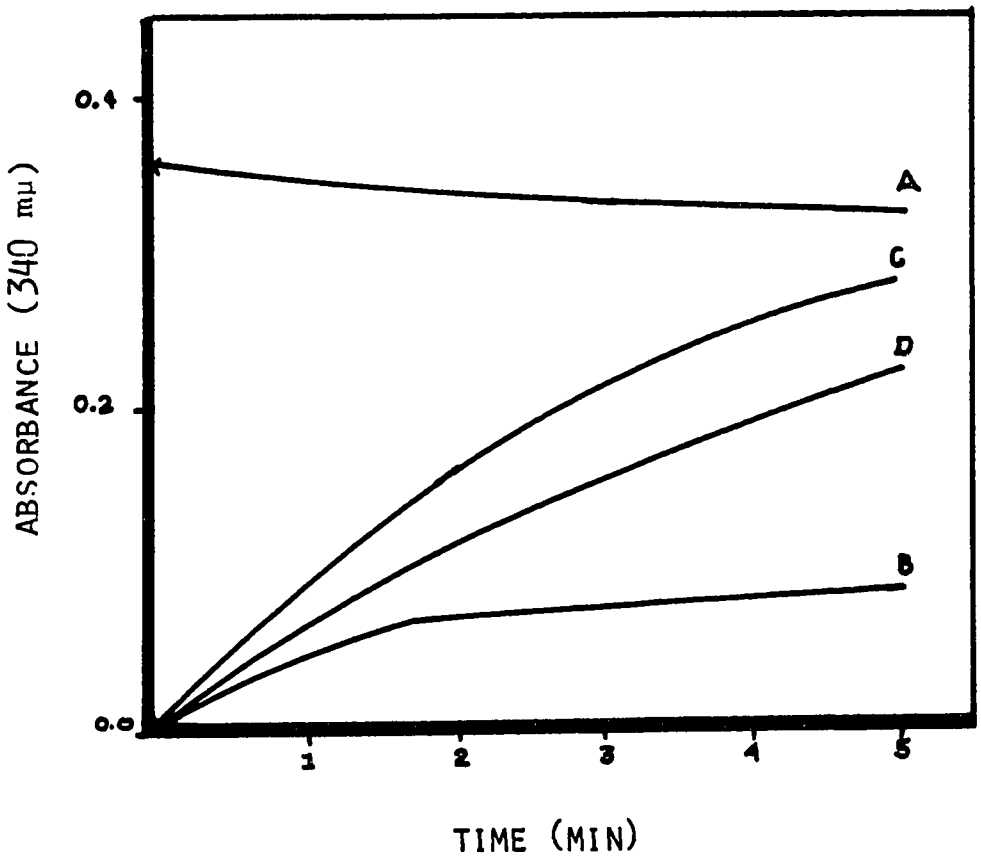


Figure 26 . LDH activity in parotid glands measured by several methods. LDH activity is defined as the rate of change of absorbance at 340 mμ at 37° due to oxidation of $NADH^+$ or reduction of NAD. The incubation conditions were: A.- 0.33 mM pyruvate, 0.067 mM $NADH^+$ and 33 mM phosphate, pH 7.4 ; B.- 0.33 mM L(+) lactate, 0.067 mM NAD^+ and 33 mM phosphate, pH 7.4 ; C.- 17 mM L(+) lactate, 1.3 mM NAD^+ and 87 mM glycine, pH 10; D.- 39 mM L(+) lactate, 5 mM NAD^+ and 50 mM pyrophosphate, pH 8.8 .

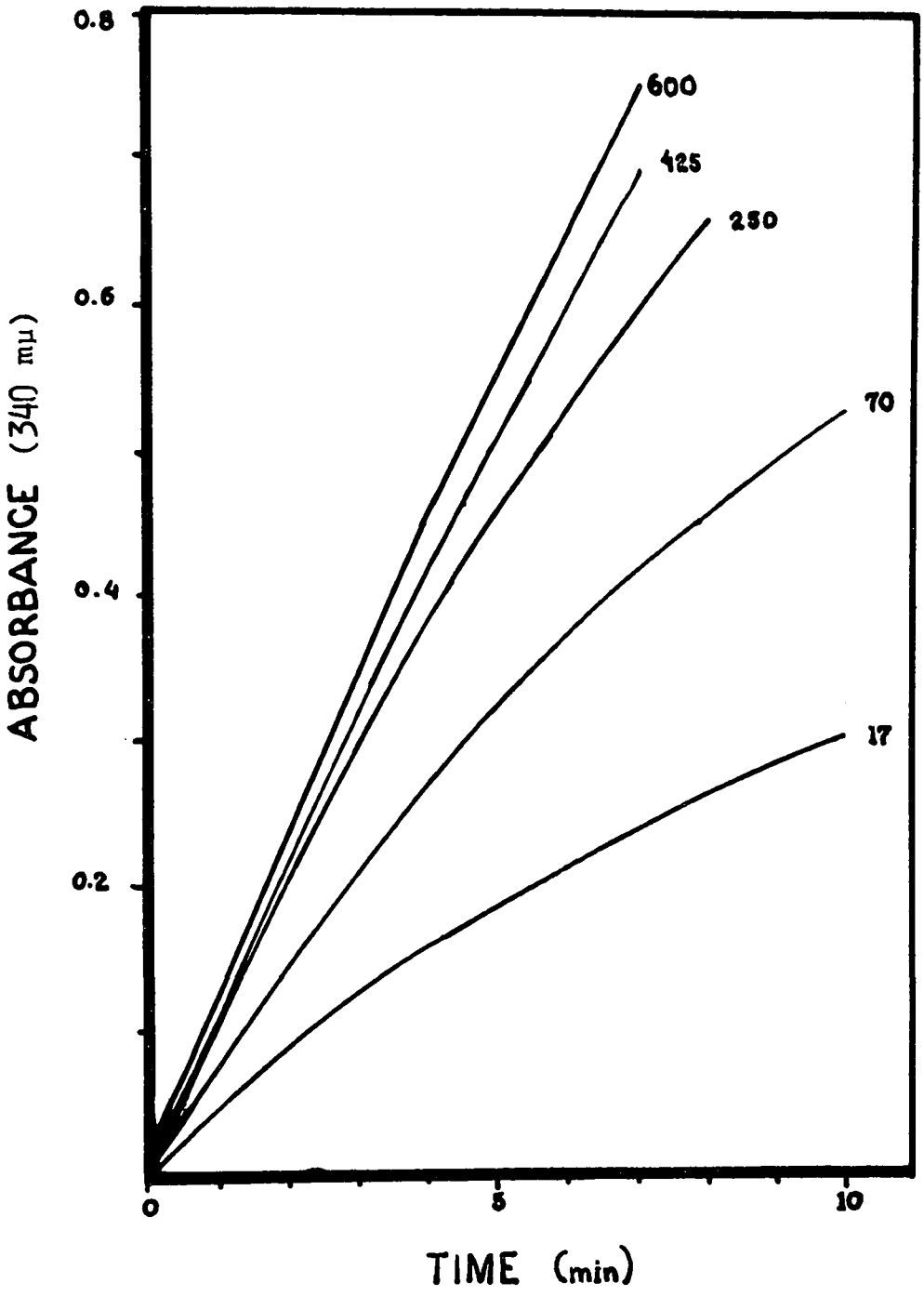


Figure 27 . Effect of lactate concentration on LDH activity in a rat parotid homogenate. LDH activity is defined as the increase in absorbance at 340 mμ due to formation of $NADH^{+}$. The L(+) lactate concentration (mM) is shown for each curve.

higher, absorbance was linear with time to at least 0.3 absorbance units. Increasing the concentration of NAD^+ had no effect on the activity curve of LDH.

Procedure

From these experiments, the following conditions were chosen for the determination of LDH in salivary glands.

Final concentrations in incubation mixture:
L(+)-Lactate - 250 mM (500 mM DL - lactate)
NAD^+ - 1.3 mM
Glycine - NaOH buffer - 93 mM pH 10.0

Since D(-)-lactate does not take part in the reaction nor interfere with the oxidation of the L(+)-isomer (338), the substrate was prepared from 85% DL-lactic acid. An equal volume of water was added to 85% lactic acid. To 20 ml of this solution, 5 N NaOH was slowly added until the solution was alkaline. The solution was heated to 80° in order to hydrolyze the inner ester (lactide). NaOH was added with heating until the solution remained neutral. For storage, the mixture was diluted to 89 ml. An equal volume of this was diluted with 0.2 M glycine - NaOH buffer, pH 10.0.

Stock NAD^+ solution (20 mM) was prepared by dissolving 133 mg of NAD (Sigma Chemical Co.) in 5 ml water and adding 1 N NaOH until pH 6.0 was reached. This was diluted to 10 ml and stored refrigerated in a dark bottle.

The activity measurements were carried out in water-jacketed cuvettes maintained at 30° in an automatic sample changer (described in the cytochrome oxidase procedure). To each cuvette was added 1.4 ml of the buffered substrate solution and 0.1 ml of the NAD^+ solution. Enzyme solutions, (5 to 50 µl), suitably diluted in 0.1 m phosphate buffer, pH 7.0, were added to each cuvette and the absorbance at 340 mµ was recorded at one minute intervals. The blank cuvette contained everything but enzyme.

LDH activity was calculated as the increase in absorbance units at 340 mµ in one minute at 30°. Assuming a molecular extinction coefficient of 6.2×10^3 for NADH (338), a $\Delta\varepsilon_{340}$ of 0.10 corresponds to the formation of 41.7 mµ moles NADH in the 1.5 ml test volume.

The LDH activity-concentration curve shown in figure 28 indicates the linearity of activity over a wide range of salivary gland concentrations.

Glucose-6-Phosphatase

The method described by Hübscher and West (339) was used for glucose-6-phosphatase estimation. Glucose-6-phosphate was incubated with the enzyme in the presence of EDTA and NaF (specific inhibitors of alkaline and acid phosphatase) and the released phosphate was determined by the method of Sumner (340).

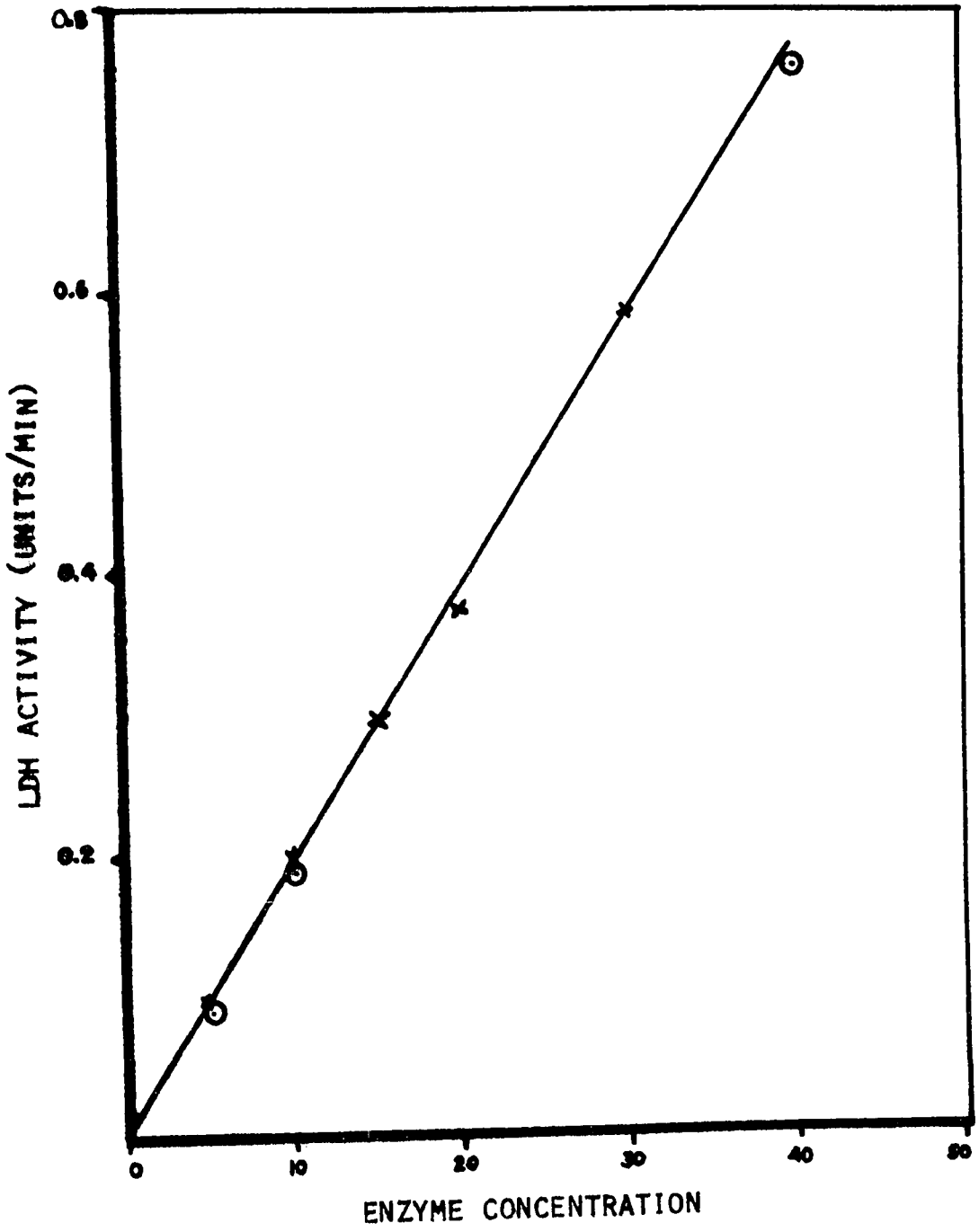


Figure 28 . Linearity of parotid LDH activity with enzyme concentration. LDH activity is expressed as the rate of change in absorbance at 340 mμ during incubation at 30°. Enzyme concentration is expressed as μl of a parotid soluble fraction preparation.

Reagents

Buffer: 43 mM maleate, pH 6.0 containing 4 mM EDTA and 2 mM NaF.

Substrate: 28.5 mM glucose-6-phosphate (Sigma Chemical Co.), pH 6.0.

Enzyme: Microsomal fraction suspended in 0.25 M sucrose.

Procedure

In a 37^{0} water bath, two sets of 10 ml test tubes were set up. They were labeled sample and blank, respectively, for each assay. To each tube, 0.2 ml buffer was added and 0.3 ml aliquots of enzyme were placed in the sample and blank tubes. To start the incubation, 0.2 ml substrate was added to each sample tube and 0.2 ml water to each blank tube. Incubation was stopped after 15 minutes with the addition of 1.3 ml of 10% TCA to each tube. A further addition of 2 ml of 12.5% charcoal suspension (Norit A, B.D.H.) in 5% TCA was followed by centrifugation at 27,000 X g for ten minutes. A reagent blank containing buffer and substrate with 0.25 M sucrose in place of enzyme was run simultaneously.

The phosphate in the supernatant was determined as follows: One ml of each supernatant was transferred to a tube containing 4.4 ml water and 0.75 ml of 6.6% ammonium molybdate. The addition of 0.75 ml of 7.5 N H_2SO_4 followed by 0.6 ml of freshly prepared ferrous sulfate solution (5 g of $FeSO_4$ in 50 ml of H_2O and 1 ml of 7.5 N H_2SO_4) completed the color development. The amount of phosphate liberated

was measured spectrophotometrically at 660 mμ. A standard curve was prepared using solutions of KH_2PO_4 at concentrations of 1 to 300 mg/ml phosphorous.

To calculate enzyme activity values for the phosphate due to the enzyme (sample blank) and the phosphate due to the substrate (reagent blank) were subtracted from the sample reading to give phosphate released from glucose-6-phosphate. Enzyme activity was expressed as μmoles of phosphate liberated at 37^0 in 15 minutes by 1 mg microsomal protein.

UDP-Glucuronyl Transferase

UDP-glucuronyl transferases have been found only in preparations containing microsomes (341). However, the specificity for certain acceptors of the glucuronate varies between tissues. Accordingly, the microsomes from salivary glands were incubated with UDP-glucuronic acid and a radioactively labeled acceptor, 17α-estradiol (342).

Reagents

Buffer: 0.15 M tris (THAM, Fischer) pH 8.0
Substrate: UDP-glucuronate, Na salt (Sigma Chemical Co.)
Acceptor: 17α-estradiol-^{3}H, 5.6 mCi/μmole
Enzyme: Microsomal pellet suspended in isotonic KCl

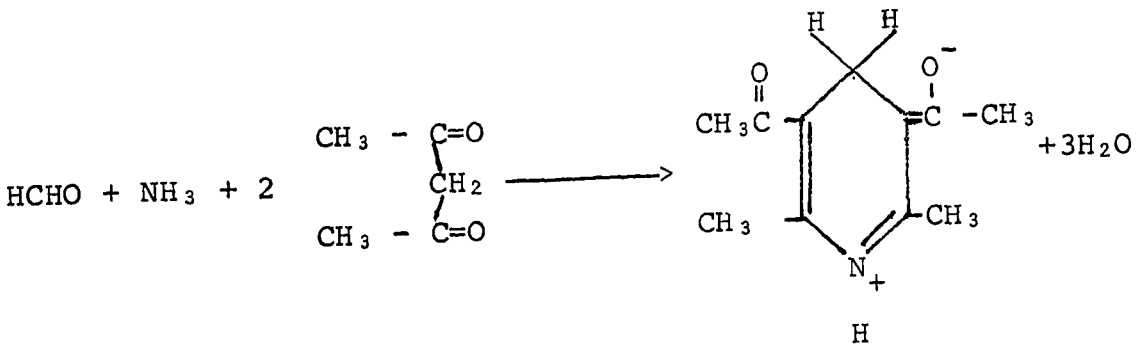
Procedure

To a 10 ml conical centrifuge tube containing 0.5 μmole of dry UDP-glucuronate, and an acceptor with 469,000 DPM, was

added 2 ml buffer and .9 ml isotonic KCl. For the blank, a similar tube was prepared but without UDP-glucuronate. Incubation at 37° was started by addition of 0.1 ml of enzyme to each tube. After one hour, the incubation was stopped by extracting the solution with 5 ml benzene. An aliquot of the benzene was transferred to scintillation vials containing 10 ml scintillation mixture and CPM was measured. Sample CPM was subtracted from blank CPM to give an estimate of glucuronide formation.

Oxidative Demethylation

The assay of the enzyme responsible for oxidative demethylation of aminopyrine was based on the methods of Orrenius (277) and Ernster (343). In these methods, enzyme was incubated with aminopyrine, NADP and a NADPH generating system, and the formaldehyde produced was then assayed by the colorimetric method of Nash (344). The product of the Nash reaction, 3,5-diacetyl-1,4-dihydrolutidine (DDL), is fluorescent (344, 345). The method we used includes solvent extraction of DDL, followed by fluorometric analysis.



In order to find the best conditions for extraction and fluorometric estimation, DDL was synthesized by the method of Nash (345) and several organic solvents were studied. Extraction with butanol followed by fluorescence measurement was so sensitive that a change in the enzyme incubation reagents was required in order to ensure a low blank value.

Reagents

The water used in all reagents was redistilled in glass.

Substrate solution A: A mixture of 11.5 mg aminopyrine, (4-dimethylamino antipyrine, Aldrich Chemical Co.)

61.05 mg nicotinamide (Niacinamide - Sigma Chemical Co.) 10.15 mg $MgCl_2.6H_2O$ and 2.8 mg NADP (TPN-Na Salt, Sigma Chemical Co.) in 5 ml of 0.1 M tris, pH 7.4.

NADPH generator solution A: 2.5 µl of glucose-6-phosphate dehydrogenase (Sigma Chemical Co.) 500 µmole/ml and 323 mg glucose-6-phosphate in 5 ml of 0.1 M tris, pH 7.4.

Substrate solution B: The same as substrate solution A except that 0.1 M K_2HPO_4 buffer pH,7.5,replaced the tris buffer.

NADPH generator solution B: 2.5 µl of glucose-6-phosphate dehydrogenase and 81 mg glucose-6-phosphate in 5 ml of 0.1 M phosphate, pH 7.5.

Nash reagent: A mixture of 30 g ammonium acetate, 0.6 ml glacial acetic acid and 0.4 ml acetylacetone was dissolved in 200 ml water.

Formaldehyde standards: Thirty-seven per cent formaldehyde (12% methanol as preservative) was diluted to final concentration between 0.02 and 2 µg/ml.

Preparation of DDL

A solution of 38 g of ammonium acetate, 3.75 ml of 40% formaldehyde, and 10 g of acetylacetone in 500 ml water was allowed to stand at room temperature for five days. The resulting yellow precipitate was filtered, washed with a few ml of water and dried. One recrystallization from alcohol gave a product with the properties shown in table 10.

Extraction of DDL

A number of non-polar solvents were tried for the extraction of DDL from aqueous solution. Benzyl alcohol, isoamyl alcohol, and 1-butanol were each able to extract most of the DDL from an equal volume of aqueous solution.

The fluorescence spectra of DDL in aqueous solution and in 1-butanol are compared in figure 29. The emission peak was 495 mμ in butanol as compared to 510 mμ in aqueous solution. The intensity was also much greater with butanol. In table 11, the relative fluorescence intensities of 0.2 μg/ml DDL in each solvent are shown along with their blank (fluorescence with solvent alone). Each of the organic solvents gives higher fluorescence with DDL than 1 M ammonium acetate solution. Benzyl alcohol had an extremely high blank value and was therefore of no use in this fluorimetric assay. DDL in 1-butanol exhibited about five-fold greater fluorescence than in aqueous solution. The butanol blank was also low.

TABLE 10

Properties of 3,5-diacetyl-1,4-dihydrolutidine (DDL) prepared by the method of Nash (344).

	M.P.	ϵmax (mμ)	I.R. Peaks (cm^{-1})
Found:	205-219° (decomp.)	415	3250(m) 1670(s) 1620(w) 1590(w) 1570(s) and 1220(s) (Nujol Mull)
Reported:			
(346)	198°	-	-
(344)	208°	412	-
(345)	190-200°	410	3480(w) 3330(m) 1680(s) 1630(m) 1570-1600(s) and 1230(s) (KBr disk)

w = weak; m = medium; s = strong

TABLE 11

Fluorescence intensity of DDL in several solvents at their individual fluorescence optima.*

	1M Ammonium Acetate	Benzyl Alcohol	Isoamyl Alcohol	1-Butanol
DDL - 0.2 μg/ml	1.35	9.65	3.70	6.20
Blank	0.15	6.00	0.10	0.25
Difference	1.20	3.65	3.60	5.95

* The fluorescence optima (excitation/emission in mμ, uncorrected) were 412/510 for 1M ammonium acetate, 412/495 for isoamyl alcohol and 412/495 for 1-butanol

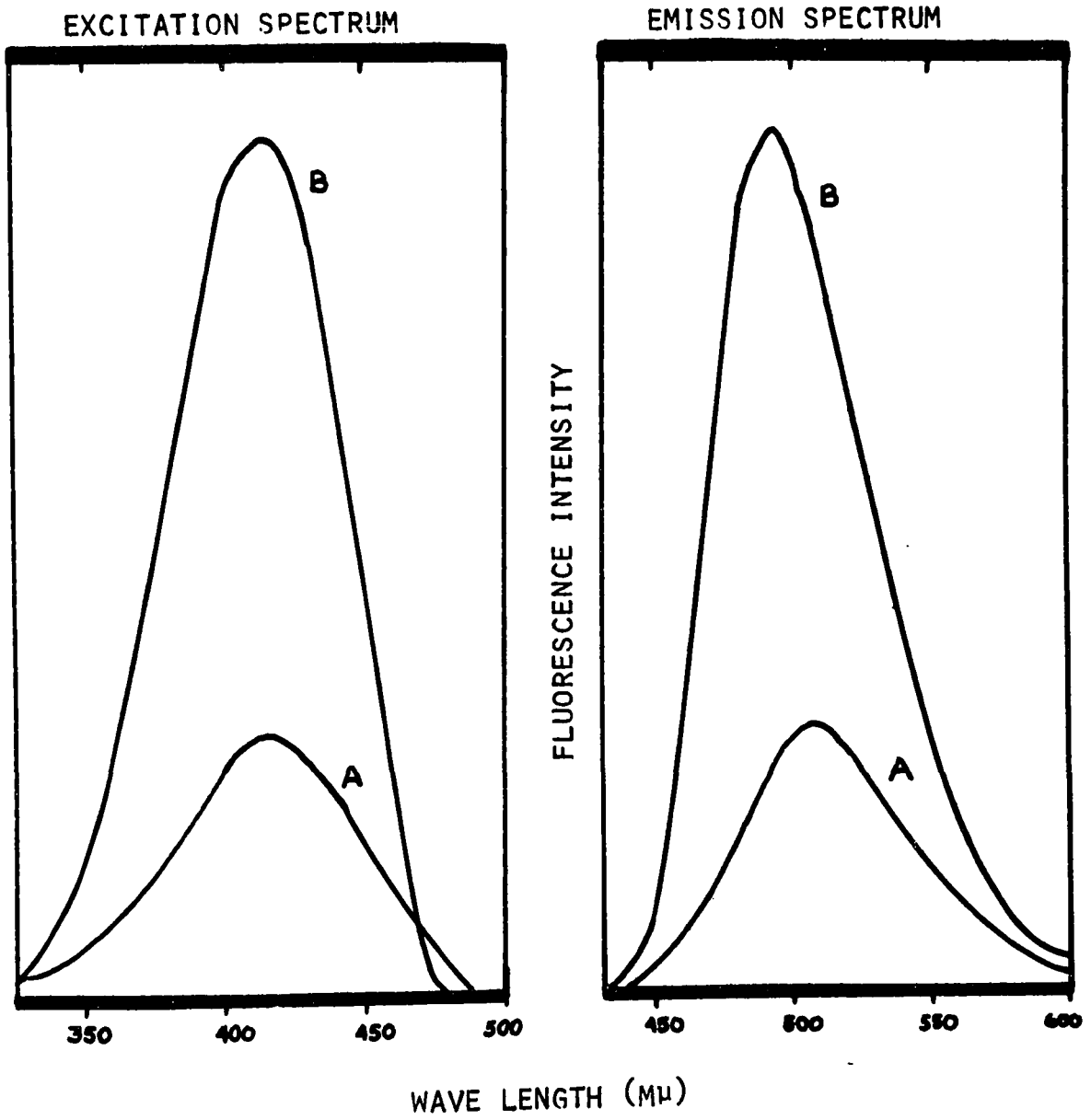


Figure 29 . Excitation and emission spectra of 3,5-diacetyl-1,4-dihydrolutidine (DDL). Curve A - 0.2 μg/ml DDL in 1 M ammonium acetate, pH 6.0 ; Curve B - 0.2 μg/ml DDL in butanol.

Determination of Formaldehyde

To 2 ml of standard formaldehyde solution or to 2 ml filtrate from the oxidative demethylation assay mixture, was added 2 ml Nash's reagent. After thorough mixing, the samples were placed in a water bath at 37° for one hour. The mixture was extracted with 1-butanol and after centrifugation, the butanol layer was transferred to cuvettes and fluorescence intensity was measured, (594 mμ emission, 412 mμ excitation, in Aminco Bowman Spectrofluorophotometer).

A standard curve for formaldehyde is shown in figure 30. The curve for fluorescence of the unextracted reaction mixture is compared to the curve for the butanol extract of the reaction mixture. The sensitivity was greatly increased by solvent extraction. The blank was also increased. (The blank could probably be greatly decreased by using freshly distilled acetylacetone in the reagent as recommended by Nash.) Using fluorescent measurement of the butanol extract, formaldehyde could be conveniently measured in concentrations between 0.02 and 1.0 μg/ml.

Assay: Oxidative Demethylation of Aminopyrine

Method A: The conditions used were essentially those described by Orrenius (277). In a 10 ml centrifuge tube, a mixture of 1.0 ml of substrate solution A, 0.1 ml NADPH generator solution A, and 1.0 ml of enzyme were incubated at 37° for 20 minutes. The enzyme was a suspension of about

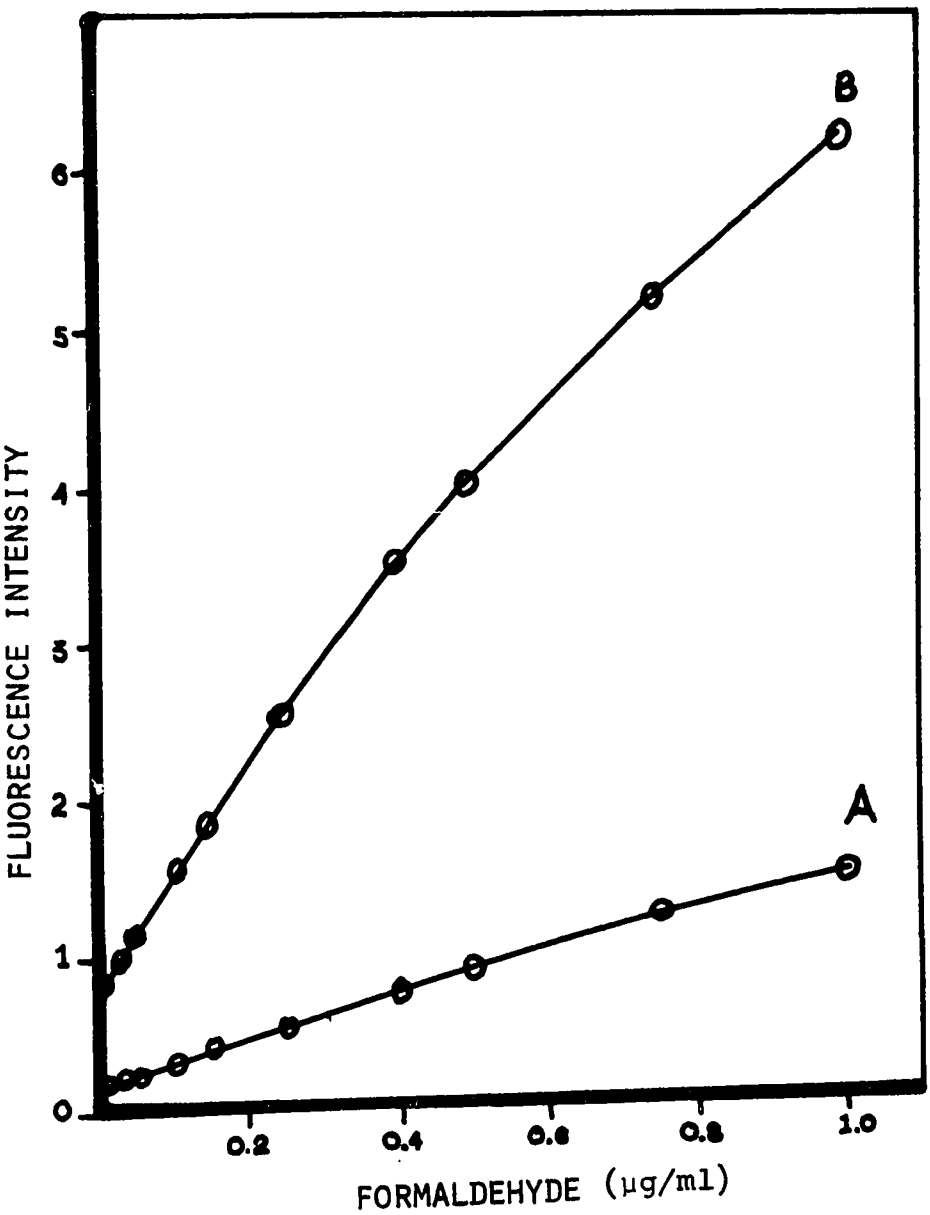


Figure 30 . Calibration curve for the fluorimetric analysis of formaldehyde : A - fluorescence intensity of the aqueous reaction mixture ; B - fluorescence intensity of the butanol extract of the aqueous reaction mixture. No blanks were subtracted.

2 mg microsomal protein in 1 ml isotonic KCl. The reaction was stopped by addition of 0.25 ml of 25% $ZnSO_4$ followed by 0.25 ml of saturated $Ba(OH)_2$ solution. After centrifugation, formaldehyde in the supernatant was determined.

Method B: This was the same as Method A except that substrate solution B and NADPH generator solution B were used in place of substrate solution A and NADPH generator solution A respectively.

Discussion

The method described by Orrenius (277) Method A, could be used in conjunction with the fluorimetric assay of formaldehyde. The reagent blank, however, was particularly high, and therefore, enzyme samples of low activity would be difficult to measure. Table 12 shows the contribution of each of the components of the assay to the high reagent blank. The tris buffer was the major source, but the NADPH generating system also contributed. In Method B, the tris buffer was replaced with phosphate buffer and the concentration of glucose-6-phosphate in the NADPH generating system was decreased. These changes resulted in a much lower reagent blank in Method B.

TABLE 12

Interference of components of Orrenius' assay for the oxidative demethylation of aminopyrine (277) in the fluorimetric determination of formaldehyde.

Assay mixture components	Formaldehyde equivalence of fluorescence (µg/ml)
Isotonic KCl	0
NADPH Generating Solution A	0.066
$BaOH_2$ and $ZnSO_4$	0.022
Aminopyrine	0.002
Nicotinamide	0.004
NADP	0
Tris Buffer	0.194
Total	0.288
Reagent Blank, Method A	0.30
Reagent Blank, Method B	0.04

Cytochrome-P-450 (P-450)

P-450, a carbon monoxide-binding hemoprotein, was estimated as described by Omura and Sato (347, 348). This was a method based on the fact that when reduced and treated with CO, P-450 exhibits a characteristic intense absorption peak at 450 mμ.

Reagents

Sample: Particulate fractions were prepared from tissue homogenates (in isotonic KCl) by differential centrifugation, and resuspended in isotonic KCl. This suspension was stored at 4^{o} and used within 24 hours.

Buffer: 0.1 M phosphate buffer pH 7.0

Carbon monoxide: CO was generated by adding 90% formic acid to 200 ml of concentrated sulfuric acid in a 1 liter filter flask. The dropping funnel containing the formic acid was connected to the flask through a reflux condenser. During dropwise addition of formic acid, the sulphuric acid was heated at 65^{o} on a water bath and the reagents were mixed with a magnetic stirrer. CO was collected from the side arm of the flask and passed through a drying tower filled with KOH pellets.

Procedure

Difference spectra of particulate preparations were measured at 25^{o} in the Bausch and Lomb Spectronic 505 spectrophotometer with cuvettes of 1 cm optical path. Sample

preparations were suspended in the phosphate buffer at a concentration of 2 mg/ml protein or greater. After recording the base-line, the content of the sample cell was reduced with solid $Na_2S_2O_4$ and the reduced-minus-oxidized difference spectrum was measured. The content of the reference cell was then reduced and CO was bubbled into the sample cell for about 30 seconds. The CO-difference spectrum was then measured. Assuming a value of 91 $cm^{-1}mM^{-1}$ for the molar extinction increment between 450 and 490 mμ for P-450 (348) the molar concentration of P-450 was calculated. From the reduced-minus-oxidized difference spectrum and also from the CO-difference spectrum, interference due to hemoglobin was detected. Correction of P-450 values on the basis of these spectra is described in the Experimental Section.

Catechol-O-Methyl Transferase (COMT)

The method used to measure COMT in salivary glands was basically that of McCaman (349) in which the substrate was 3,4-dihydroxybenzoic acid and the methyl donor was ^{14}C -methyl-S-adenosylmethionine.

Reagents

Buffer: 0.4 M phosphate pH 7.8
Substrate: 5mM 3,4-dihydroxybenzoic acid (K and K Laboratories).
$MgCl_2$ solution: 25 mM $MgCl_2$
HCl solution: 2.4 N HCl

Methyl donor solution: This was prepared immediately before each assay and contains both labeled and unlabeled S-adenosyl-L-methionine (SAM). Labeled SAM was S-adenosyl-L-methionine-1-methyl-^{14}C (New England Nuclear) at a concentration of 21 mμmole/μCi, 1 μCi/50 μl. It was stored frozen and only thawed long enough to prepare the working solution. Unlabeled SAM 2 mμmole/μl S-adenosyl-L-methionine (Calbiochem) was also stored frozen. The working solution was prepared by mixing 25 parts of labeled SAM solution and 10 parts unlabeled SAM with 65 parts of water. The final concentration was 61.1 mμmole, 1 μCi in 0.2 ml.

Procedure

At the start of each assay, an incubation medium was prepared from equal volumes of buffer, substrate, $MgCl_2$ and methyl donor solutions. Plastic centrifuge tubes (0.4 ml) were placed on ice, and to each was added 20 μl of incubation medium and an enzyme solution. The tubes were mixed with the aid of a vortex mixer, then placed in a 37° heating bath. After 30 minutes, the tubes were placed on ice and 0.5 μl of HCl solution was added. Ethyl acetate (100 μl) was added and thorougly mixed to extract the product, 3-methoxy-4-hydroxy-benzoic acid. After a brief centrifugation to separate the phases, 50 μl portions of ethylacetate were removed and placed in scintillation vials. Fifteen milliliters of Spectrofluor solution (New England Nuclear) and 0.2 ml of NCS solubilizer (New England Nuclear) were added to each vial and the radioactivity determined in a scintillation counter.

A blank value, obtained by running simultaneously, a tube containing water in place of enzyme solution, was subtracted from the sample radioactivity values.

Enzyme activity was calculated on the basis of the known specific activity of the ^{14}C-SAM and was expressed as µmole of labeled product formed per g tissue per hour. The enzymatic reaction was linear with time up to about 40 minutes (figure 31) and with enzyme concentration from both parotid and submaxillary glands, (figure 32) to about 10,000 DPM or about 1.1 mµmole/hour.

Electrophoresis

In order to study the purity of amylase preparations, electrophoresis on polyacrylamide gel was carried out by the techniques of discontinuous electrophoresis (disc electrophoresis), described by Ornstein and Davis (350, 351) and isoelectric focusing (352, 353).

Disc Electrophoresis

Reagents

The following solutions were prepared, and all except G could be stored refrigerated for weeks. (G was prepared each day.)

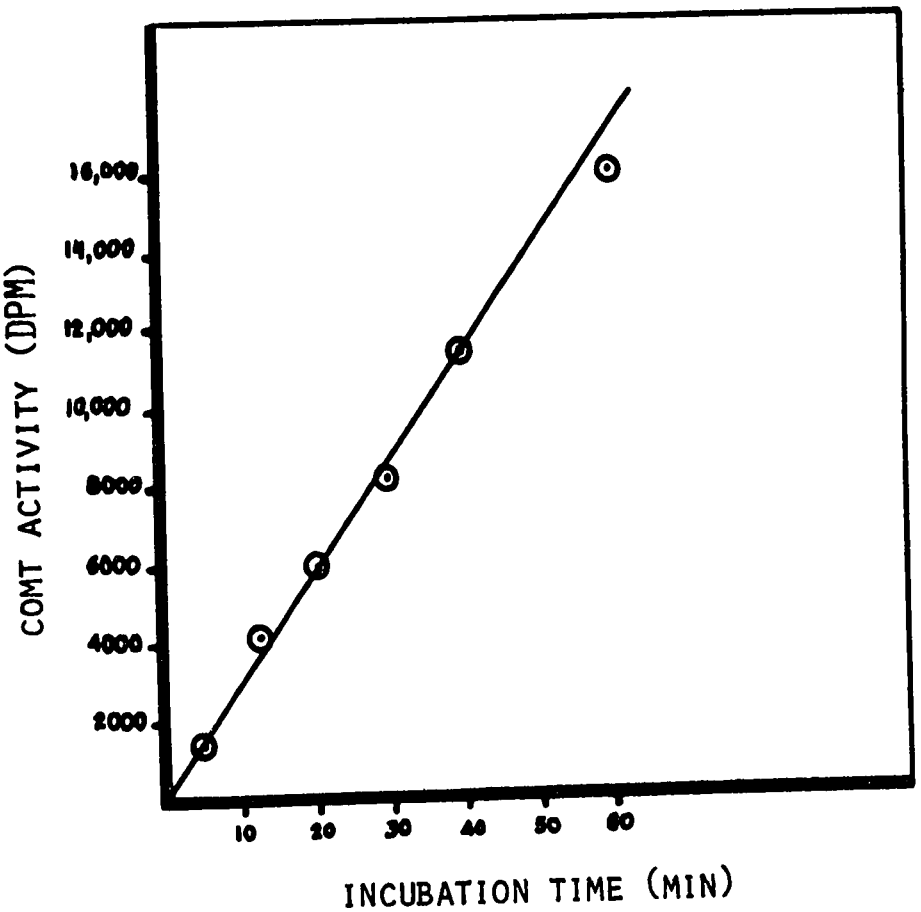
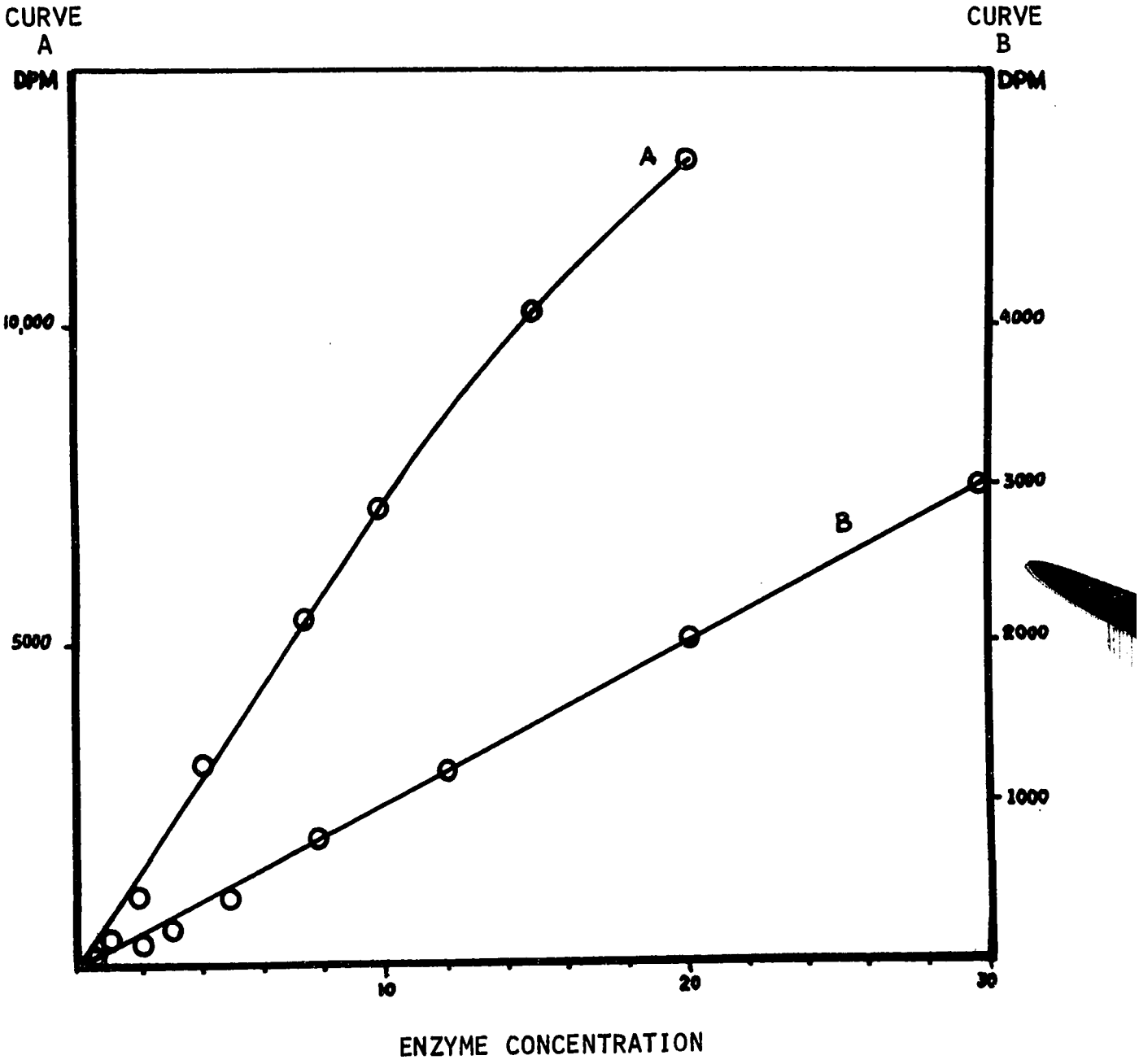


Figure 31 . Linearity of submaxillary COMT activity with time. Activity is expressed as DPM of ^{14}C-methyl group transferred to 3,4-dihydroxybenzoic acid by 20 µl of a submaxillary gland homogenate at 37^{o} in 30 minutes.



Figre 32 . Linearity of parotid and submaxillary COMT with enzyme concentration. COMT activity is expressed as DPM of ^{14}C-methyl transferred to 3,4-dihydroxybenzoate in 30 minutes at 37^{o}. Enzyme concentration is expressed as µl of enzyme solution added to the incubation medium. The enzyme solutions were: A - submaxillary supernatant fraction ; B - parotid total homogenate.

A. Buffer for separating gel (pH 8.9): 48 ml 1 N HCl, 36.3 g tris and 0.23 ml TEMED (N,N,N',N'-tetramethylethylene diamine; Eastmen Organic Chemicals) made to 100 ml with water.

B. Buffer for stacking gel (pH 6.7): 48 ml 1 N HCl, 5.98 g tris and 0.46 ml TEMED made to 100 ml with water.

C. Acrylamide monomer for separating gel: 28 g acrylamide (Eastman Organic Chemicals) and 0.735 g bis (N,N'-methylene bisacrylamide: Eastman Organic Chemicals) made to 100 ml with water.

D. Acrylamide monomer for stacking gel: 10 g acrylamide and 2.5 g bis made to 100 ml with water.

E. Catalyst for stacking gel: 4 mg riboflavin in 100 ml water.

F. 40% sucrose solution.

G. Catalyst for separating gel: 0.14 g ammonium persulfate in 100 ml water.

H. Eluting buffer: 28.8 g glycine and 6 g tris made to 2 l with water.

I. Tracking dye: 2.5 mg bromophenol blue in 50 ml water.

Procedure

A Canalco Model 12 apparatus was used for disc electrophoresis. Gel columns consisted of two separately polymerized gels, the stacking gel (3% acrylamide) and the separating gel

(7% acrylamide). They were prepared in glass tubing (64 mm x 5 mm id) in the following way: The lower end of each glass tube was plugged with a rubber stopper and 250 µl 40% sucrose was placed in the bottom by means of a syringe. Stacking gel was prepared by mixing the solutions B, D, E and F, in the proportions 1:2:1:4. One hundred and fifty µl of this solution was layered on top of the sucrose and overlayered with water to prevent curvature of the gel due to the meniscus. Polymerization was carried out by exposing the gels to a fluorescent light for 30 minutes. The separating gel was formed immediately before use by mixing solutions A, C, F and G in the proportions 1:2:1:4. The layer of water in the tube was removed, the tube was filled with the separating gel solution to 3 mm from the top and overlayered with water. Polymerization was complete in about 30 minutes. At the end of this time, the rubber stoppers were removed and the tubes were fixed in the upper buffer reservoir, with the stacking gel at the top. The two reservoirs of the electrophoresis apparatus were filled with solution H (eluting buffer). Care was taken to expel air bubbles from the ends of the tubes.

Enzyme solutions were prepared in 40% sucrose and to 1 ml of each was added 50 µl of the tracking dye (solution I). The enzyme solutions (50 to 250 µl) were carefully layered into the space on top of the stacking gel in the columns. Electrophoresis was started at a constant current of 2 ma per gel with the positive electrode connected to the lower buffer

reservoir. Electrophoresis was carried out in a refrigerator at 4° and the progress of electrophoresis could easily be followed by watching the disc of tracking dye form in the stacking gel and then move into the separating gel ahead of the bands of protein. When the dye had travelled near the end of the gel, current was turned off and gels were removed with the aid of a fine wire (syringe cleaner).

Isoelectric Focusing on Polyacrylamide

Reagents

Several of the reagents were the same as the reagents for disc electrophoresis.

C. Acrylamide monomer: 28 g acrylamide and 0.735 g bis made to 100 ml with water.

E. Catalyst: 4 mg riboflavin in 100 ml water.

F. 40% sucrose solution.

I. Tracking dye: 2.5 mg bromophenol blue in 50 ml water.

J. 0.46 ml TEMED in 100 ml water.

K. 40% ampholine, pH3-pH10 (LKB, Sweden)

L. Cathode solution: 35 ml ethanolamine in 1750 ml water.

M. Anode solution: 25 ml phosphoric acid in 1750 ml water.

Procedure

The apparatus was the same one described for discontinuous polyacrylamide electrophoresis. The lower end of each glass tube was plugged with a rubber stopper, and 100 µl of 40% sucrose was placed in the bottom by means of a syringe. A gel solution was prepared by mixing solutions C, F and J in the proportions 2:2:1. For each sample, the following solution was prepared: Between 5 and 50 µl sample, 100 µl solution J, 25 µl solution K and water to a final volume of 0.4 ml. Immediately before use, 0.6 ml of the prepared gel solution was mixed with the 0.4 ml of the sample solution and carefully layered over the 40% sucrose in the tube. It was overlayered with water and photopolymerized by exposure to fluorescent light for one hour. The sucrose and water at each end were removed and the tubes inserted in the upper buffer reservoir. Solution L was placed in the upper reservoir and solution M in the lower reservoir. The whole apparatus was run in a refrigerator at 4^{o} with the cathode in the upper reservoir.

Voltage was set at 150 v and an initial current of 2 ma/gel resulted. After about one hour, the current was about 0.5 ma/gel which indicated the carrier ampholytes were at their final equilibrium position. (This was further indicated by the movement of the bromophenol blue to the anode end of the gels.) Electrophoresis was continued for four hours. The gels were removed with the aid of a thin wire.

Stains for Acrylamide Gels

Detection of Protein

Staining of protein was carried out with Amido black dye or with Coomassie blue dye (354).

When staining was with Amido black, the gels were fixed immediately after removal from the tubes by immersion in 15% acetic acid for 15 to 30 minutes. They were transferred to the staining solution which contained 1 g of Amido black in 200 ml 7% acetic acid for one hour. Destaining was achieved with running tap water overnight. The protein bands stained blue-black with an almost colorless background.

When staining was with Coomassie blue, the gels were fixed immediately after removal from the tubes by immersion in 10% TCA for one hour. They were transferred to 0.025% Coomassie blue in 10% TCA for three hours, then transferred in 10% TCA. The protein bands stained a bright blue. On standing 24 hours, the background became almost colorless.

After isoelectric focusing, the fixing step is particularly important since the ampholine in the gel binds protein stains, particularly Coomassie blue. For Coomassie blue, it is necessary to wash the gels many hours with several changes of 10% TCA in order to obtain a good background.

Detection of Amylase

Amylase activity in gels can be detected by the starch-iodine reaction. Several techniques have been proposed.

Direct staining of the polyacrylamide gel has been accomplished by incubation with soluble starch followed by treatment with iodine reagent (50, 355). In another technique, starch was incorporated in the polyacrylamide gel before electrophoresis. After electrophoresis (in the cold), the gel was incubated and then treated with the iodine reagent (46). An indirect method involves taking a "contact print" of the polyacrylamide gel (49, 53). This technique consists of allowing the amylase to diffuse from the gel onto a plate containing starch, then treating the plate with the iodine reagent.

These methods were all tried. Direct staining of the gel with soluble starch was very poor since very little starch could diffuse into the acrylamide gel resulting in a very weak background and poor definition of activity bands. When 0.2% starch was incorporated into the polyacrylamide gel, the electrophoretic migration of amylase was greatly reduced, and a "smear" of amylase activity was seen between the origin and the main amylase band. The indirect, "contact print" method was the most successful in detecting amylase activity in a 7% polyacrylamide gel. The bands of amylase activity were very sharp.

A further advantage of this method was that after a "contact print" was taken, the polyacrylamide gel could be used for other analyses such as staining for protein, isolation of enzyme or radioactive counting. The following adaptation of Wolf and Taylor's (49) method was used for amylase detection in polyacrylamide gels.

Reagents

Starch-agar substrate: 1 g agarose (Mann Research Laboratories) 0.25 g soluble starch and 0.25 g amylopectin were dissolved in 100 ml 0.02 phosphate buffer pH 6.9, 0.067 M NaCl by heating in a boiling water bath.

Iodine reagent: I_2 (0.01 M) and KI (0.01 M) were dissolved in distilled water.

Procedure

Plates were prepared by spreading 2 ml of starch agar substrate (heated in a water bath) onto microscope slides. Polyacrylamide gels were removed from their glass tubes immediately after completion of electrophoresis, rinsed with water, blotted and placed on starch agar plates. Gels were compressed to about 3 mm by grooved plexiglas plates and incubated at room temperature for 3 to 15 minutes. The slide was placed in the iodine reagent for 20 - 30 seconds, rinsed with water, then with 7% acetic acid. The amylase position was indicated by clear zones on a dark purple field. The stained plate could be stored by placing a microscope slide on top, and setting it in an upright position in a dark jar containing 7% acetic acid.

Preparation of Polyacrylamide Gels for Liquid Scintillation Spectrometry

The solubilization of polyacrylamide gels with hydrogen peroxide has been described by Tishler and Epstein (356). Solubilized gel slices were then easily dispersed in toluene based scintillation fluid with NCS solubilizer for efficient counting of radioactivity. In the procedure described below, this method was used to permit the counting of gels which had been stained for protein.

Polyacrylamide gels which had been stained for protein were sliced transversely into thin discs. This was done either into equal segments of 1.27 mm by the device of Chrambach (357) (Canalco gel slicer) or else each stained protein zone was sliced with a razor blade. In either case, the thin slices were placed in conical plastic sample cups and allowed to dry in a drying oven (80 - 90^{o}) for 3 - 4 hours. The dried, shrunken discs were covered with 0.1 or 0.2 ml of 30% hydrogen peroxide. The cups were tightly capped and placed in a water bath at 50^{o} until solubilization of the gel was complete (2 to 8 hours). The solubilized gels were dispersed in 15 ml of scintillation fluid with the aid of 1.0 ml NCS solubilizer. The resulting clear solutions were counted in the usual way and quench correction was made by external standardization or with the aid of a quench correction curve. Efficiency in counting ^{14}C was high (75 - 80%). The dyed slices became completely colorless on incubation with hydrogen peroxide.

Liquid Scintillation Counting

All liquid scintillation counting was carried out using a solution of 0.4% Omnifluor (New England Nuclear) in toluene. The counting was done in a Mark 1 liquid scintillation counter (Nuclear Chicago). Any aqueous samples or protein samples were dissolved in NCS solubilizer (New England Nuclear) before being dispersed in 15 ml of scintillation fluid. Other organic samples were readily soluble in 10 ml of scintillation fluid. Two channels were monitored in order to detect any quenching effects by the channels ratio method. Using sets of quenched ^{14}C and ^{3}H standards (New England Nuclear), quench correction curves were made. The NCS reagent which quenches moderately was used routinely in the solubilization of isolated ^{14}C-labeled amylase samples. In this case, the scintillation counter was operated at the balance point for ^{14}C with NCS quenching. At this point, counting efficiency was insensitive to quenching over a fairly wide range (figure 33). Thus most samples were counted at maximum efficiency.

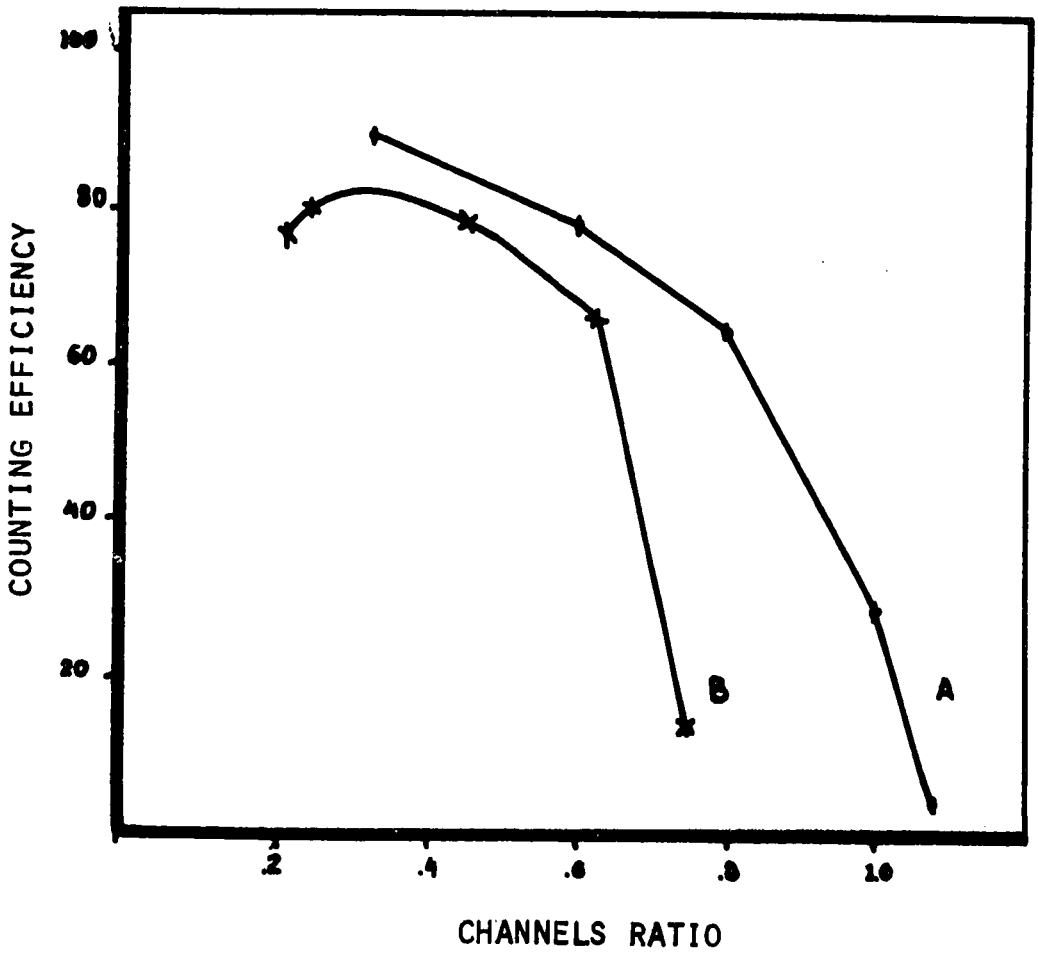


Figure 33 . Quench correction curves for ^{14}C scintillation counting : A - counting at maximum efficiency for unquenched ^{14}C samples ;
B - counting at maximum efficiency for ^{14}C samples, quenched with NCS solubilizer.

SECTION III

EXPERIMENTAL

PART I

AMYLASE BIOSYNTHESIS IN THE RAT PAROTID GLAND

Preliminary Experiments

Introduction

Under normal conditions, the secretory components of the rat parotid gland vary in a cyclic manner. Since eating is a known stimulus for salivation, and since rats feed mainly at night, the secretory proteins and amylase in parotid were lowest in the early morning and highest in the late afternoon (184). The amylase content in the morning was close to half the amylase level in the afternoon. Much greater differences in parotid amylase have been produced by artificial stimulation of salivary secretion. Injection of pilocarpine into starved rats resulted in a 60% decrease in amylase (148). The minimum was reached about ten hours after injection. This was followed by a slow recovery of amylase until it leveled off at about 48 hours to 90% of the original level. A different depletion-repletion cycle was observed after injection of isoproterenol (133). Isoproterenol-stimulated salivation resulted in a 98% depletion at two hours and complete recovery at about 18 hours after stimulation.

Experiment I

The rats used in this experiment were Sprague-Dawley males weighing between 190 and 310 g. Salivary secretion was induced by intra peritoneal injection of 10 mg of isoproterenol sulfate. (Solutions of isoproterenol sulfate were prepared at a concentration of 10 mg/ml in isotonic saline immediately before injection.) All rats were starved at least 24 hours before injection. At various times between 1 and 20 hours after injection, rats were killed by a sharp blow on the head, followed by exsanguination. The parotid glands were rapidly removed and placed in 1 ml of ice-cold saline. Within four hours, each gland was minced and homogenized in 0.02 M phosphate buffer pH 6.9 containing 0.007 M NaCl and 0.05% triton X-100. Homogenization was accomplished with a motor driven ground glass tissue grinder with a conical pestle. Cellular debris was removed by centrifugation at 2000 x g for ten minutes. Amylase was determined by the automated Bernfeld method and protein was determined by the automated biuret method as described in the Analytical Section.

The results of this experiment are summarized in figure 34 and Table 13. Injection of isoproterenol resulted in an immediate drop in amylase content from the control level of 13766 ± 722 units to 716 ± 117 at 1 hour. The minimum value, 342 ± 39 at 2½ hours, represented a 97% depletion of parotid amylase. Amylase activity increased at an almost steady rate after 4½ hours. At 20 hours, however, it was still less than half the control level. Protein levels

TABLE 13

Amylase and protein levels in rat parotid glands after isoproterenol injection: Experiment I

Time (hr)	Number of rats	Rat Wt. (g)	Gland Wt. (mg)	Total Amylase (units/gland)	Total Protein (mg/gland)
0	8	230±11	256±9.5	13766±722	35.0±1.5
1	6	255±12	175±5.5	716±117	17.7±0.8
1½	6	218±10	182±10	436±53	17.9±1.4
2	8	245±19	180±9	460±50	16.0±0.9
2½	6	214±13	179±5	342±39	16.3±0.7
4½	6	234±21	217±8	399±31	17.1±1.1
8	6	253±10	182±8	2141±476	21.4±1.0
12	8	313±4	185±7	3066±270	20.8±0.6
16	6	302±11	215±5	5190±380	23.1±0.8
20	5	280±19	217±11	6113±549	26.4±0.5

Parotid glands were removed from rats at the stated time after i.p. injection of 10 mg isoproterenol.

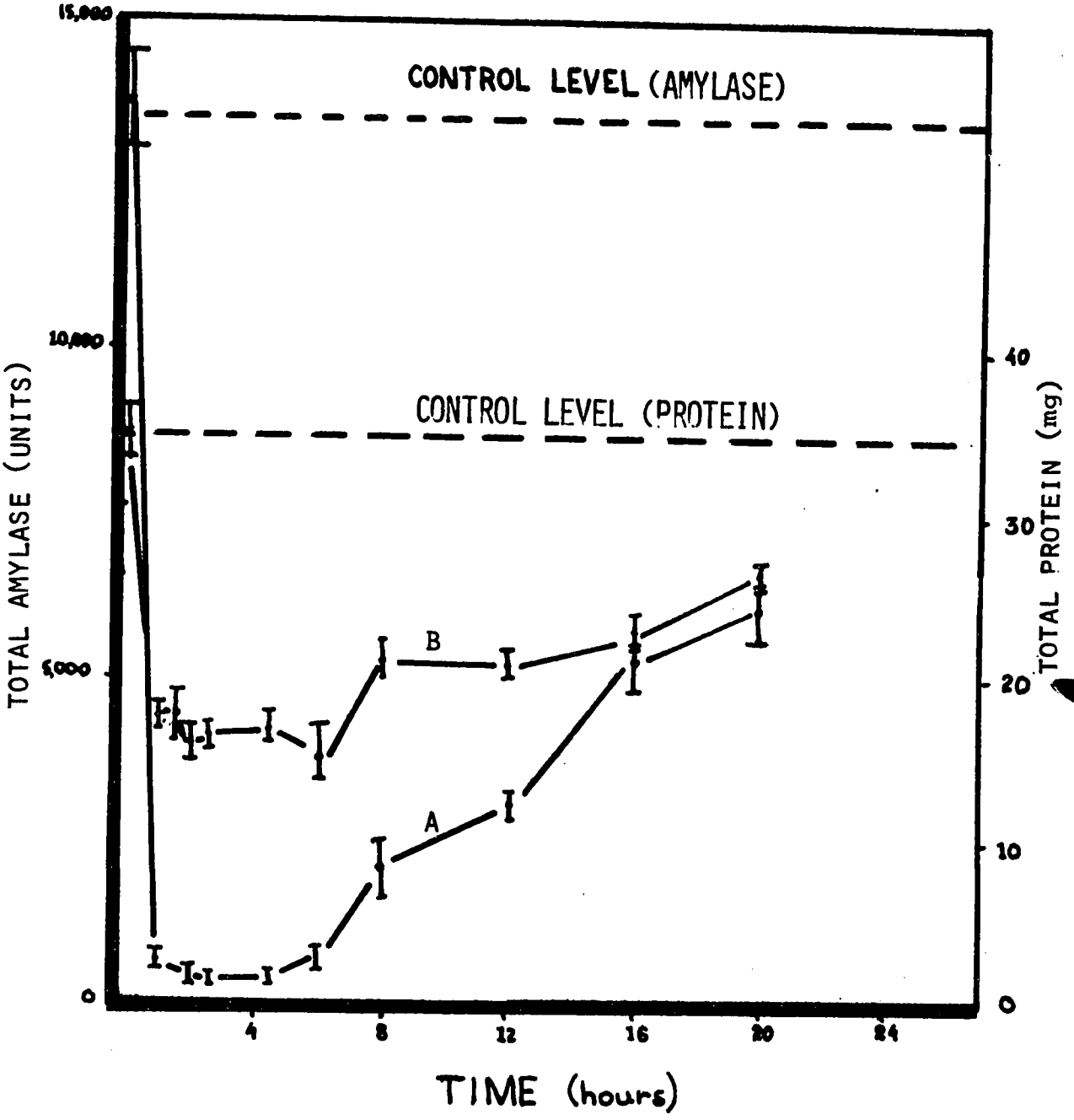


Figure 34 . Total amylase and protein in rat parotid gland homogenates at different times after injection of 10 mg of isoproterenol. (Results of Experiment I): A - amylase ; B - protein. Mean values with standard errors of the mean are indicated.

changed similarly, except that the changes were much less dramatic. The minimum protein content was at 2 hours when it was about half the control value. It also increased after 4½ hours, but did not recover completely to the control level.

These results were in some ways similar to previous studies. The immediate response to isoproterenol was the same as that described by Byrt (133). Recovery of amylase content was not, however, complete by 18 hours. The recovery part of the curve was more similar to the recovery from pilocarpine injection (148). Several explanations are possible for this. The experimental conditions for the times 12, 16 and 20 hours were slightly different from the others. In the times, 0 to 8 hours, the rats were injected exactly 24 hours after being starved. This was in the morning. The rats at 12, 16 and 20 hours were injected either in the afternoon or evening after having been starved since the previous morning. (This difference in injection time was to enable the rats to be killed and the subsequent amylase and protein analysis to be carried out at a more convenient time.) This means that the time of starvation was greater for these times. The slow recovery of parotid amylase and protein may reflect this. It is of interest that in an experiment in which rats were injected with pilocarpine after 48 hours starvation (148), repletion of amylase was very slow. This slow recovery may have been influenced by the long starvation period used.

Several changes in procedure were suggested by this experiment. The size of the rats was considered to be too large. Smaller rats were easier to handle, and their parotid glands were also much more easily removed. The amount of isoproterenol (10 mg per rat) was possibly too large to allow complete metabolism of the drug. It was noted in several rats that some effects of the drug, such as raised hair, tachycardia and frightened appearance, were detectable after two hours. The excess isoproterenol could possibly affect the general metabolism of the rat enough to decrease enzyme biosynthesis. The excess drug might also result in secretion of newly synthesized amylase. For these reasons, in subsequent experiments, 5 mg isoproterenol/rat was used.

Experiment II

The experimental set-up was the same as for Experiment I except for the following: The rats were much smaller, between 140 and 200 g, but mostly close to 160 g. The rats were starved for 18 hours and injection of 5 mg of isoproterenol was always between 8:30 and 9:30 A.M. The glands from times 12 and 15 hours were stored in ice-cold saline until the following morning when they were homogenized and assayed. (This was possible because no change in amylase activity during storage for up to 18 hours in ice-cold saline was observed, as indicated in Table 4.)

The results of this experiments are summarized in Table 14 and figure 35. Depletion of salivary amylase (from 17667 to 472 units was 97% complete at four hours. Repletion occurred at an ever increasing rate between 4 and 15 hours and the control level had been reached by 24 hours. Protein levels were similar, reaching a minimum (50% of control level) at two hours, and returning to the control level by 15 hours.

Analysis of Variance

In order to ascertain the magnitude of variance attributable to several levels of variation in this type of experiment, a nested (hierarchic) analysis of variance was made on data of Experiment II as described by Sokal and Rohlf (358). Values of total parotid amylase and protein, parotid weight and rat weight, for four rats in each of the groups, 0, 2 and 12 hours were analyzed. (In Table 15, the values of group mean, standard deviation, standard error and coefficient of variation are shown for each group.) Total variance of amylase, protein and gland weight measurements, was partitioned into three levels: 1. between glands, within animals (due to the differences between the pair of parotid glands in each animal); 2. between animals within times, (due to the variation among glands from different animals which have received the same treatment; 3. between times, (due to the treatment alone with no contribution from the other two levels).

TABLE 14

Amylase and protein levels in rat parotid glands after isoproterenol injection: Experiment II

Time (hr)	Number of Rats	Rat Wt. (g)	Gland Wt. (mg)	Total Amylase (units/gland)	Total Protein (mg/gland)
0	6	164±6	178±5	17667±1032	27.6±1.5
2	5	163±9	149±13	513±66	13.4±0.9
4	5	161±9	142±8	472±51	14.5±0.7
8	3	188±14	160±15	2437±264	14.2±1.5
12	4	166±7	148±9	4625±270	17.2±0.9
15	5	160±3	216±23	10500±1129	27.3±2.6
24	5	158±8	207±12	15700±1243	32.1±1.4

Parotid glands were removed from rats at the stated times after i.p. injection of 5 mg isoproterenol.

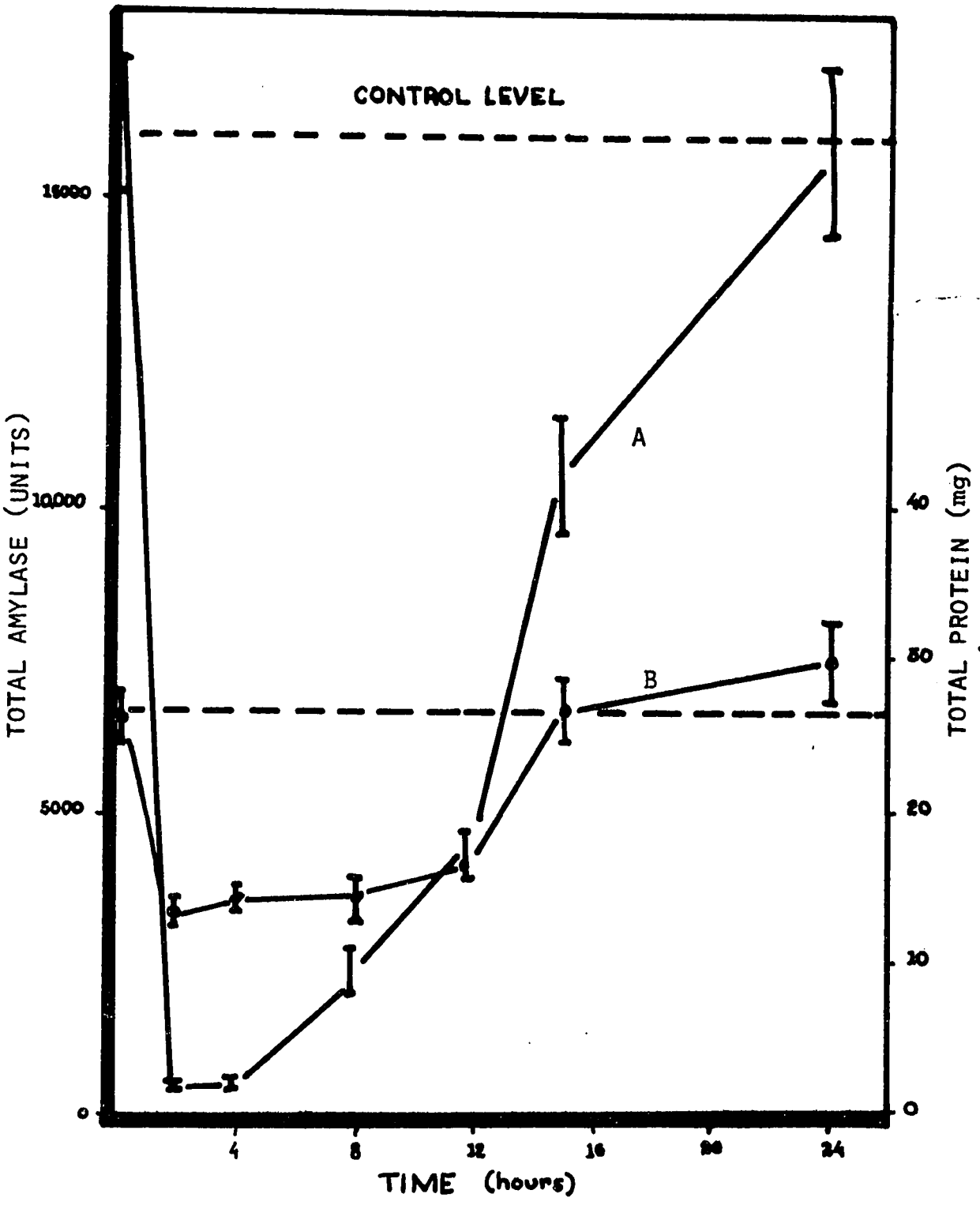


Figure 35 . Total amylase and protein in rat parotid gland homogenates at different times after injection of 5 mg of isoproterenol. (Results of Experiment II): A - amylase ; B - protein. Mean values with standard errors of the mean are indicated.

TABLE 15

Comparison of variations in total amylase, total protein, parotid weight and body weight, 0, 2 and 12 hours after injection of isoproterenol into starved rats.

	N	Mean	S.D.	S.E.	C.V.
AMYLASE (units/gland)					
0 hour	8	16625	3688	1304	22.2
2 hour	8	578	172	61	29.7
12 hour	8	4625	765	270	16.5
Total	24	7276	7265	1483	99.8
PROTEIN (mg/gland)					
0 hour	8	25.3	3.3	1.1	12.9
2 hour	8	14.8	1.8	0.6	12.4
12 hour	8	17.2	2.6	0.9	15.1
Total	24	19.1	5.2	1.1	27.4
GLAND WEIGHT (mg)					
0 hour	8	174.8	16.9	5.6	9.7
2 hour	8	164.2	27.3	9.6	16.6
12 hour	8	147.6	26.2	9.2	17.7
Total	24	162.2	25.5	5.2	15.7
RAT WEIGHT (g)					
0 hour	4	160	5.8	2.9	3.6
2 hour	4	171.2	11.1	5.5	6.5
12 hour	4	166.2	14.4	7.2	8.6
Total	12	165.8	11.0	3.2	6.7

S.D. - standard deviation ; S.E. - standard error ;
C.V. - coefficient of variation ; N - number of measurements

The analysis of variance is summarized in Table 16. The variance among animals within times is a significant component of variance in each of the three sets of measurements. This is a reflection of biological variation. (Analytical variation was included in variance between glands, within animals). Variance due to treatments (between times) was highly significant for amylase ($P<.01$), significant for protein ($P<.05$), but not significant for gland weight. These results were similar to the changes found during the normal diurnal variation of the parotid gland (184). It is of interest that while no significant difference was found in gland (wet) weight, differences were found in dry weight (184). The percentage distribution of variance is also shown in Table 16. It is apparent that variance between glands is low in comparison to the other sources of variance (generally about one-third the variation among animals). This suggested that in future experiments, it would be of advantage to pool the glands from each rat. A greater numbers of rats could then be conveniently treated and assayed.

The coefficients of variation for each group in Table 15 indicate that variations within each treatment were similar for both protein and gland weight, greater for amylase and lower for rat weight. The variation in gland weight may have been due to the difficulty in removing all the surrounding connective tissue from the glands before weighing. Because of this relatively large variation, we consider it less reliable to express the enzyme activity as amylase per gram

TABLE 16

Analysis of variance of total amylase, total protein and parotid gland weight in rats at the times 0, 2 and 12 hours after injection of isoproterenol.

Source of Variance	df	S.S.	M.S.	F	Variance	Variance (% of total)
GLAND WEIGHT						
Between times	2	2,964	1,482	1.36	49.4	7.2
Between animals, within times	9	9,780.5	1,086.7	5.87*	451.0	65.8
Between glands, within animals	12	2,218	184.8		184.8	27.0
Total	23	14,962.5			685.2	100
AMYLASE						
Between times	2	1,114,412,033.4	557,706,016.7	515.965**	69,578,139.93	99.1
Between animals, within times	9	97,928,075	1,080,897.22	8.146**	474,108	.7
Between glands, within animals	12	1,592,175	132,681.25		132,681.25	.2
Total	23	1,213,932,283.4			70,184,929	100
PROTEIN						
Between times	2	485.78	242.89	18.583**	28.73	78.8
Between animals, within times	9	117.66	13.07	5.538	5.36	14.6
Between glands, within animals	12	28.35	2.36		2.36	6.4
Total	23	631.79			36.44	100

$F_{0.05}$ (2,9) = 4.26 $F_{0.05}$ (9,12) = 2.80 df - degrees of freedom
$F_{0.01}$ (2,9) = 8.02 $F_{0.01}$ (9,12) = 4.39 S.S. - sums of squares
* P<0.05 M.S. - mean square
**P<0.01

wet weight. When the rats used in any experiment varied only slightly in size, amylase was best expressed as total units per gland (or per pair of glands). Amylase values were normalized in rats of a wide size range by expressing them as amylase units per rat weight.

Repletion of Parotid Amylase: Incorporation of Amino-Acids into Amylase

Experimental Design

This experiment was similar to the two preliminary experiments. The parotid amylase levels were followed during the depletion and repletion of amylase caused by isoproterenol injection. Amino acid incorporation was followed by injection of ^{14}C-amino acids 30 minutes before killing and by isolation of amylase.

Rats were starved overnight (about 18 hours) and were injected with 5 mg isoproterenol in the morning. They were killed (a blow on the head followed by bleeding) at various times after injection of isoproterenol, and the parotid glands immediately removed. Exactly 30 minutes before killing, an i.p. injection of 5 μCi of ^{14}C-mixed amino acids (New England Nuclear) in 1 ml isotonic saline was made. The excised parotid glands were stored in 1 ml ice-cold isotonic saline until it was convenient to homogenize them, generally within one hour. Each pair of parotid glands was minced with scissors, then thoroughly homogenized in phosphate buffer (0.02 M phosphate

0.007 M NaCl, 0.5% triton X-100, pH 6.9), and made to 10 ml. After centrifugation at 2000 x g for 10 minutes to remove cell debris, an aliquot was taken for amylase determination. Total amylase (per rat, both parotid glands) was calculated. The remaining homogenate was treated with ethanol and glycogen as described in the Analytical Section to isolate amylase.

The amylase-glycogen complex was dissolved in a small volume of phosphate buffer by incubation for one hour at room temperature. An aliquot was taken and suitably diluted for amylase and protein determinations. The remaining solution was dissolved in NCS solubilizer then dispersed in toluene-base scintillation liquid (Omnifluor). Scintillation counting was carried out as described in the Analytical Section, and the channels ratio method was used to calculate counting efficiency.

Amylase was determined by the automated Bernfeld method, and protein, by the automated Lowry method. (Pre-weighed centrifuge tubes were used at all stages of amylase isolation so that sample volume could be accurately estimated by weighing.)

Total amylase was calculated from the amylase assay of the whole homogenate. Amylase activity in the isolated enzyme preparation as well as total isolated protein and DPM were also calculated. Total DPM (DPM incorporated in the complete amylase content of the parotid glands) was calculated from the DPM of isolated amylase and the recovery of amylase after isolation. A summary of the results is shown in Table 17.

TABLE 17

Total amylase activity and radioactivity of amylase isolated from parotid homogenates at different times after isoprenaline injection and 30 minutes after injection of ^{14}C-amino acids.

Time (hr:min)	Number of Rats	Rat Wt. (g)	PAROTID AMYLASE			^{14}C-AMINO ACID INCORPORATION		
			Total*	Units / Rat Wt.	Specific† Activity	Total (DPM)	DPM / Amylase units	DPM / mg Amylase
0	10	175±6	27725±1852	158±16	3192±120	4172±289	0.15±.012	491±43
2:10	9	168±10	756±80	4.77±.62	1930±167	4056±803	5.09±.70	9736±1533
4:09	7	165±13	1072±158	6.58±.94	2258±104	9298±1081	9.40±1.30	20686±2807
6:05	11	167±5	1612±146	9.63±.65	2705±125	11139±1064	7.92±.88	21506±2533
8:06	7	148±4	4349±452	30.1±3.7	3039±114	14681±966	3.61±.52	10900±1450
11:02	3	169±8	9464±932	55.8±2.9	3218±48	13738±243	1.48±.16	4622±301
12:10	4	180±6	12937±1418	71.5±6.5	2972±63	13249±890	1.16±.24	3787±608
15:34	6	178±7	22429±1378	126±5.0	2820±42	11634±1544	0.53±.062	1526±194
18:33	4	158±1	27631±3156	174±19	2878±48	10672±938	0.39±.027	1161±85
22:38	7	165±11	28194±3587	162±16	3014±110	8620±1339	0.36±.063	1122±216
24:20	7	158±6	22187±1845	141±12	3025±76	7097±767	0.33±.034	989±120
25:42	4	154±9	22418±1670	146±13	3463±59	5037±538	0.23±.032	793±108
28:03	4	167±8	27605±2643	166±18	2950±35	6720±1160	0.25±.012	727±49
30:13	4	147±5	21677±2993	150±24	3424±28	5924±1892	0.24±.042	830±137
22:38 30:13	26	159±4		153±7.1	3139±64	7013±615		

Mean values are given with the standard deviation of the mean. Amylase units are expressed as mg maltose monohydrate released/3 min at 37°, ^{14}C-amino acid incorporation is expressed as DPM of isolated parotid amylase.

* Amylase units/pair of parotid glands.

† Specific activity of isolated purified amylase, units/mg protein.

Results

Changes in amylase levels: The changes in amylase levels at various times after injection of isoproterenol are represented in figure 36. Amylase was expressed as total parotid amylase per weight of animal (units/g). This was necessary since the size of rats varied between groups.

The general pattern of amylase depletion and repletion in figure 36 was similar to that shown in figure 35. Amylase was lowest two hours after injection, slowly increased between 2 and 6 hours and rose at an increasing rate to 18 hours when the control level was reached. Amylase remained at close to the control level from 18 to 30 hours. This pattern of repletion was very similar to the one described by Byrt (133) as exponential or logarithmic.

The mathematical relationship of amylase levels during the repletion phase (2 to 18 hours) was explored. The linear, exponential, and quadratic regression equations of best fit were estimated from the amylase/rat weight values from Table 17. The following equations were used:

Linear equation; $A = a - bt$

Exponential equation; $A = e^{(a - bt)}$

$\log A = a - bt$

Quadratic equation; $A = k + at - bt^2$

$\frac{A - k}{t} = a - bt$

where; A = Amylase/rat wt (units/g)

t = time (minutes after isoproterenol injection)

a, b, k = constants

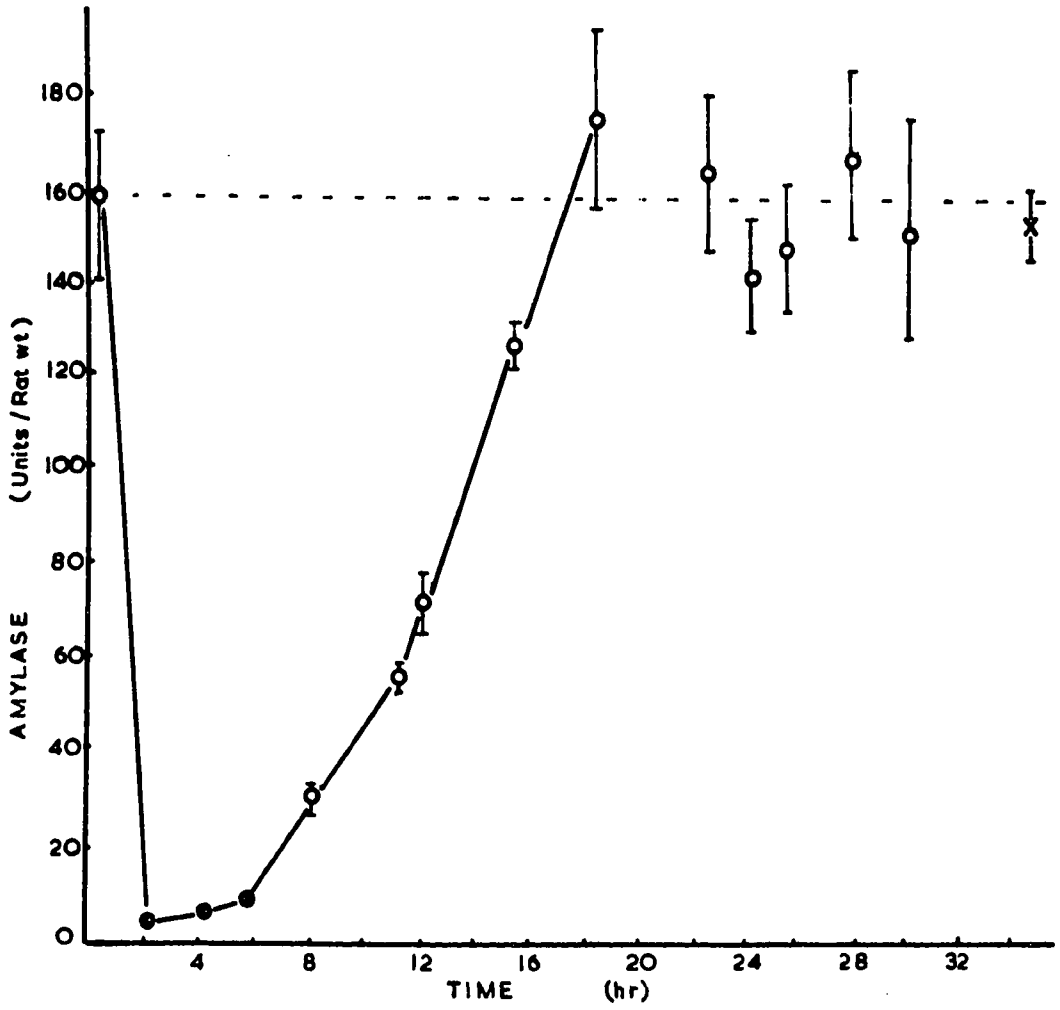


Figure 36 . The change in total parotid amylase following injection of starved rats with 5 mg of isoproterenol. Mean values with 5% confidence limits are shown. The last point, (|–x–|) is the pooled value for times between 22 and 30 hours.

The best linear regressions of A, log A and $\frac{A - k}{t}$, on t were found. Calculations were made on an Olivetti Underwood Programma desk-top computer using the least squares program (359). The general regression formula is $Y = a - bt$. The values A, log A, or $\frac{A - k}{t}$ were substituted for Y in this formula and the statistics; a, b, k, r (correlation coefficient) and Sy.x (standard error of estimate) were calculated. These data are listed in Table 18.

These data were used to calculate the variance due to regression and variance about regression for each of the above regressions. The following calculations were made:

$$SS = (Y - \bar{Y})^2$$

$$SS_R = r^2(Y - \bar{Y})^2$$

$$SS_r = (1 - r^2)\ (Y - \bar{Y})^2$$

where; Y = individual value

$\bar{Y}$ = mean value

SS = total sums of squares

SS_R = SS due to regression

SS_r = SS about regression

Variances between times and within times were calculated by computer with an analysis of variance program (360).

The analysis of variance for the regressions and times is shown in Table 19. The closeness of fit of the data to the regression lines, was tested by comparing the variance about the regression coefficient with the sample variance (variance within times). If the variance about the

TABLE 18

Regression of parotid amylase on time.

Regression Formula	a	b	r	Sy.x
LINEAR				
$A = a - bt$	-38.017	1.682	0.937	1.930
EXPONENTIAL				
$\log A = a - bt$	0.431	0.177	0.955	0.169
QUADRATIC				
$\frac{A - k}{t} = a - bt$				
where; K = 6	-3.909	1.727	0.950	1.718
K = 7	-4.532	1.788	0.954	1.699
K = 8	-5.158	1.850	0.958	1.691
K = 9	-5.782	1.912	0.961	1.690

The data of Table 17 for the times 2:10 to 18:33 were used to calculate linear, exponential and quadratic regression lines from the basic regression formula $Y = a + bt$ by the method of least squares, where:

Y = amylase values expressed as A, log A or $\frac{A - k}{t}$

A = amylase activity (units/rat wt)
a, b, k = constants
r = correlation coefficient
Sy.x = standard error of estimate

TABLE 19

Analysis of variance of regression lines for repletion of amylase.

Source of Variance	df	S.S.	M.S.	F†
LINEAR				
Total	49	1535.40		
Due to Regression	1	1348.90	1348.90	2.53*
About Regression	48	186.50	3.8855	
Between Times	7	1470.80	210.11	
Within Times	42	64.60	1.536	
EXPONENTIAL				
Total	49	16.405		
Due to Regression	1	14.973	14.973	1.52
About Regression	48	1.432	0.02983	
Between Times	7	15.586	2.227	
Within Times	42	9.820	0.01953	
QUADRATIC				
Total	49	1886.68		
Due to Regression	2	1743.67	871.84	1.078
About Regression	47	143.03	3.043	
Between Times	7	1768.10	252.58	
Within Times	42	118.575	2.823	

df - degrees of freedom; S.S. - sums of squares; M.S. - mean square

† $F = \frac{\text{M.S. About Regression}}{\text{M.S. Within Times}}$

* $P<0.01$

regression is significantly higher than sample variance, then the regression is not a good fit.

The best fit for the linear equation is shown in Table 18; $A = 1.68t - 38.02$. The F value of 2.53 (Table 19) was highly significant ($P<0.01$). This indicates that the best linear fit to the data is significantly different from the data. In other words, the repletion of amylase was not linear with time.

The best fit of the exponential equation was; $\log A = 0.431 + 0.177\ t$. The F value of 1.52 was not significant. This means that the data for amylase repletion are consistent with an exponential regression.

The quadratic regression equation was more complicated than either the exponential or linear equations, since one more constant value (k) needed to be estimated. Several values of k were therefore used in calculating the regression equations. It was only after analysis of variance and calculation of F ratios for each of these equations that the equation of best fit could be found. In figure 37, these F ratios are plotted against values of k. The F values decreased between k = 6 and k = 9, but leveled off between k = 8 and k = 9. The value of k = 9 was chosen as being very close to the best fit. The quadratic equation of best fit, from Tables 18 and 19, is:

$$\frac{A - 9}{t} = -5.782 + 1.912\ t$$

$$\text{or } A = 9 - 5.782\ t + 1.912\ t^2$$

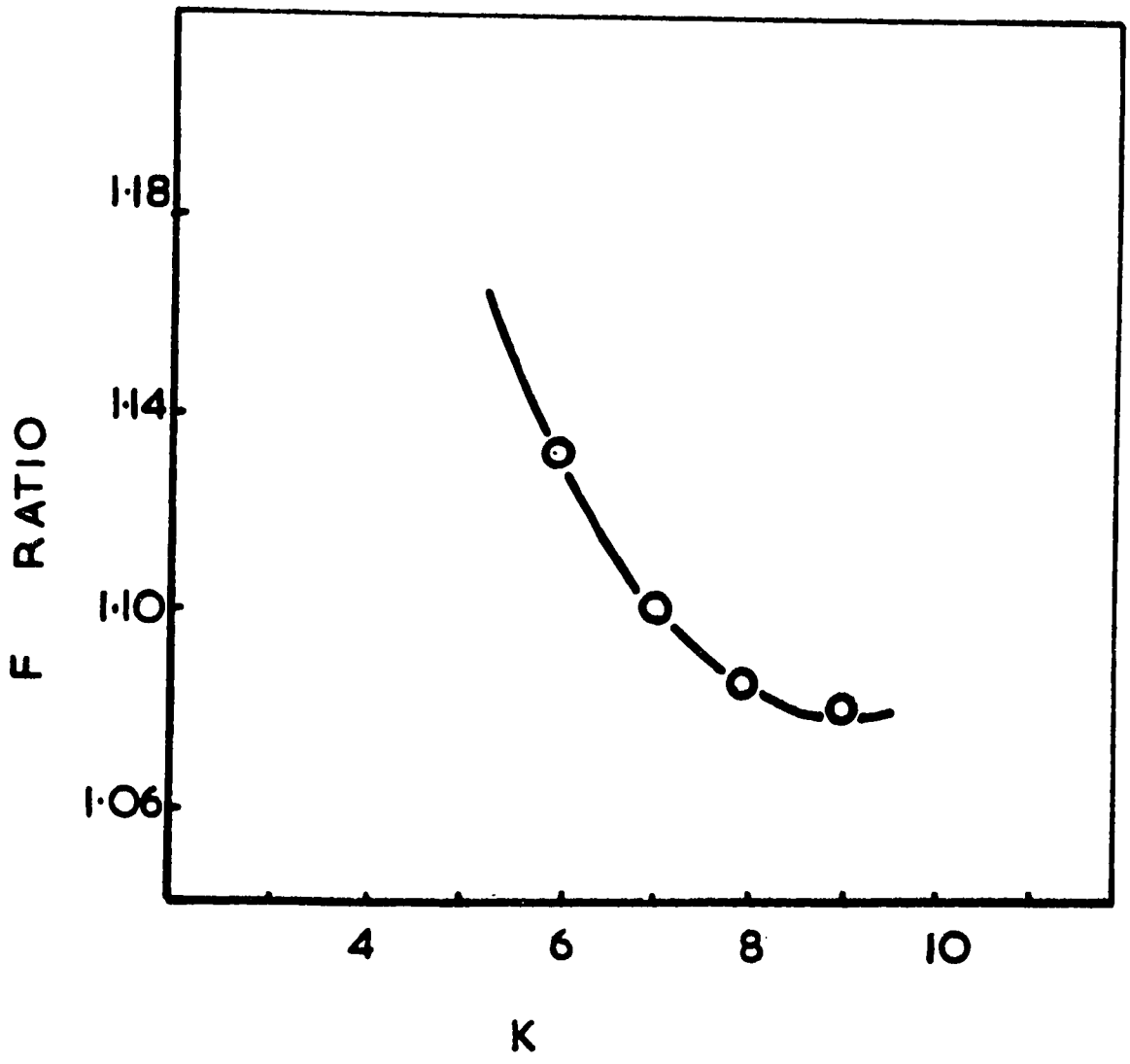


Figure 37. Determination of constants of the quadratic regression formula for amylase repletion: Variation of F ratio with K ;

$$\frac{A - K}{t} = a + bt, \quad F = \frac{\text{MS regression}}{\text{MS error}}$$

The F value of 1.078 was not significant and thus the data of amylase repletion were consistent with the quadratic regression.

Linear regression lines with their 5% confidence limits were plotted with the aid of a modified Olivetti Underwood program (361). Figures 38, 39 and 40 show the regression and confidence limits for the linear, exponential and quadratic equations respectively. Experimental points from Table 17 are also shown in these figures. The exponential and quadratic regression lines (figures 39, 40) obviously fit the experimental points much better than the linear regression line (figure 38).

Changes in amino acid incorporation: The change in incorporation of ^{14}C-amino acids into total parotid amylase after isoproterenol stimulation is shown in figure 41. No change in incorporation was observed during the secretion phase (first 2 hours). During the early repletion phase, the radioactive incorporation increased rapidly to a level about 3 times the basal level, 8 hours after isoproterenol injection. A decrease in incorporation occurred between 8 and 24 hours, but even after 24 hours, the incorporation was about 40% above the basal level. All points between 4 and 24 hours were significantly higher than the basal level. The points at 25 hours and 30 hours were not significantly different from the basal level. When the values for the points 25 hours, 28 hours and 30 hours were pooled, the mean was 5798±730, which was significantly higher than the control (4172±289).

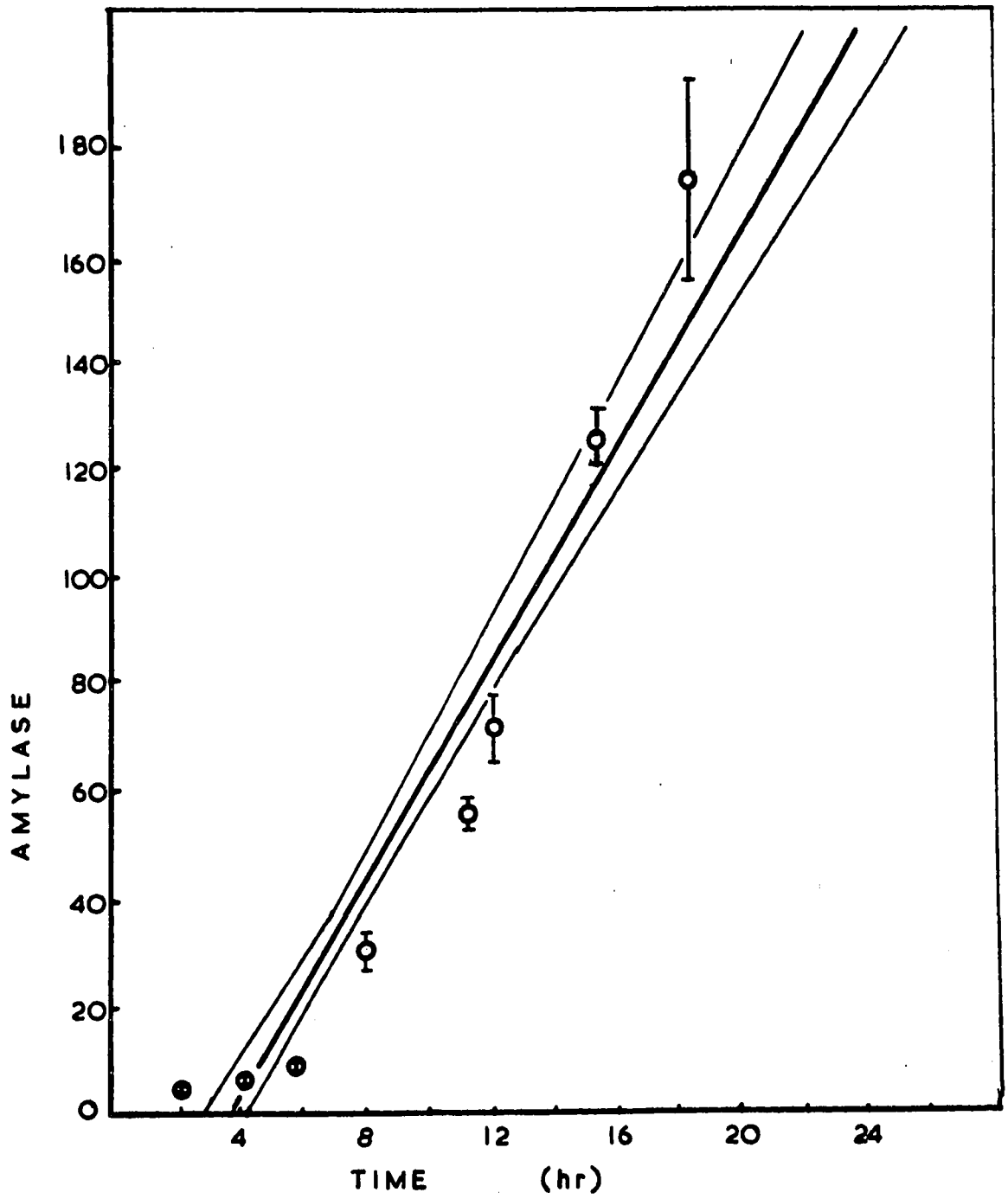


Figure 38 . Linear regression line of amylase repletion, with 5% confidence limits. Sample means with standard errors of the mean are also shown.

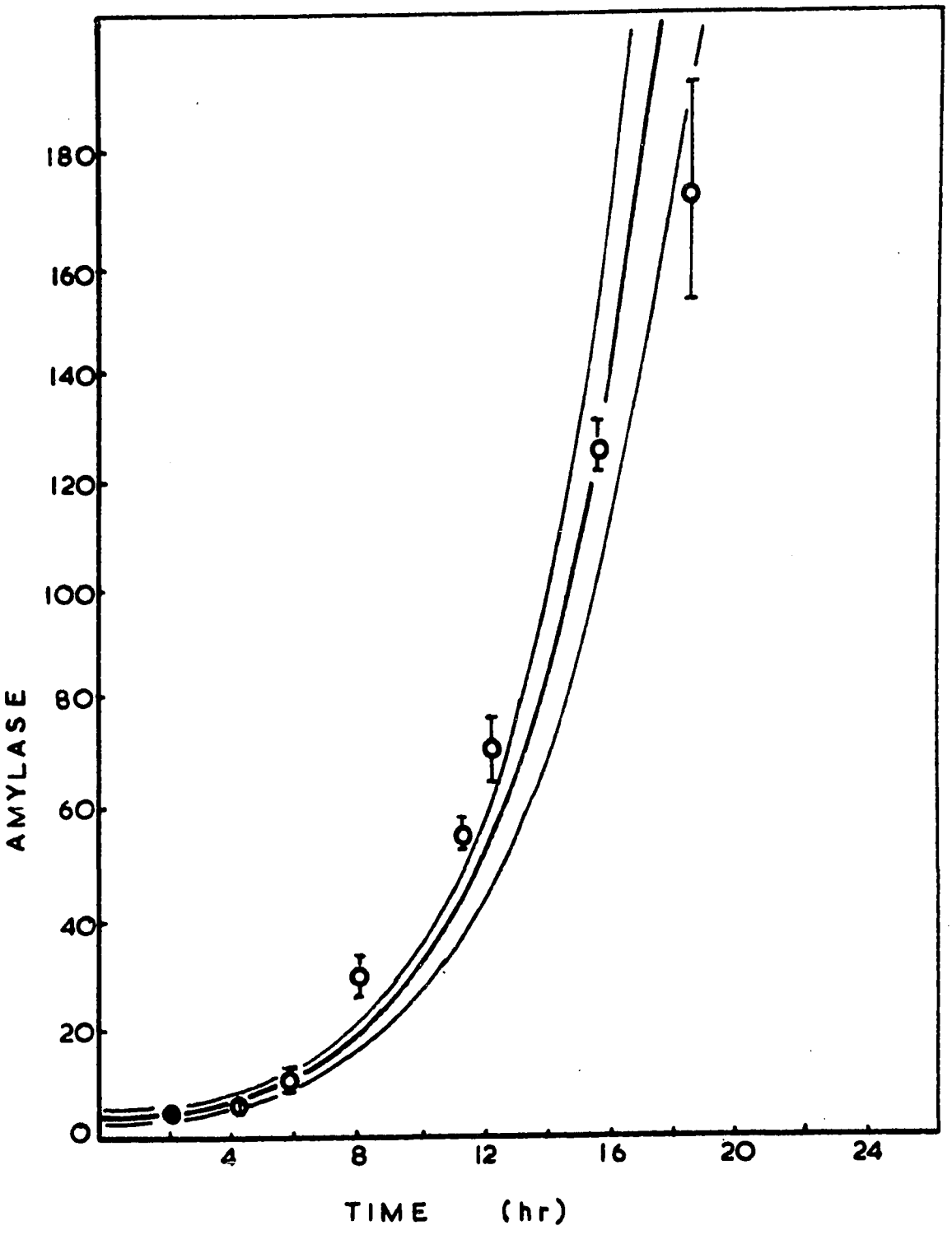


Figure 39 . Logarithmic regression line of amylase repletion, with 5% confidence limits. Sample means with standard errors of the mean are also shown.

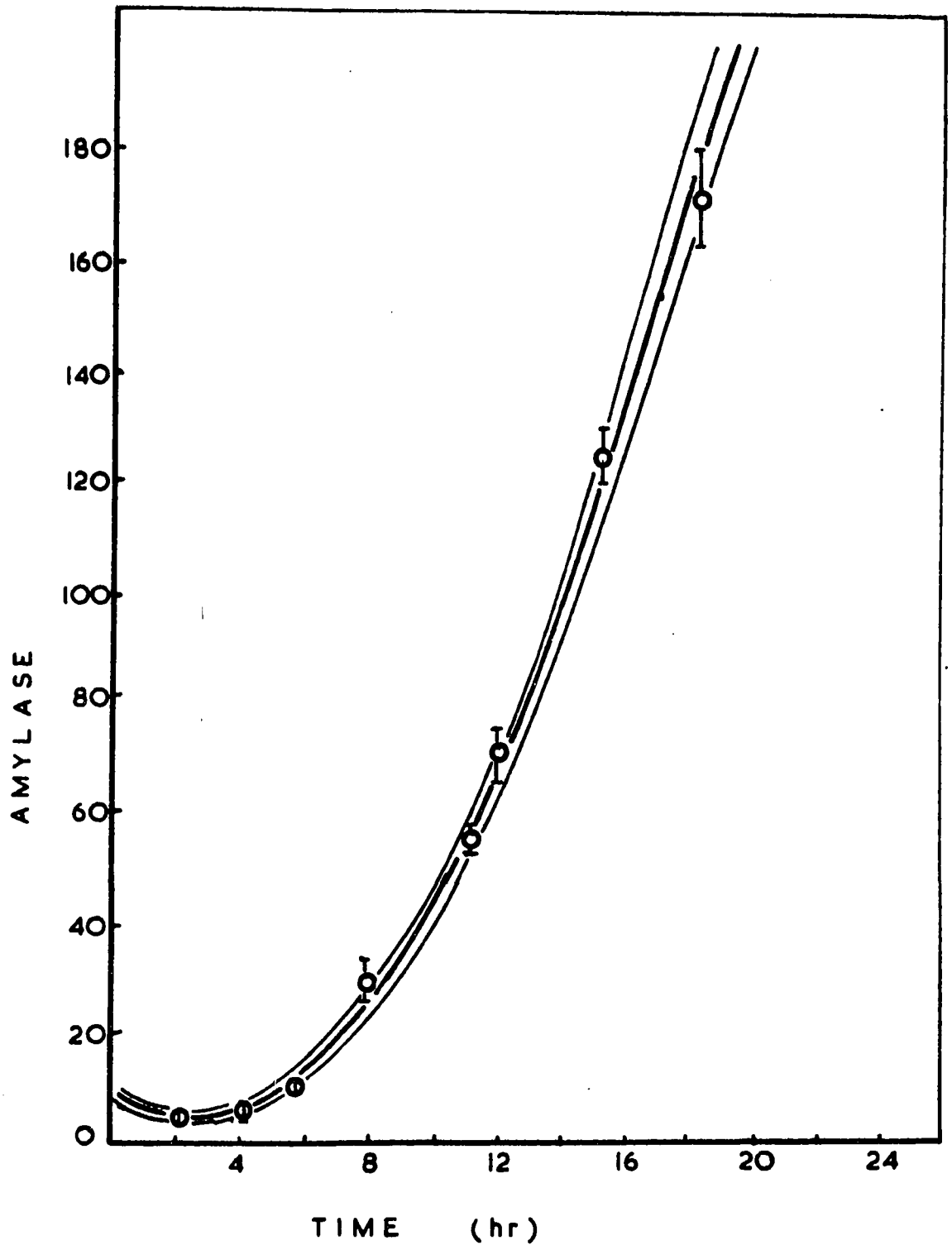


Figure 40 . Quadratic regression line of amylase repletion, with 5% confidence limits. Sample means with standard errors of the mean are also shown.

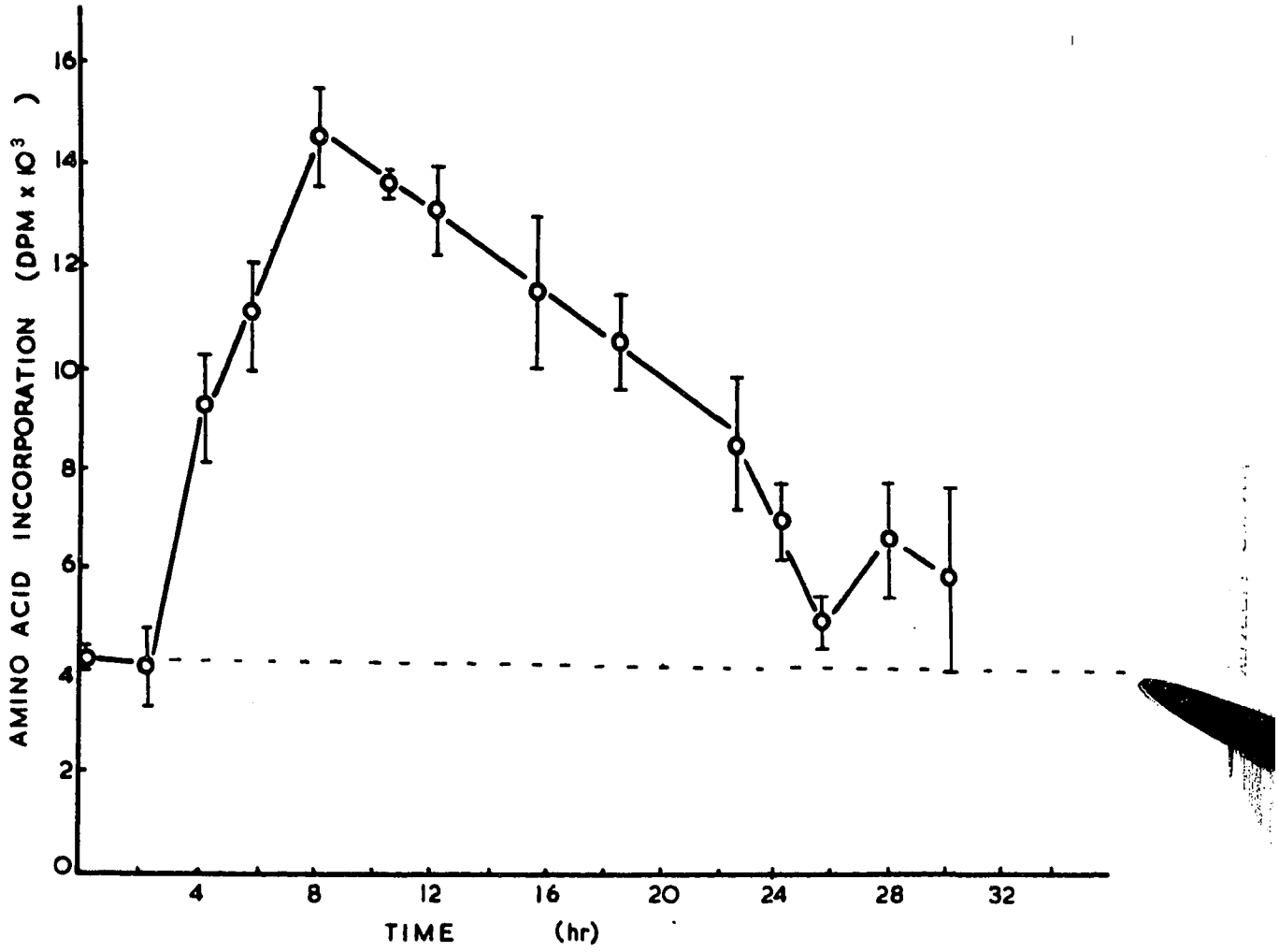


Figure 41 . Total incorporation of amino acids into rat parotid amylase at various times after injection of isoproterenol. Mixed ^{14}C-amino acids (5 μC) were injected 30 minutes before the rats were sacrificed.

Gromet-Elhanan and Winnick (148) have followed amino acid incorporation into total rat parotid protein. The pattern they described was very different from the one described here. Salivary stimulation was followed by an immediate decrease in amino acid incorporation. At 8 hours, the incorporation was only slightly above basal, but reached maximum (a 2-fold increase) at 18 hours.

In figure 42, the striking difference between amino acid incorporation and the repletion of parotid amylase is shown. Eight hours after isoproterenol, when amino acid incorporation was maximum, the increase in amylase was low. Maximum increases in amylase occurred when amino acid incorporation was falling off. To explain changes in parotid amylase level by de novo enzyme biosynthesis alone, one would expect to observe a linearly increasing incorporation of amino acids during the logarithmic increase of amylase levels (2 to 18 hours). This should be followed by an immediate return to basal levels. The relationship between amylase levels and amino acid incorporation is more easily seen in figure 43, in which increments in the amylase levels of 100 minutes have been plotted against time. These increases describe a straight line which indicates a steadily increasing rate of amylase biosynthesis. Assuming that de novo synthesis, alone, is responsible for the increase in amylase, one would expect the rate of amino acid incorporation to parallel the incremental increase in amylase levels.

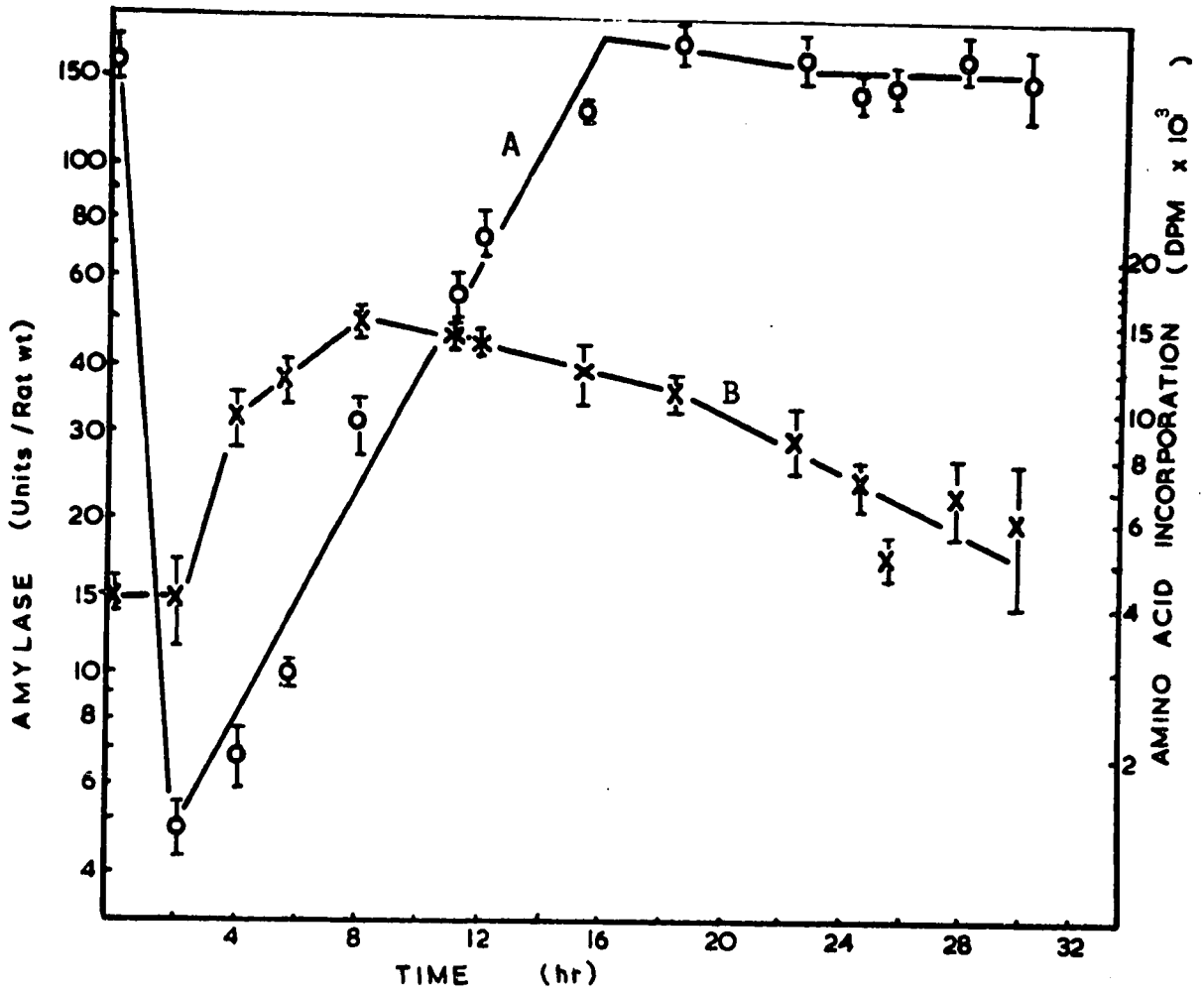


Figure 42 . Comparison of total amylase and amino acid incorporation : A - total parotid amylase ; B - total incorporation. Both scales are logarithmic.

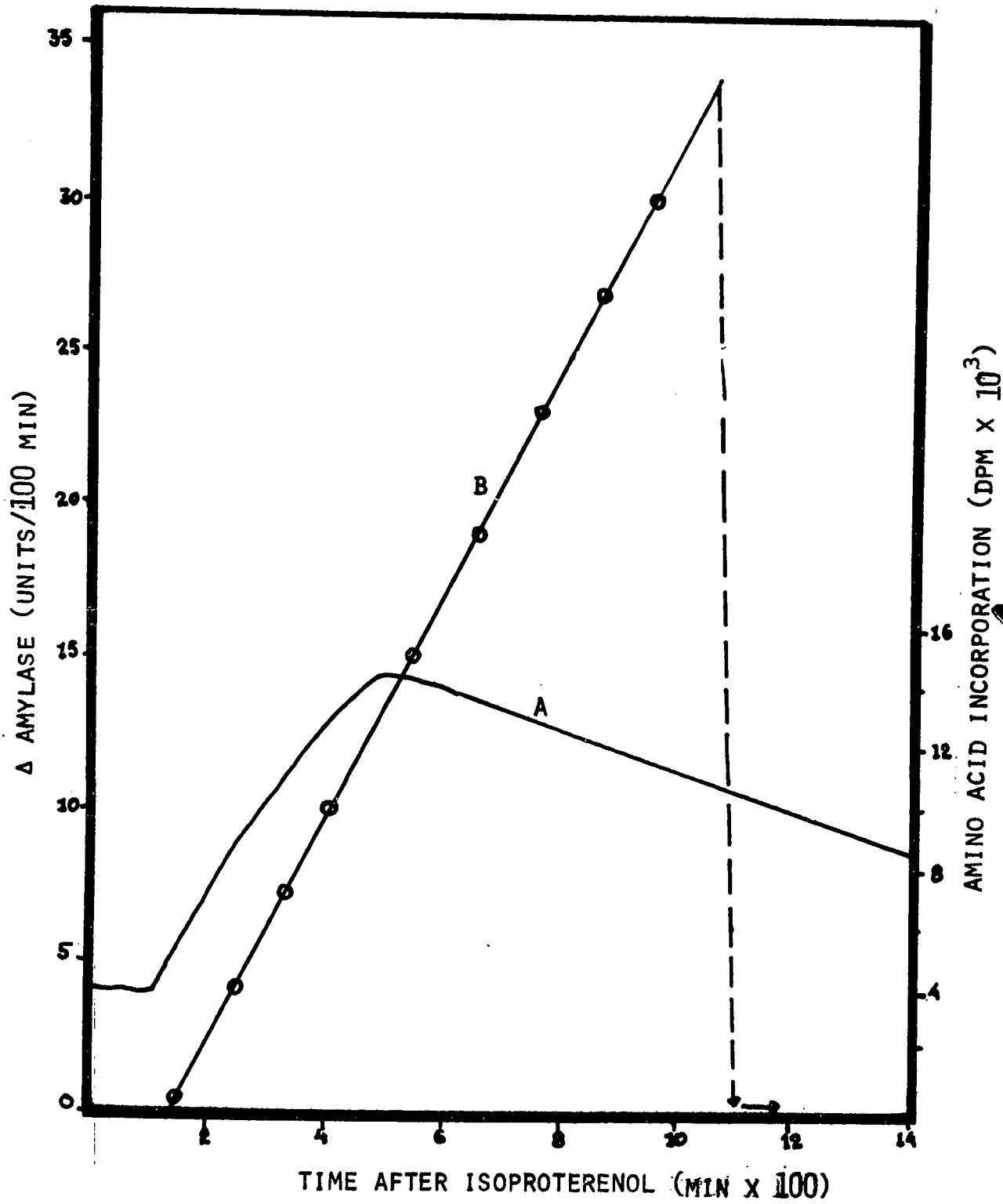


Figure 43 . Comparison of the incremental increase in amylase with incorporation of amino acids into amylase: A - DPM ; B - $_{\Delta}$ Amylase, the change in total amylase during 100 minute intervals.

Contamination of Isolated Amylase

The isolation of amylase contaminated with excess ^{14}C-amino acids would, of course, affect the results shown in figure 41. Contamination of the isolated amylase-glycogen preparations was tested in the following way: Rats were injected with ^{14}C-amino acids, and amylase isolated in the usual way. Each isolated amylase preparation was divided into two equal volumes. One was counted in the usual way. The other was precipitated with an excess of cold 20% TCA containing unlabeled casein hydrolysate. The precipitate was centrifuged, resuspended in TCA and recentrifuged It was finally washed with absolute alcohol and ether, dried, dissolved in NCS solubilizer and counted in the usual way.

Table 20 shows that a recovery of nearly 95% of radioactivity after precipitation with TCA was usually attained, suggesting a non-protein, ^{14}C-contamination of 5 or 6 per cent. Even in the depleted gland (sample #5), the maximum contamination was 12 per cent. This contamination may, in fact, have been less since it is likely that during the extra manipulations of TCA precipitation, (especially the resuspension of the TCA precipitate) there would be a small loss of protein.

In Table 17, the specific enzymatic activity of the isolated amylase was usually close to 3000 amylase units/mg protein. At the times, 2 and 4 hours, however, the values were close to 2000. This suggested contamination of amylase

TABLE 20

Isolation of amylase: contamination of labeled parotid amylase with ^{14}C-amino acids.

Sample Number	Total Amylase (units/2 glands)	TOTAL DPM IN AMYLASE		
		Glycogen Precipitate	Re-precipitated with TCA	% Recovery in TCA ppt.
1	24300	1382	1301	94
2	28700	2502	2242	89
3	45200	3456	3240	95
4	37700	4150	3936	95
5	2080	3030	2660	88

by other proteins. This would result from decreased efficiency of amylase isolation at the low concentrations of amylase found in the depleted gland.

Contamination of isolated amylase with heavily labeled contaminant could seriously affect any studies of amino acid incorporation into amylase. In order to find the contribution of contaminating proteins to the total sample radioactivity, two sets of experiments were performed. In one, labeled amylase was isolated in the usual manner by precipitation on glycogen, except that a higher than usual level of contamination was ensured by using an excess of glycogen, and by not washing the glycogen-amylase precipitate. This experiment represents greater contamination than could be ordinarily expected to occur. In the other set of experiments, amylase was isolated with the usual care, in order to obtain low contamination.

Protein and amylase determinations were performed on all preparations to determine specific activity, and aliquots were taken for scintillation counting and for disc electrophoresis. After staining for protein, the disc electrophoresis gels were sliced and radioactivity was determined in the slices as described in the Analytical Section. Typical results are shown in figure 44.

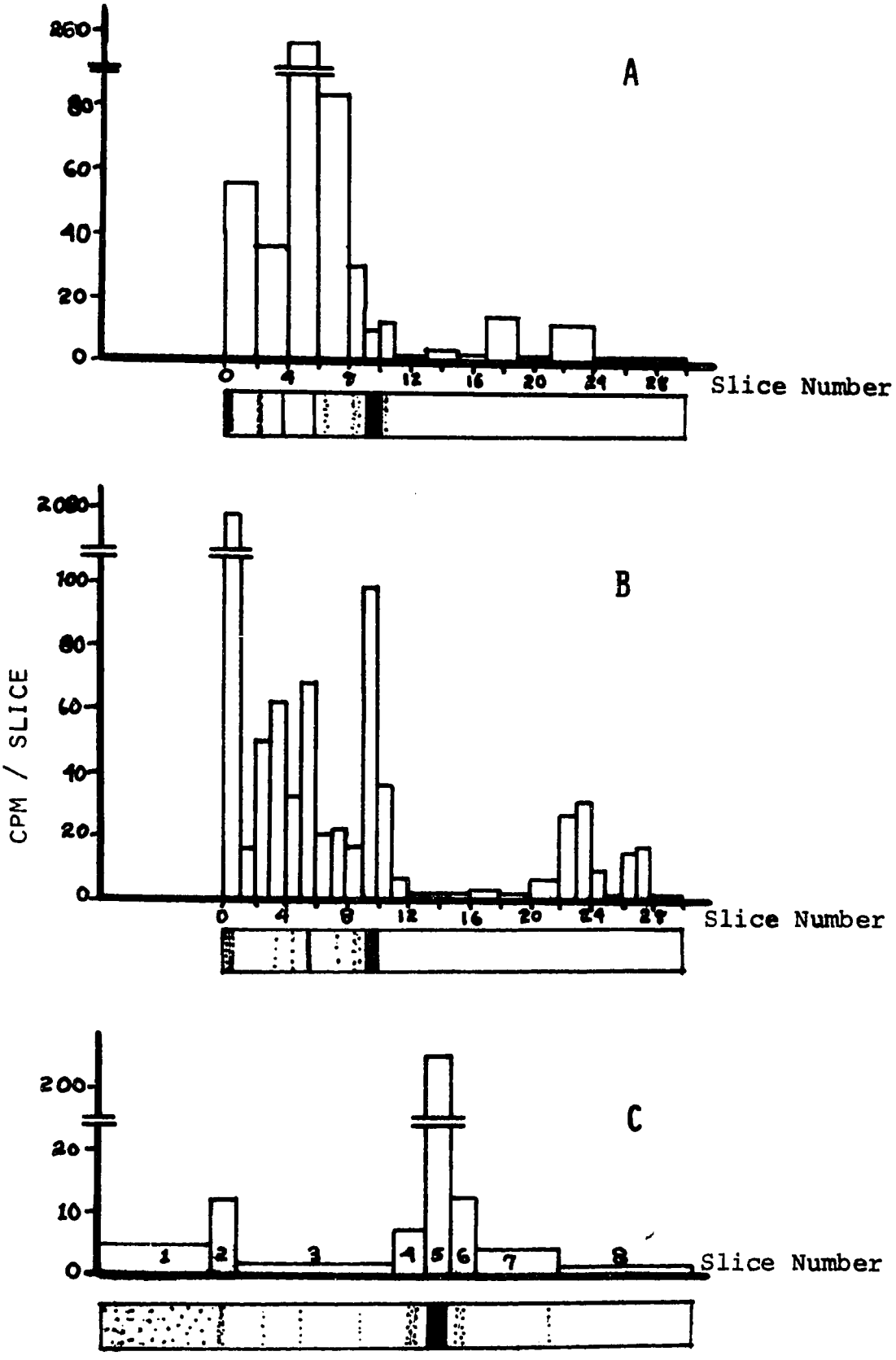


Figure 44 . Disc electrophoretic separation of ^{14}C-labeled amylase. Gels are shown stained for protein with amido black (A and B) or Coomassie brilliant blue (C). Samples A and B contained more non-amylase protein than sample C.

Crude parotid homogenates revealed between 12 and 16 distinct bands, most of them very faint. The strongest band was amylase. When amylase was isolated with care, then subjected to disc electrophoresis and stained with amido black, only a single band was observed. Amylase, isolated with high contamination, separated into a very strong amylase band plus three or four other faint bands, (figure 44, A and B). The contamination resulted in a high proportion of radioactivity outside the heavily stained amylase band. The per cent of radioactivity in the amylase band is shown in Table 21. The two contaminated preparations (Rats 1 and 2) were much less pure than was normally obtained (as could be seen by the low specific activity (892 and 1057 units amylase/mg/rat). Gel C in figure 44 is a typical amylase preparation, isolated in the same way as gels A and B, except with the usual care to ensure low contamination. The sensitive stain, Coomassie blue, was used since only a single band could be observed after staining with amido black. In this case, each identifiable band was cut out and measured separately. Band 1 was the stacking gel, bands 4 and 6 were lightly staining bands on either side of the major band, 5, which was amylase.

In Tables 21 and 22, the results of these experiments are summarized. Total DPM is a sum of all the radioactivity found in all the bands. On this basis, amylase contained 81, 88 and 91% of the radioactivity when isolated from rats, 2 hours, 4 hours and 0 hours after isoproterenol injection. When gels

TABLE 21

Isolation of amylase: distribution of radioactivity of labeled amylase after disc electrophoresis.

Amylase Sample	Rat Number	Radioactivity in Gel (DPM) Total	In Major Amylase Bands	In Minor Amylase Bands	% in Amylase	Amylase Specific Activity (units/mg Protein)
HIGH CONTAMINATION						
2 hours	1	1008	53	-	5.2	1057
	2	3434	148	-	4.3	892
LOW CONTAMINATION						
2 hours	3	271	204	20	83	1442
	4	571	475	36	90	1305
	5	301	255	16	90	1673
	6	76	63	4	88	2293
4 hours	7	837	766	22	94	2142
	8	1001	874	27	90	2559
	9	344	302	10	92	2072
	10	584	507	18	90	2184
0 hours	11	392	525	16	91	3194
	12	712	646	16	93	3187
	13	738	690	13	95	3092

The amylase samples were isolated from parotid glands by precipitation on glycogen at the stated times after isoproterenol injection. The amylase was either isolated with care, to ensure low contamination (high specific activity) or with high contamination (low specific activity). Rats were given a 30 minute pulse of ^{14}C-amino acids before being sacrificed.

TABLE 22

Isolation of amylase: correction of amylase DPM due to contaminants.

	Rat Number	Rat Wt	TOTAL DPM IN AMYLASE Uncorrected	TOTAL DPM IN AMYLASE Corrected*	Total †	Specific‡ Activity
HIGH CONTAMINATION						
2 hours	1-2	245±7	74500±51300	3313±2108	2.31±0.05	975±83
LOW CONTAMINATION						
2 hours	3-7	152±2	5266±1800	4657±1600	5.49±1.00	1678±218
4 hours	8-12	143±5	7771±672	7166±485	7.06±1.17	2339±109
0 hours	13-15	139±8	3024±282	3024±282	93.6 ±25.4	3158±33
VALUES FROM TABLE 17						
2 hours		168±10	4395±793	-	4.77±.62	1930±167
4 hours		165±13	9298±1082	-	6.53±.94	2259±104
0 hours		175±6	4193±294	-	158±15.5	3192±120

* Correction of total DPM in amylase was on the basis of the % DPM in the main electrophoretic band of amylase shown in Table 21.

† Amylase units/2 glands

‡ Amylase units/mg protein

Results are mean values ± standard deviation of the mean.

were assayed for amylase activity (described in the chapter, Isoamylases), some slight amylase activity was found in bands other than the strongly staining, main amylase band. These bands corresponded to fractions 3, 4 and 6 of figure 44, gel C. The radioactivity of the minor amylase bands 2 and 4 are shown in Table 22 and are included in the calculation of per cent DPM in amylase.

The results of Table 21 are summarized in Table 22 and compared to values of Table 17. The correction of amylase DPM was made on the basis of per cent DPM in the main amylase band (Table 21). It is apparent that under the methods of amylase isolation used to obtain the results summarized in Table 17, the contamination was low. With amylase isolated from rats, 2 and 4 hours after isoproterenol injection, specific enzymatic activity was low. However, the contaminating protein contributed very little radioactivity.

De Novo Synthesis of Amylase and Non-Amylase Protein

Introduction

It has been suggested by Goldstein and Reddy (362, 363) that the change in amino acid incorporation into rat skeletal muscle is due to changes in the specific radioactivity of the precursor amino acid pool. This can be the result of changes in the composition of the amino acid pool, a change in the size of

the pool or a change in the rate of uptake of labeled amino acids by the tissue. It is possible that similar changes in rat parotid gland could account for some of the changes in incorporation into amylase.

Kridakorn (364, 365) observed a decrease in the free amino acid pool of both parotid and submaxillary glands in rats treated with epinephrine. This was under conditions which resulted in an increase of submaxillary amylase but no change in parotid amylase. Grand (152) found that in parotid gland slices, the composition of the amino acid pool remains constant during stimulation with epinephrine. Under the conditions of the experiment, secretion of amylase was induced, and two-fold and four-fold increases were found in incorporation of labeled amino acids into total cell protein and amylase respectively (150).

Epinephrine in the medium inhibited the uptake of radioactive precursors into the slices. However, when the slices were washed after exposure to epinephrine, prior to pulse labeling, uptake was normal (152). Grand showed that the increase in specific radioactivity in amylase and TCA insoluble protein three hours after treatment with epinephrine was accompanied by a 50% decrease in specific radioactivity of the amino acid pool. He concluded that changes in the labeling of the total protein and amylase represented changes in the rate of protein synthesis rather than precursor transport.

The changes he found in amylase and protein specific radioactivity could, on the other hand, be explained by the loss of preformed (unlabeled) amylase and protein due to secretion.

Changes in the RNA precursor pool in mouse salivary glands have also been shown (249). The specific radioactivity of the UTP pool increased more than two-fold one hour after injection of isoproterenol and remained higher than the control for 20 hours. The increased incorporation into RNA, which was found 12 hours after injection of isoproterenol, could be explained by changes in precursor pool rather than increase in RNA biosynthesis. There was, however, a significant decrease in RNA synthesis one hour after isoproterenol.

In a review of methods of measuring rates of enzyme biosynthesis, Shimke and Doyle (366) recommended the use of a single pulse-labeling with isotopic amino acid and subsequent isolation of enzyme to determine whether there is an accelerated rate of synthesis. They suggested that results be expressed as total incorporation of radioactivity into a specific protein relative to incorporation into total protein. That would, therefore, make a correction for any differences in dose of isotope or in the time-integrated amino acid precursor pool.

When applying this method to amylase in the parotid gland, it must be emphasized that amylase makes up a very large portion of the soluble protein, and also of the total amino acid incorporation into protein. If changes in incorporation into amylase are being investigated, then expressing total radioactivity incorporated into amylase,relative to that

incorporated into total protein, will not make a complete correction for changes in precursor pool because changes in amylase biosynthesis will also affect total protein incorporation. It should, therefore, be preferable to express results as incorporation into amylase relative to incorporation into total non-amylase protein.

Experimental Design

In many of the previously described experiments in which incorporation of ^{14}C-amino acids into total amylase was determined, an estimate of non-amylase amino acid incorporation was also made. In these experiments, amylase was precipitated on glycogen in a 40% alcohol homogenate of the parotid glands. Amino acid incorporation into amylase was determined from the precipitated amylase. The 40% alcohol supernatant was used for the determination of non-amylase incorporation. Starved male Sprague-Dawley rats were injected with 5 mg isoproterenol and killed at various intervals as previously described. They were treated with 5 μCi ^{14}C-amino acids exactly 30 minutes before killing. Amylase was isolated from the parotid glands in the usual way. The 40% alcohol supernatants from each group (time after isoproterenol) were pooled. Solid TCA was dissolved in the cold 40% ethanol supernatant until a concentration of 10% TCA was reached. It was centrifuged after 30 minutes.

The precipitated protein was resuspended in 10% TCA and centrifuged. The precipitate was washed with absolute alcohol. It was transferred quantitatively to scintillation vials and allowed to dry under vacuum. For counting, the dried TCA precipitate was moistened with one or two drops of water. About 1 ml of NCS solubilizer was added. This was usually sufficient to dissolve the protein on standing overnight. It was sometimes necessary to add more NCS solubilizer and gently warm the mixture to ensure complete solubilization. After addition of scintillation fluid, DPM was determined in the usual manner.

Results

The amino acid incorporation is shown in figure 45 and compared with incorporation into amylase in Table 23. There is no increase in incorporation into the non-amylase protein two hours after isoproterenol but incorporation almost doubles at four hours and a moderate increase occurs between four hours and 18 hours when it reaches maximum. This pattern was very similar to that found by Grommet-Elhanan and Winnick (148). They followed amino acid incorporation into total parotid protein after rats were treated with pilocarpine.

The incorporation into amylase relative to the incorporation into non-amylase protein is shown in figure 46, curve B. There was a two-fold increase in relative incorporation between 0 and 7 hours after isoproterenol. The level remained approximately double the basal level between 7 and 16 hours,

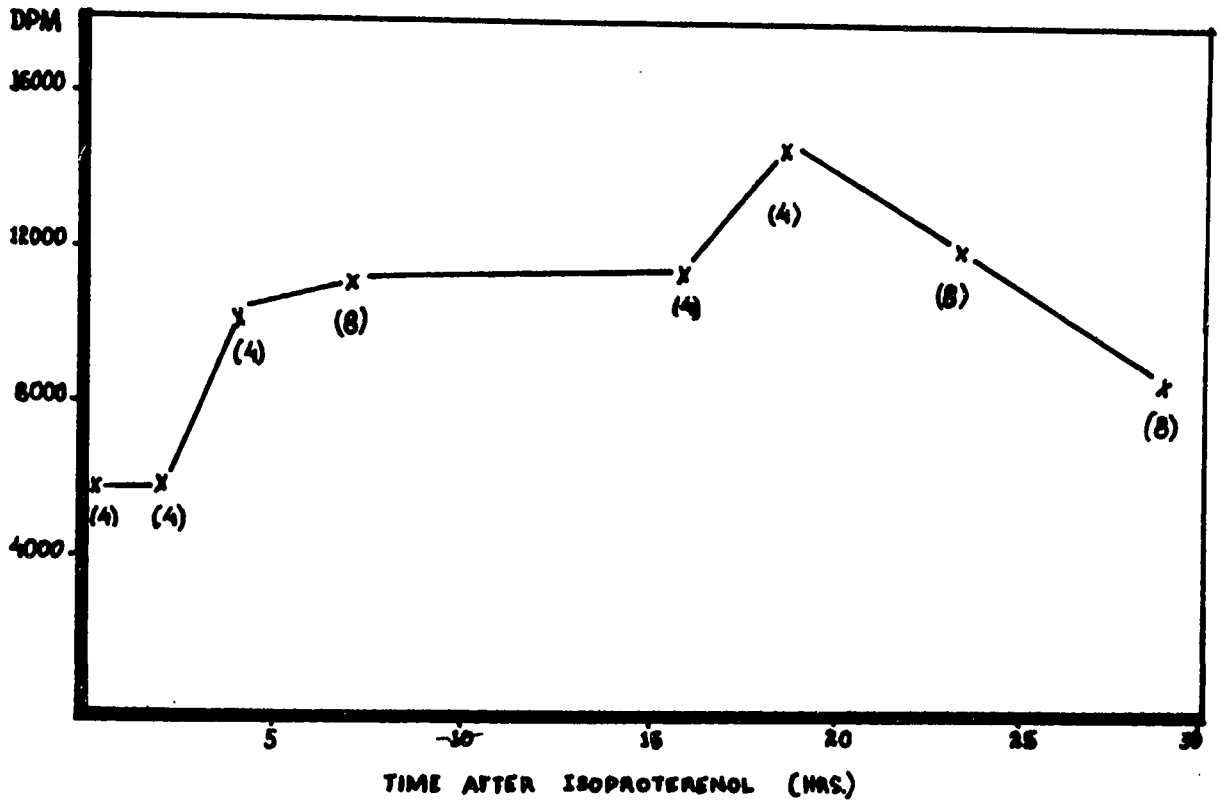


Figure 45 . Total incorporation of ^{14}C-amino acids (DPM) into non-amylase protein of the rat parotid gland, after isoproterenol injection. The number of animals used for each experimental point are indicated.

TABLE 23

Amino acid incorporation into amylase and non-amylase protein of rat parotid glands.

Time after Isoproterenol (hours)	Number of Rats	Total Incorporation (DPM)		Ratio Amylase DPM / Non-Amylase DPM
		Amylase	Non-Amylase	
0	4	2872	5960	.482
2	5	4815	6329	.761
4	4	7777	10220	.740
7	8	10679	11091	.963
16	4	10471	11379	.919
18.5	4	10672	14787	.722
23-24	8	6078	12124	.513
28-30	8	4791	8988	.533

The values of DPM are in most cases from the pooled parotid homogenates of several rats.

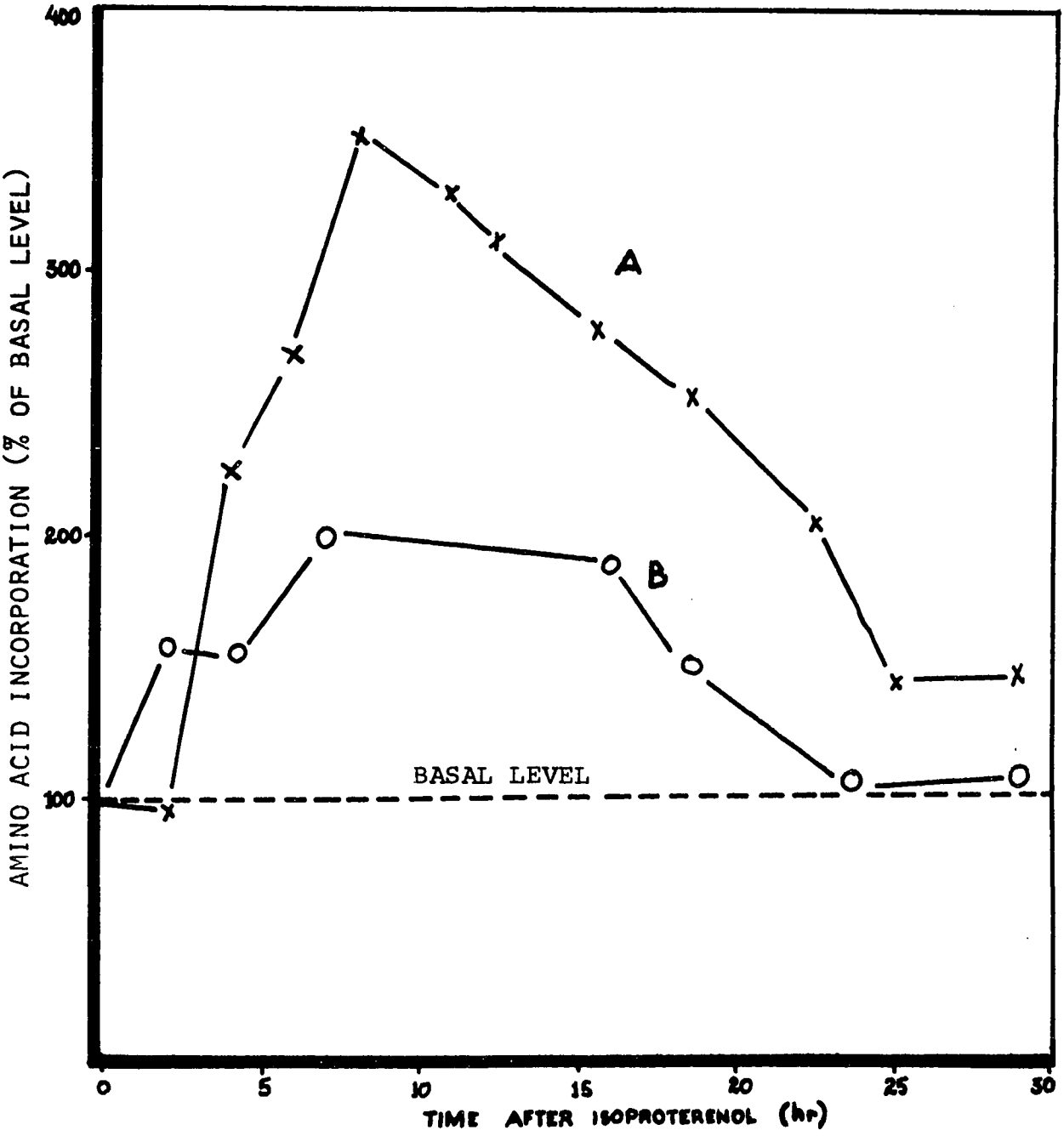


Figure 46 . De novo amylase biosynthesis during the depletion and repletion of parotid amylase : A - Amino acid incorporation into total parotid amylase (per cent of basal level); B - Ratio of incorporation into amylase to incorporation into non-amylase protein (per cent of basal level).

decreased at 18 hours and returned to almost the basal level at 23 hours. In figure 46, the total incorporation of labeled amino acids into amylase relative to non-amylase protein (curve B) is compared to the total incorporation into amylase (curve A). It is apparent that the changes in incorporation are similar but smaller when expressed relative to non-amylase protein.

The incorporation of ^{14}C-amino acids into non-amylase protein, shown in figure 45, most likely reflects a change in precursor amino acid specific radioactivity. It may, however, reflect to a certain extent, changes in general protein synthesis. Assuming these changes are due to changes in precursor, then the incorporation into amylase relative to incorporation into non-amylase protein (figure 46, curve B) represents changes in rates of amylase biosynthesis. If it is assumed that the changes in non-amylase incorporation reflect general changes in parotid gland protein synthesis, then the total incorporation of ^{14}C-amino acids into amylase reflects the rate of amylase biosynthesis.

In either case, a real increase in the rate of amylase biosynthesis occurs in the parotid gland during the repletion phase. This increase is between 2 and 3.5 fold.

Secretion of Amylase in Anaesthetized Rats

Introduction

Schucher and Hokin (366) questioned the assumption that stimulation of enzyme secretion in the pancreas leads to an

accelerated rate of enzyme synthesis. They suggested that "the so-called cycle of secretion and resynthesis of pancreatic enzymes, based on fall and subsequent rise in zymogen granule content of the pancreas following food or cholinergic stimulation may merely reflect a constant rate of enzyme synthesis with superimposed variations in secretion rate". The exponential rate in the concentration of amylase in the parotid gland (figure 42) might, by the same logic, be an artifact resulting from a decreasing rate of secretion superimposed on a more or less linear rate of amylase synthesis. It has been shown that amylase is secreted from the parotid gland only in response to a definite stimulation (367), and that the secretion of amylase after isoproterenol injection continues for only two hours (132). The latter study was made with whole saliva, however, rather than parotid saliva.

In order to study the direct response of amylase secretion to isoproterenol stimulation, parotid amylase secretion was continuously monitored in anaesthetized rats.

Experimental Design

Rats were prepared for continuous monitoring of amylase secretion as described in the Analytical Section. They were anaesthetized by i.p. injection of Nembutal or urethane. The parotid duct was exposed and attached to the collector and amylase was measured continuously as described. The rats were injected with isoproterenol during the course of

the experiment. The rats were sacrificed after completion of secretion, the parotid glands were removed and assayed for amylase.

Results

The flow of amylase from the parotid glands of several rats is shown in figures 47, 48 and 49. Conditions for these experiments are outlined in Table 24, which also indicates the total amylase secreted from the parotid gland and the amylase remaining at the end of the experiment. The percentage of the total gland amylase secreted at various times after injection of isoproterenol is also shown in figures 47, 48, and 49.

In these experiments, the results were quite variable, but a few observations were the same in all cases. When the parotid duct was first placed in the collector, no amylase could be detected. The apparatus was capable of detecting an amylase secretion rate of 1 unit/min. Secretion was initiated by i.p. injection of isoproterenol. This was detected from 1 to 3 minutes after injection. In most cases, the maximum secretion rate was observed 5 to 10 minutes after stimulation. These maximum rates were between 200 and 400 units/min.

Rats 1, 2, 3 and 4 were anaesthetized with Nembutal (Table 24, figure 47, 48). When 5 mg of isoproterenol was injected (figure 47), the amylase secretion rate very quickly reached a maximum (near 250 units/min). Rat 1 continued to secrete for 65 minutes, during which 90% of the parotid

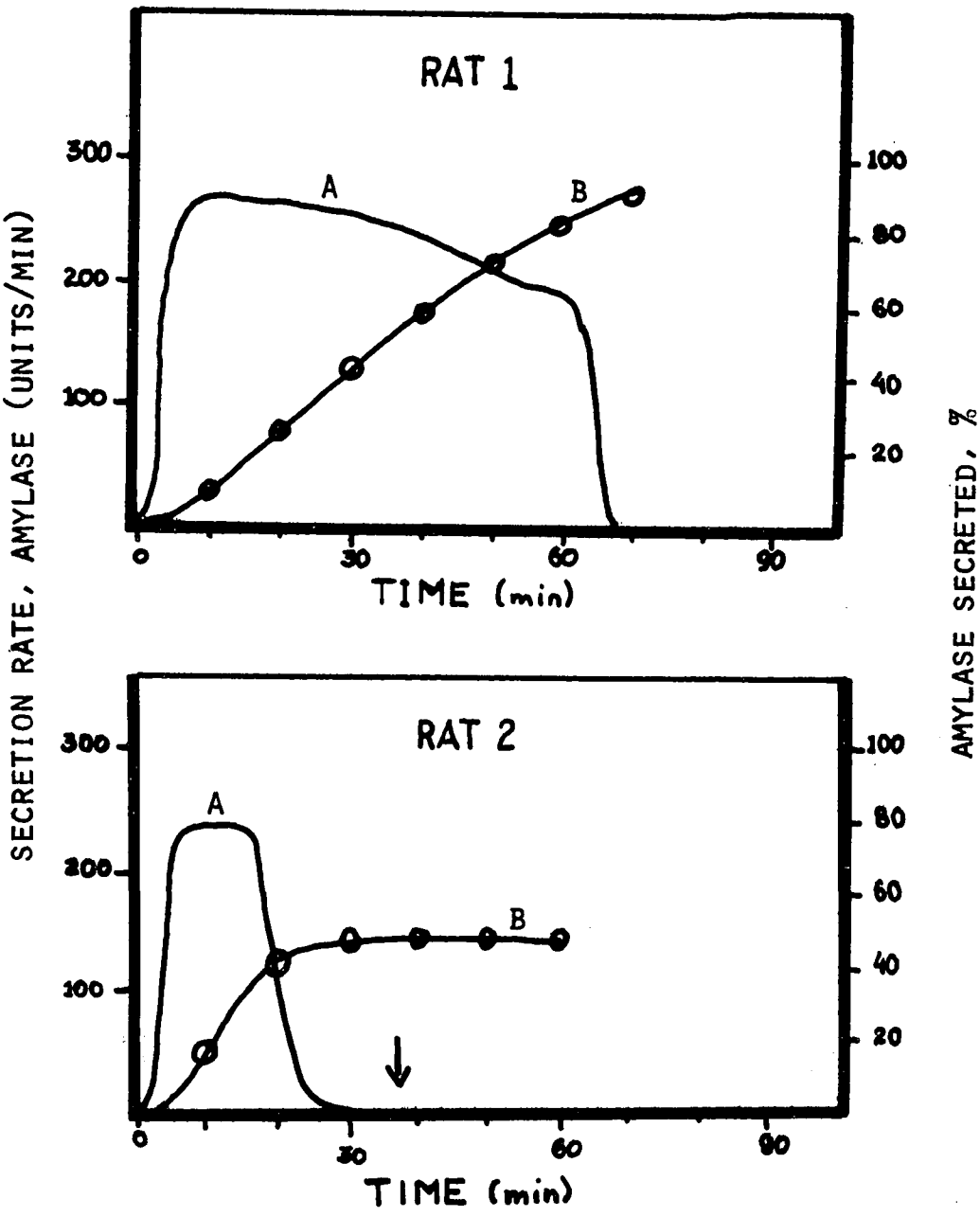


Figure 47 . Amylase secretion in Nembutal anaesthetized rats : Rats were anaesthetized and injected with 5 mg isoproterenol at time 0. The arrow indicates a subsequent injection of 5 mg of isoproterenol. A - secretion rate; B - amylase secreted as % of initial parotid amylase.

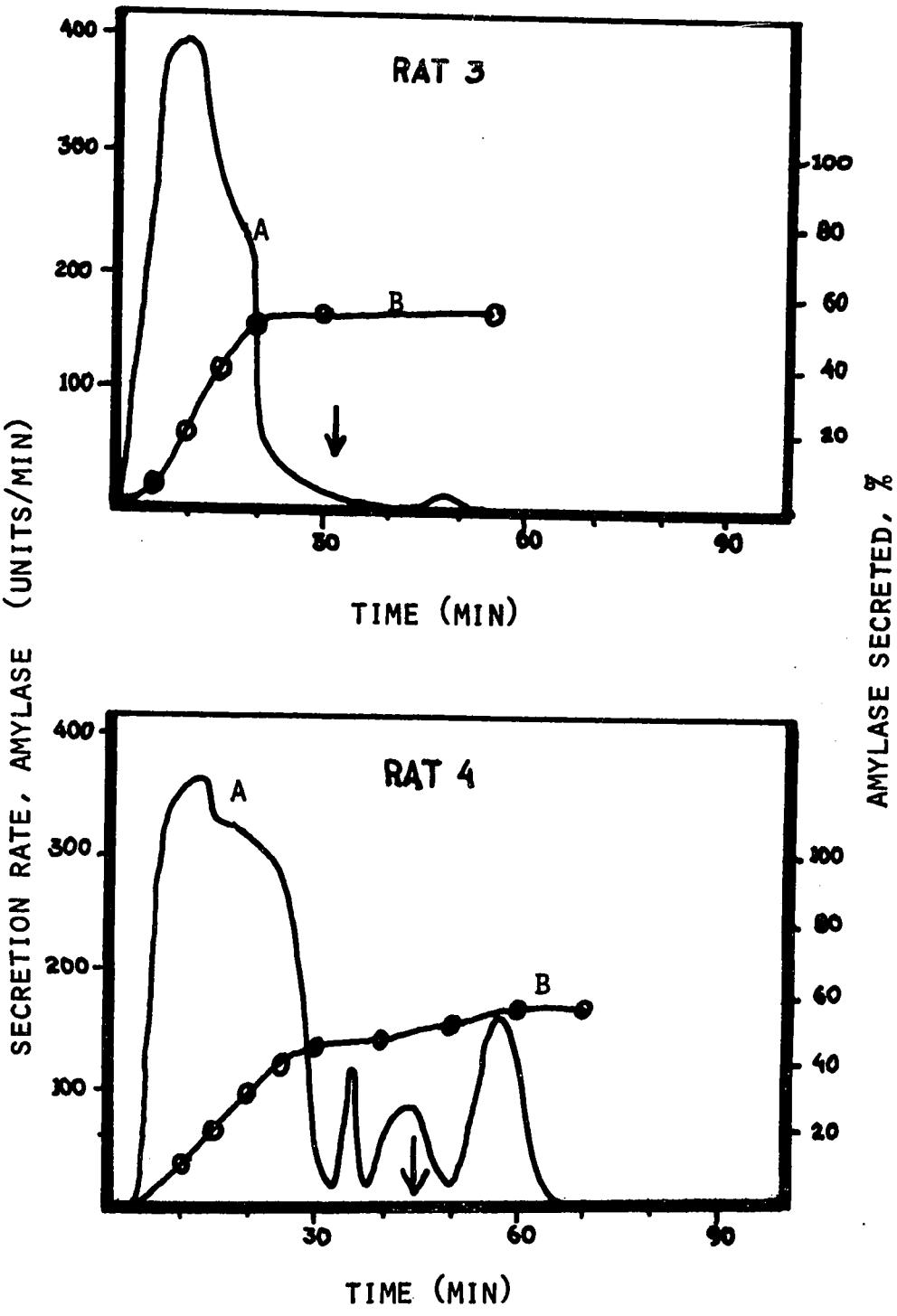


Figure 48 . Amylase secretion in Nembutal anaesthetized rats. Rat 3 - 2.5 mg of isoproterenol was injected at 0 and 32 minutes. Rat 4 - 1 mg was injected at 0 and 45 minutes. A - secretion rate; B - % of initial total parotid amylase secreted.

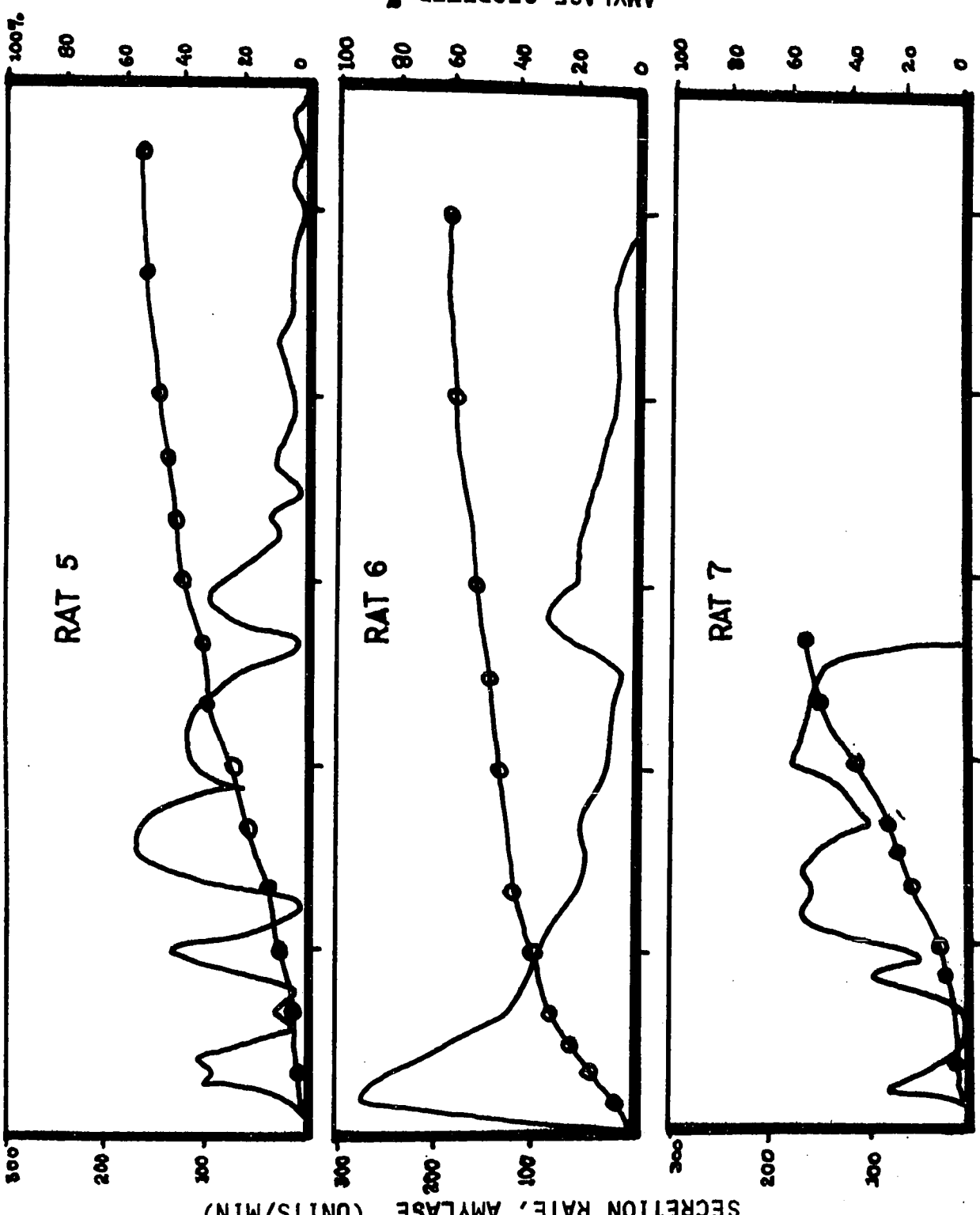


Figure 49 . Amylase secretion in urethane anaesthetized rats. Rats were injected with isoproterenol, (1, 2 and 2 mg for rats 5, 6 and 7 respectively).

TABLE 24

Amylase secretion in anaesthetized rats

Rat Number	Anaesthetic	ISOPROTERENOL Dose (mg)	ISOPROTERENOL Injection* Time (min)	AMYLASE (units) In Gland	AMYLASE (units) Secreted	AMYLASE (units) Total	Duration of Secretion (min)
1	Nembutal	5	0	1625	14600	16225	65
				1900	-	-	
2	Nembutal	5	0	4600	4100	8700	30
		5	37	1240	-	-	
3	Nembutal	2.5	0	8600	5870	14470	35
		2.5	32	5000	-	-	
4	Nembutal	1	0	6800	9495	16295	65
		1	45	4600	-	-	
5	Urethane	1	0	7800	8970	16770	180
				9500	-	-	
6	Urethane	2	0	5800	9660	15460	145
				6000	-	-	
7	Urethane	2	0	6100	7900	14000	80
				6800	-	-	

* Injection time is the time after the start of monitoring amylase secretion that the stated dose of isoproterenol was injected.

amylase was secreted. Rat 2 secreted 48% of its amylase during 30 minutes. An additional injection of 5 mg isoproterenol had no effect on amylase secretion. A lower dose of isoproterenol in rats 3 and 4 (figure 48) resulted in a higher maximum rate of amylase secretion (between 350 and 400 units/min). Injection of 2.5 mg isoproterenol resulted in 55% depletion of the gland in 30 minutes. An additional injection did not stimulate further secretion. Rats 1, 2 and 3 were similar in that secretion increased to maximum, decreased, then fell to zero without further secretion. Rat 4, however, was very unsteady, with several peaks of amylase secretion. In this case, injection of 1 mg isoproterenol at 0 and 45 minutes resulted in 45% secretion during the first 30 minutes, and 56% during 70 minutes.

Urethane anaesthesia produced results which were varied but slightly different from Nembutal anaesthesia (figure 49). With injection of 1 or 2 mg isoproterenol into urethane-anaesthetized rats, secretion up to three hours was detected. In each case, secretion rates were very unsteady, and between 54 and 64% amylase was secreted.

The anaesthetic probably affects the response of the gland to secretory stimulation. This was suspected when anaesthetized rats died after injection of 10 mg isoproterenol. There seemed to be a difference in the effect of urethane and Nembutal. In one case, 90% of amylase was secreted after 65 minutes in Nembutal-anaesthetized rats. Lower depletions

were found in other cases. There were greater fluctuations in secretion rates in urethane-anaesthetized rats, and secretion continued for a longer time. Inhibition of secretion was indicated by the general low percentage secretion in these experiments. In unanaesthetized rats, 5 mg isoproterenol induced 98% secretion and 1 mg induced 85% secretion. Additional injections of isoproterenol did not induce further secretion in anaesthetized rats after initial partial depletion.

These experiments point out the problems of using data from anaesthetized animals to represent normal physiological or pharmacological responses. This type of experiment cannot conclusively prove as claimed, (132) that little or no secretion of amylase takes place more than two hours after injection of isoproterenol. In two cases (rats 5 and 6), secretion continued more than two hours at a low rate. This might be explained by interference of anaesthetic in metabolism of isoproterenol. Under normal circumstances, isoproterenol was rapidly metabolized (294), and unmetabolized isoproterenol in salivary glands was very low as soon as 30 minutes after injection (217). In the absence of free isoproterenol to stimulate secretion, it is unlikely that secretion takes place during the repletion stage of the amylase cycle

Microsomal Amylase

Introduction

It has been reported by Brosemer and Rutter (44) that the amylase in rat liver is microsomal. Most of the liver amylase is present in a latent form which becomes active on addition of detergents. Mordoh et al (23) suggested that the concentration of detergent used by Brosemer and Rutter was suboptimal for the purpose of activation. They found that when larger concentrations of Triton X-100 were used, microsomal amylase was solubilized and that this activation was reversible.

In our experiments on repletion of amylase and incorporation of ^{14}C, Triton X-100 was used in both the homogenizing medium and the incubation medium, but the concentration was lower than that recommended by Mordoh et al (23). While parotid amylase is found mainly in the zymogen granules and in the soluble fraction, it was thought that membrane-bound amylase might represent "amylase precursor". The following experiment was done to explore this possibility.

Experimental Design

Eleven rats were treated with 5 µg isoproterenol and killed five hours later. The parotid glands were homogenized in 0.25 M sucrose and fractionated by centrifugation as outlined in Table 25. Each fraction was assayed without detergent to

measure free amylase and then with 0.1% Triton X-100 to measure total amylase. The difference was considered to be "latent amylase".

The microsomal fraction (100,000 x g for 30 minutes) was further purified by recentrifuging at 10,000 x g for 10 minutes and the supernatant was centrifuged at 100,000 x g for 30 minutes to reprecipitate the microsomal fraction. The purified microsomal fraction was dissolved in 0.25 M sucrose and incubated with either water or Triton X-100 (0.1% final concentration) for 15 minutes at 37°. The preparations were recentrifuged (100,000 x g for 30 minutes) and the resulting precipitates and supernatants were assayed for both free and total amylase (without and with Triton X-100). These results are shown in Table 26.

Results

Table 25 indicates that at a high concentration of Triton X-100 (0.1%) the amylase activity of bound amylase (the amylase in the precipitates) was increased. This was particularly true in the case of the microsomal fraction (about 100% increase in activity). The latent amylase, however, was very low compared to total amylase (about 2600 units out of a total of 44,000, or about 240 units per rat). It should be noted that at high concentrations of Triton X-100, the amylase activity of soluble amylase was slightly decreased.

TABLE 25

Distribution of free, total, and latent amylase among subcellular fractions of rat parotid glands.

Fraction	Amylase Activity*			Protein (mg)
	Free	Total	Latent	
Total Homogenate	44080	41180	-2900	201.6
9000 x g,10 min. ppt	9417	10273	856	61.3
27000 x g,10 min. ppt	3816	4305	489	48.6
100,000 x g,30 min. ppt	1135	2426	1291	55.6
100,000 x g,30 min. Supernatant	23237	20791	-2446	120.6

* Amylase activity is expressed as mg maltose monohydrate released in 3 minutes at 37°. Free amylase was measured without detergent. Total activity was measured with Triton X-100 (0.1%) in the incubation mixture, and latent amylase activity was the difference between total and free activity.

TABLE 26

Effect of Triton X-100 on the solubilization and activation of microsomal amylase.

	Pretreatment with Triton X-100	Amylase Activity*		Total as % of Sum (S + P)
		Free	Total	
Total Microsomal Homogenate	+	1312	1281	
	-	969	1250	
Supernatant, (S)	+	1359	1309	96.9%
	-	161	151	11 %
Precipitate, (P)	+	14	42	3.1%
	-	389	1111	88 %
Sum, (S + P)	+		1351	100 %
	-		1263	100 %

* The free and total amylase activity is defined in Table 25.

Table 26 indicates that the detergent almost completely solubilized the microsomal amylase. With detergent, 96.9% of the amylase was found in the soluble fraction but without detergent, only 11% was solubilized.

These results parallel those of Mordoh et al (23) for liver amylase. The difference is that in liver over 70% of the amylase is microsomal whereas in the depleted parotid, only about 5% was found. Although activation of microsomal amylase by detergent was found in the parotid gland, the levels were much too low to account for the "amylase precursor" suggested by our incorporation studies. Furthermore, the preparation of parotid homogenates for the repletion studies was such that the microsomal amylase would have been present in the assay mixture even if the Triton X-100 concentration was too low for complete solubilization. The detergent was sufficient for activation of insoluble amylase.

Attempted Activation of Amylase Precursors: Hydrolytic Enzymes

Introduction

It was thought that amylase might be synthesized in an inactive form and require some sort of hydrolytic cleavage for activation. This was suggested by the following evidence: Amylase isolated from Aspergillus oryzae (taka-amylase A) was incubated with taka-proteinase. The amylase was 50% degraded

after 24 hours at 37°. From the hydrolized mixture was isolated a fraction with higher specific activity than the original taka-amylase A (368). The possibility that similar activation might take place in the parotid gland was suggested by the finding of protease activity in the parotid (70). The following experiments were undertaken to explore the possibility of this type of activation.

Experiment A

Eight rats were killed between 4 and 5 hours after injection of 5 µg of isoproterenol. The parotid glands were homogenized in the usual way and cell debris was removed by centrifugation at 4000 x g for 10 minutes. One ml aliquots were incubated at 37° with Ca^{++} (0.002 M) or EDTA (0.002 M) plus 0.1 ml of one of the following solutions: trypsin, 2 mg/ml; papain, 10 mg/ml; pepsin, 2 mg/ml; phospholipase C, 5 mg/ml; digitonin, 4%; or water. Amylase determinations were made on small aliquots of each solution at many time intervals between 0 and 5 hours incubation. The results are shown in figure 50.

Experiment B

Since pancreatin (a crude pancreatic extract) contains numerous hydrolytic enzymes, it was decided to test the effect of incubation of pancreatin with homogenates from rats at various times after depletion of amylase with isoprenaline. Pancreatin was incubated at 37° with homogenates from rats killed at the following times after injection of 5 µg isoprenaline; 0, 3½, 5, 6½ and 10 hours. The supernatant

and the residue after centrifugation (4000 x g for 10 minutes) were similarly analyzed. The results are shown in figure 51.

Results

In figure 50, it is shown that during five hours of incubation, there was no increase in amylase activity. There was a decrease with EDTA (18% in 4 hours) ; pepsin + EDTA (20%) ; papain + EDTA (25%) ; trypsin + EDTA (85%) ; and chymotrypsin + EDTA (60%). The slow loss of activity with EDTA (30% over 24 hours) was seen with phospholipase c + EDTA and Digitonin + EDTA. There was no loss in activity during six hours incubation when Ca^{++} was present or when no EDTA was present. Without EDTA, the levels of amylase remained steady over 48 hours for the control (no additions), phospholipase c, and digitonin. There was a slight decrease in 48 hours with trypsin (7%), chymotrypsin (20%) and pancreatin (35%). The decrease in stability of amylase in the presence of EDTA was due to chelation of Ca^{++} which is necessary for full activity and stability. In the presence of sufficient Ca^{++}, amylase is remarkably resistant to proteolytic attack (70).

In Experiment B, (figure 51) incubation of the various homogenates with or without pancreatin did not result in any increase in amylase activity. Without pancreatin, the amylase activities remained constant for 24 hours. With pancreatin, there was a loss (between 20% - 80% loss at 24 hours).

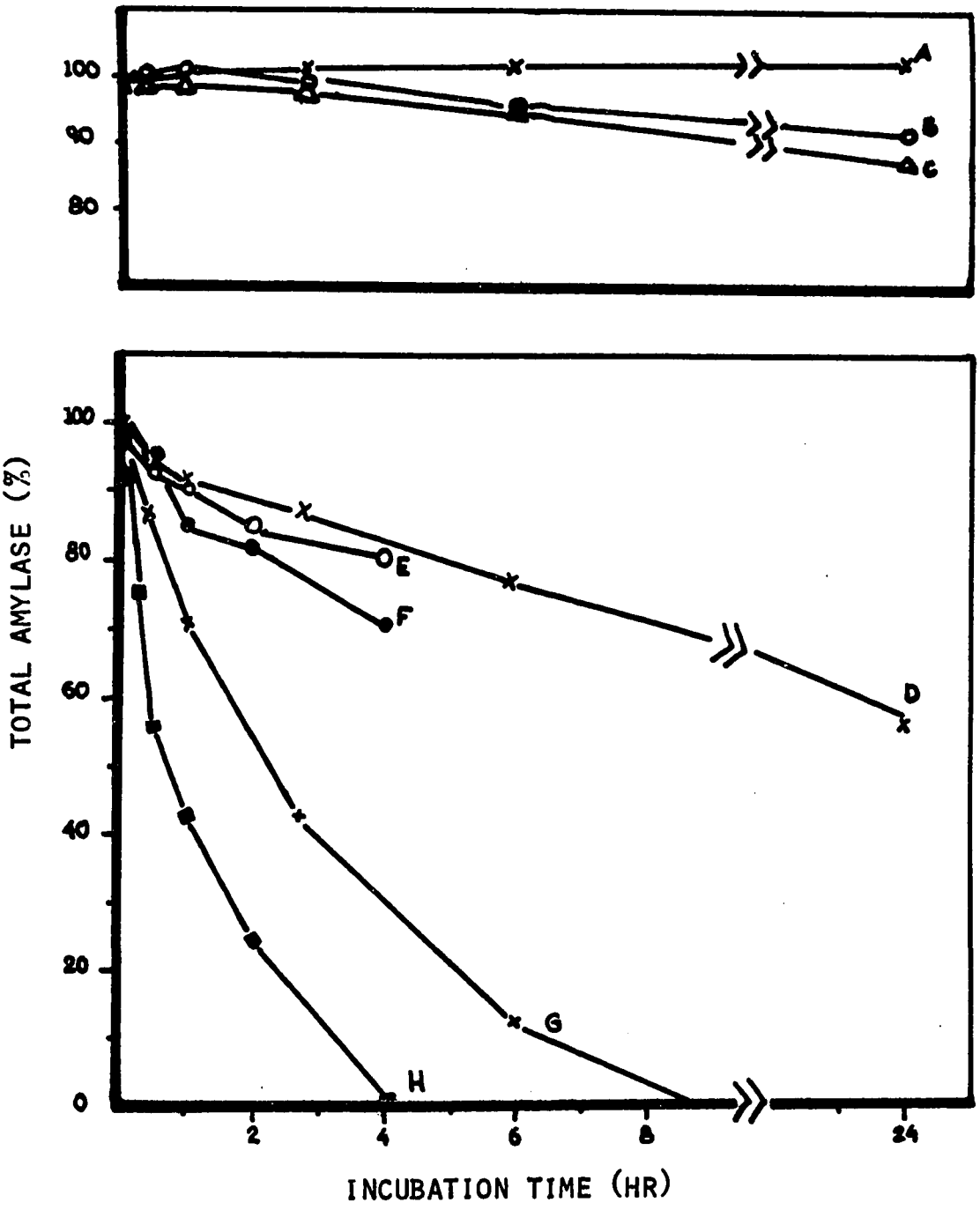


Figure 50 . Attempted activation of "amylase precursor" with hydrolytic enzymes. Incubation (37°, 0.02 M phosphate pH 6.9) of parotid gland homogenates was carried out as described in the text with the following additions: A - no addition, or addition of pepsin, phospholipase c, or digitonin ; B - trypsin ; C - chymotrypsim ; D - EDTA ; E - pepsin + EDTA ; F - papain + EDTA ; G - chymotrypsin + EDTA ; H - trypsin + EDTA.

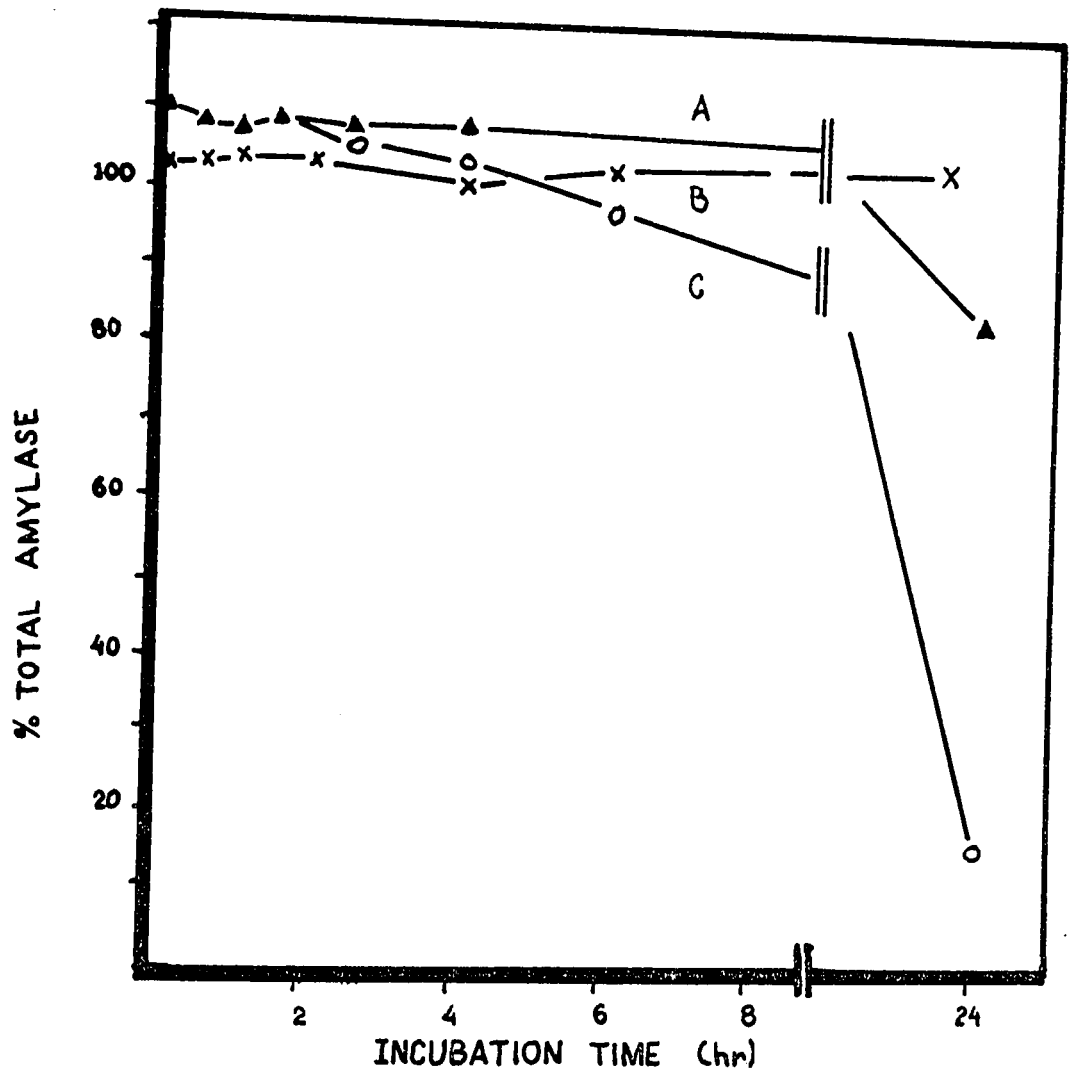


Figure 51 . Attempted activation of "amylase precursor" with pancreatic extracts and calcium. Incubation of parotid homogenate (37° in 0.02 M phosphate pH 6.9) was carried out as described in the text with the following additions :

A. 3 x concentrated homogenate (unstarved rat) with pancreatin and Ca^{++}.

B. homogenate (4 hours after isoproterenol) with pancreatin and Ca^{++}.

C. homogenate (5 hours after isoproterenol) with pancreatin, no Ca^{++}.

These results indicate that if some amylase is stored in the parotid gland in the form of an inactive precursor, it is different from other pro-enzymes. Those are easily activated by proteolytic attack, and cleavage of specific peptide bonds (369).

Attempted Activation of Amylase Precursors: Protein Kinase

Introduction

The role of protein kinase in several tissues was discussed in the Literature Review. It appears to be one of many enzymes activated in the complex series of reactions taking place following adrenergic stimulation, starting with adenyl cyclase activation and resulting in a specific physiological response. The elevated salivary protein kinase activity following isoproterenol stimulation (214) suggested its possible role in the activation of amylase during the repletion stage of the secretory cycle. This possibility was investigated in the experiments described below.

Conditions for Activation of Protein Kinase

Protein kinase activity has been determined by measuring the transfer of ^{32}P from γ-^{32}P-ATP during incubation with a protein substrate, such as bovine serum albumin (220), casein (215), histone (213, 219), protamine (213, 219), and

phosphorylase kinase (215). The conditions for maximum activation of protein kinase by Mg^{++} (219, 220), cyclic AMP (215, 216, 217, 219, 220), glycogen (370) and ATP (220), have been reported. Optimum pH was 6.5 (220). Other substances used in incubation mixtures have been theophylline, an inhibitor of phosphodiesterase activity (215), and sodium fluoride, a specific inhibitor of the phosphatase which attacks phosphorylase kinase (214).

Several incubation mixtures were used in the study of protein kinase of rat parotid gland. These mixtures, listed in Table 27, were based on optimum conditions described above. An ATP regenerating system (phosphoenol pyruvate and pyruvate kinase), was included because incubations were carried out for extended periods of time. As can be seen in Table 27, only incubation mixture A contained γ-^{32}P-ATP. This was the only mixture in which protein kinase activity was actually measured. No protein substrate was included in any of the incubation mixtures because we were interested in the phosphorylation of normal cell components rather than in the actual levels of protein kinase activity.

An estimation of protein kinase activity was made after incubation of medium A. At a specific time after initiation of incubation, a 1.0 ml aliquot was mixed with 2.0 ml of bovine serum albumin (6 mg/ml) and 6 ml of cold 10% TCA was immediately added. The precipitated protein was twice washed with 4 ml of 10% TCA and drained. After solubilization in 0.3 M KOH, it was dispersed in scintillation fluid (Aquasol, New England Nuclear)

TABLE 27

Conditions used for the activation of protein kinase in salivary glands.

Components	MEDIUM*		
	A	B	C
Potassium Phosphate pH 6.5	10	-	-
Sodium Glycerol Phosphate pH 6.5	-	21	16
Magnesium Acetate	5	16	13
Sodium Fluoride	-	9	9
Theophylline	2	1.8	1.8
ATP	1.2	0.45	0.45
γ-^{32}P-ATP	10 µCi	-	-
Cyclic-AMP	.005	.022	.022
Phosphoenol Pyruvate	8	7	7
Pyruvate kinase	50 µl	25 µl	10 µl
Protein Kinase	-	-	200 µl
Parotid Homogenate	1 ml	2 ml	0.5 ml
Final Volume	5 ml	2.7 ml	1.1 ml
Number of Parotid Glands in each incubation	1	0.4	0.1
Incubation Temperature	37°	37°	30°

* The conditions in each medium are expressed as final concentration (mM) unless otherwise specified.

and radioactivity was measured. From the specific activity of γ-^{32}P-ATP in the incubation mixture and the DPM of the isolated protein, protein kinase activity could be calculated as $\eta M^{32}P$ transferred from γ-^{32}P-ATP to total incubation mixture protein.

The protein kinase used in medium C was prepared by Mrs. Dorothy Horwood. It was isolated from rat parotid gland according to the general method of Myamoto et al (226) and was purified by the first three steps described by these authors (extraction of the soluble enzyme with an EDTA solution followed by precipitation of pH 4.8 and ammonium sulfate fractionation). The amount of this preparation (0.2 ml) in medium C contained 2 mg protein and under the conditions of the assay (10 minutes at 30^{o} with .550 mg histone as substrate) had an activity of 3.9 ηM. The preparation also contained some amylase (575 amylase units in 0.2 ml or approximately 0.2 mg).

Experiment 1

Rats were starved overnight and injected with 5 mg isoproterenol except for the control which was untreated. Parotid glands were immediately removed and homogenized in distilled water, and made to a final volume of 1 ml. The whole homogenate was incubated at 30^{o} under the conditions described in Table 27,medium A. These conditions were very similar to those used by Corbin and Krebs (217) except that no substrate was added and an ATP regenerating system was used.

In all cases, 10 µl was removed for amylase assay at 10 minutes and one hour after initiation of the incubation. With the control, amylase was also assayed after 2, 4 and 6 hours of incubation. Protein kinase activity was determined on 1.0 ml aliquots removed from all incubation mixtures after 10 minutes incubation and in the control, after 10 minutes, 1 hour, 2 hours, 4 hours and 6 hours.

Amylase was isolated (by precipitation on glycogen) after one hour of incubation. Incorporation of ^{32}P into the isolated amylase was measured. Aliquots of the amylase were subjected to disc electrophoresis, and the ^{32}P in electrophoretically pure amylase was measured.

The results of this experiment are summarized in Table 28. It was apparent that there was significant protein kinase activity in parotid glands, and that the normal cell contents of the glands could serve as a substrate for this enzyme. However, amylase was not phosphorylated. The low levels of ^{32}P found in the amylase prepared by glycogen precipitation were probably due to contamination with small amounts of phosphorylated protein. The report that some phosphorylated proteins such as phosphorylase-b and phosphorylase-b-kinase are bound to glycogen (371) suggests that similar phosphorylated enzymes could be contaminating our amylase preparation.

TABLE 28

The amylase activity and ^{32}P incorporation into protein by protein kinase in rat parotid homogenates (Experiment A).

Isoproterenol	Incubation Time	Total Amylase*	Total incorporation of ^{32}P into protein		
			Total Parotid Protein†	Amylase‡	Electrophoretically separated Amylase
Control (no isoproterenol)	10 min	27,750	9.32	-	
	1 hr	27,500	9.27	-	
	2 hr	26,750	5.84	-	
	4 hr	28,000	5.39	-	
	6 hr	28,250	5.09	-	
1 hr	10 min	3,182	7.81	-	0
	1 hr	3,202	-	0.74	
2 hr	10 min	686	11.84	-	0
	1 hr	675	-	0.52	
4 hr	10 min	731	4.17	-	0
	1 hr	765	-	0.39	
6 hr	10 min	3,249	3.81	-	0
	1 hr	3,491	-	0.37	

* Amylase units/pair of parotid glands.

† mM of phosphate incorporated into total parotid protein during incubation with γ-^{32}-P-ATP.

‡ mM of phosphate incorporated into total parotid amylase isolated by precipitation on glycogen.

Experiment 2

A series of rats were starved overnight and treated with 5 mg isoproterenol in the morning. After six hours, the rats were killed, the parotid glands were removed, immediately homogenized in 0.025 M sodium glycerol phosphate buffer pH 6.5 and made to a final volume of 5 ml. Incubation mixture B, Table 27, was prepared for each homogenate. At the same time, a control made the same way but without ATP, cyclic AMP, or the ATP generating system, was prepared for each homogenate. Aliquots were removed for amylase assay at times 0, ½, 2½ and 16 hours after initiation of incubation at 37^{o}.

The results from two typical experiments are shown in figure 52. There were no significant changes in amylase activity during incubation, with or without ATP and cyclic AMP.

Experiment 3

Essentially the same conditions for incubation were used (Table 27, medium C) except that protein kinase was added and incubation was carried out at 30^{o}. Eight rats were starved overnight, injected with 5 mg isoproterenol in the morning and killed after 8, 10 or 11 hours. The parotid glands from each rat were homogenized in 0.025 M sodium glycerol phosphate pH 6.5 and made to a final volume of 5.0 ml.

Two sets of incubations were carried out for each parotid homogenate one containing everything in medium C, the other without the protein kinase. Aliquots were removed for amylase assay

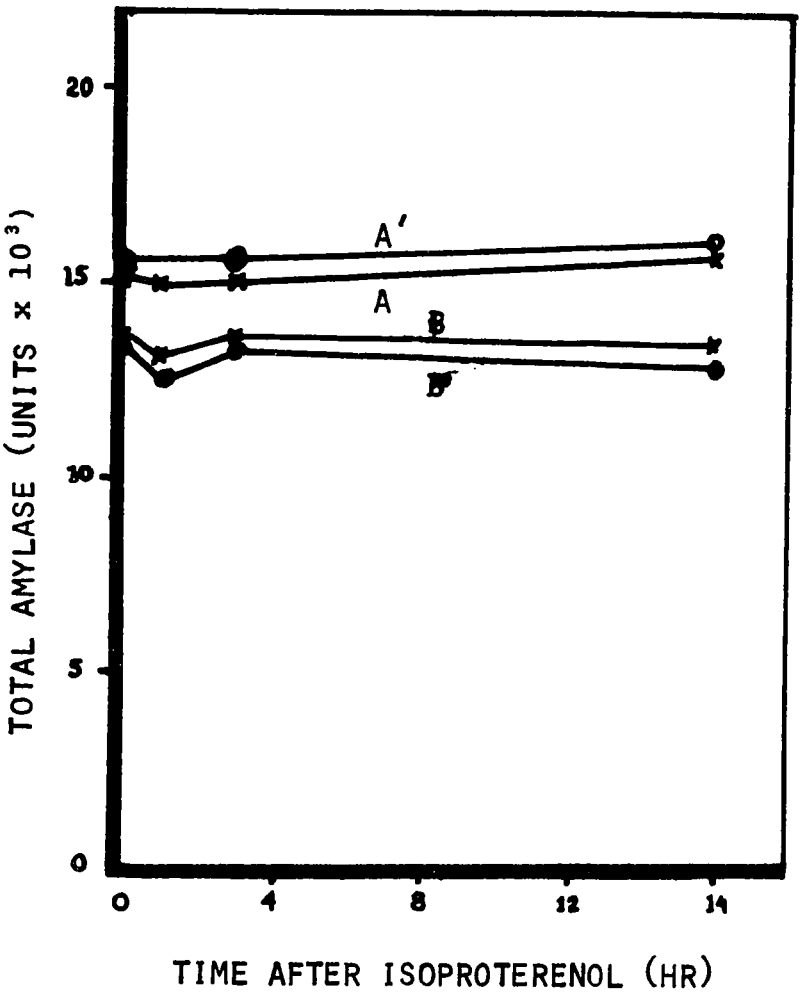


Figure 52 . Attempted activation of amylase by activation of protein kinase. A and B are two parotid homogenates, incubated with ATP and cyclic AMP. A' and B' are their respective control incubations (no ATP or cyclic AMP).

at times 0, ½, 1½ and 4 hours after initiation of incubation at 30°.

The results of this experiment are shown in figure 53. The lower points are from the incubation without protein kinase. The upper points are for the incubation mixtures containing 0.2 ml protein kinase. The difference between these two sets is due to contamination of the protein kinase preparation with amylase. In neither incubation mixture was any significant change in amylase levels observed.

Inhibition of Protein Synthesis

Introduction

Many compounds have been used to inhibit the biosynthesis of protein in intact animals. Actinomycin-D, which indirectly affects protein synthesis by inhibiting the synthesis of messenger RNA has been shown to inhibit the incorporation of ^{3}H-uridine into salivary gland RNA (249). At near lethal dose of actinomycin-D, the pilocarpine induced increase in rat submaxillary gland amylase was significantly augmented (157).

Cycloheximide inhibits an early step in protein synthesis, probably the interaction of m-RNA with ribosomes (372). At a dose of 1 mg/Kg in adrenalectomized rats, it causes a 95% inhibition of induction of liver tyrosine transaminase by hydrocortisone (373), and a dose of 30 mg/Kg inhibits ^{3}H-leucine incorporation into mouse salivary gland proteins (150).

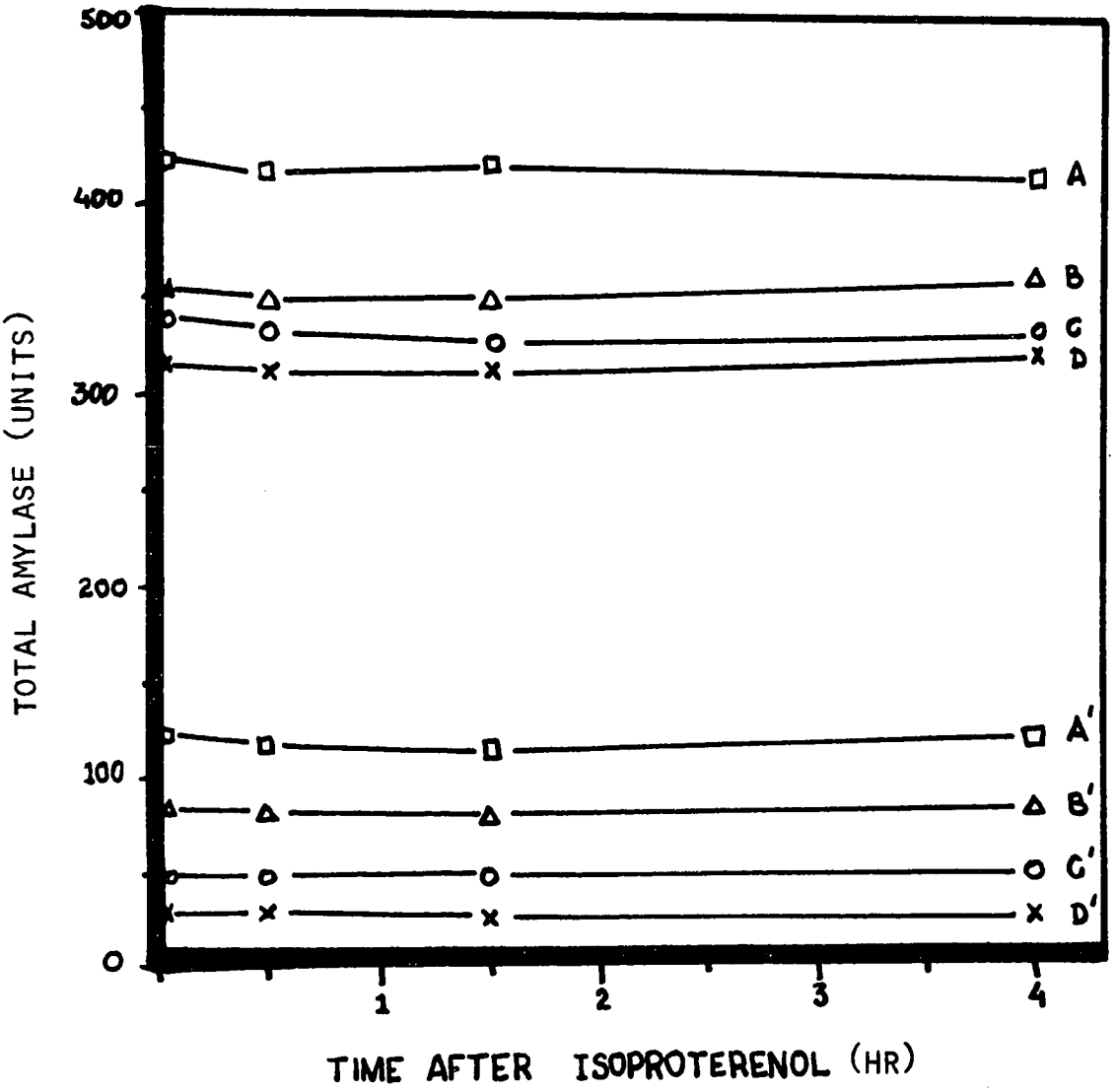


Figure 53 . Attempted activation of "amylase precursor" by incubation with protein kinase. A, B, C, and D are incubations of different homogenates with protein of kinase. A', B', C', D' are their respective control incubations without protein kinase.

Cycloheximide in rat parotid gland slices inhibited ^{14}C-amino acid incorporation into amylase and protein by 90% but did not affect epinephrine induced secretion of amylase (150).

Puromycin, which is an analog of amino acyl-transfer-RNA, causes the release of partially synthesized protein from ribosomes (374), which results in the inhibition of synthesis of specific proteins. A dose of 85 mg/Kg into rats each hour for three hours resulted in 99% inhibition of amino acid incorporation into protein and 75% inhibition of tryptophan pyrolase synthesis in the liver. At the same time, a 3 to 4-fold increase in free amino acids was observed (375). Within 30 minutes of injection of puromycin (50 mg/Kg) into mice, ^{3}H-leucine incorporation into proteins was inhibited by 70% (376). In rat submaxillary glands, injection of 150 mg/Kg puromycin did not inhibit the pilocarpine induction of amylase (157).

Ethionine has been shown to inhibit leucine incorporation into liver proteins of female rats but not in males (377, 378). It stimulated protein synthesis in the pancreas of rat (379). Ethionine has also caused degeneration of acinar tissue in the pancreas, stomach and submaxillary glands (380).

Experimental Design

A series of experiments were carried out to study the effect of the inhibitors, discussed above, on the repletion of amylase in the rat parotid gland, and on amino acid

incorporation into proteins and amylase. Rats were starved overnight, injected with 5 mg isoproterenol in the morning and injected with an inhibitor six hours later. Some rats were injected with 5 μCi of ^{14}C-leucine or ^{14}C-mixed amino acids at the same time as the inhibitor. The rats were killed ten hours after injection of isoproterenol (4 hours after injection of inhibitor). Control rats were also injected with isoproterenol and six hours later were injected with isotonic saline. The control rats were killed six hours or ten hours after isoproterenol injection. Parotid glands were homogenized and amylase content was determined. In experiments in which labeled amino acids were injected, an aliquot of the homogenate was treated with TCA to precipitate protein. Both the TCA supernatant and precipitate were dispersed in scintillation fluid with the aid of NCS solubilizer and DPM was determined. Amylase was also isolated from the homogenates and DPM determined. The dosage of the inhibitors used in these experiments are the following:

Actinomycin-D	- 1.0 mg/Kg	(single injection)
Actinomycin-D	- 1.5 mg/Kg	(single injection)
DL-Ethionine	- 1350 mg/Kg	(675 mg/Kg, 6 and 8 hrs after isoproterenol)
Puromycin hydrochloride	- 100 mg/Kg	(40, 20, 20 and 20 mg/Kg at 6,7,8 and 9 hrs after isoproterenol)
Puromycin hydrochloride	- 200 mg/Kg	(80, 40, 40 and 40 mg/Kg at 6, 7, 8 and 9 hrs after isoproterenol)
Cycloheximide	- 0.6 mg/Kg 1.1 mg/Kg	(single injection)

Results

Table 29 summarizes the results of these experiments. The total activity of parotid amylase in each group of rats is compared with the total radioactivity incorporated into the total amylase, the TCA precipitable protein, and the TCA supernatant. The radioactivity in the TCA supernatant may be mostly in free amino acids, but some of it may be due to mucoprotein (TCA soluble) and to short peptides. Short peptides could result from interrupted protein synthesis particularly in animals treated with puromycin. In Table 29 (Group A), ^{14}C-leucine was used and in Table 29 (Group B), mixed ^{14}C-amino acids were used, so that amino acid incorporation cannot be compared between groups. Amylase can be compared between A and B.

Parotid amylase content increased almost four-fold (from 16.62 to 63.78 units/g rat wt) between six hours and ten hours after injection of isoproterenol. Approximately the same rates of amylase increase were observed in rats treated with actinomycin-D and ethionine since the amylase activities in these rats were significantly higher than the six hour control but not significantly different from the ten hour control. The amylase biosynthesis in the cycloheximide treated rats was apparently inhibited. Although there was a significant increase in amylase it was much lower than in the ten hour control. Amino acid incorporation into total amylase and TCA precipitable protein were also decreased about five-fold. There were no significant differences in

TABLE 29

Inhibition of protein biosynthesis in rat parotid gland: effect on amylase repletion.

Treatment	Dose (mg/kg)	No.of Rats	Total Amylase*	INCORPORATION OF ^{14}C-AMINO ACIDS (DPM) In Total Amylase	In TCA Precipitate	In TCA Supernatant
A. Control - 6 hr	0	7	16.6±1.0			
10 hr	0	3	63.8±6.4	5438±980	34900±6114	7816±2200
Actinomycin D	1.0	4	87.2±20.1			
Actinomycin D	1.5	3	66.6±6.9	4474±306	29650±7249	6933±426
Actinomycin D	(average)		78.4±11.8			
Ethionine	1350	2	72.2±2.2	5600±236	49500±15800	7125±3325
Puromycin	200	1	4.6	7.4	2500	5000
B. Control - 10 hr	0	4	63.8±6.4	21743±70	64775±11525	48925±6525
Cycloheximide	0.6-1.1	6	27.6±2.4	4674±478	17438±1013	22914±1846
Puromycin	100	1	54.0	18739	46000	124,000

* Total amylase is expressed as total units/pair of glands/g body weight.

amino acid incorporation between the ten hour control and the actinomycin and ethionine treated rats.

A large dose of puromycin (200 mg/kg) completely inhibited protein biosynthesis and amino acid incorporation into amylase. The fact that the amylase level in this rat was much lower than in the six hour control, indicated either a very rapid destruction of amylase or induced amylase secretion. When a lower dose of puromycin was used (100 mg/kg), neither the amylase level, nor the incorporation of amino acids was significantly different from the ten hour control. The TCA soluble fraction had much higher radioactivity. This may be due to release of partially synthesized protein from ribosomes. It is therefore possible that disruption of protein biosynthesis may have taken place without effect on the increase in amylase activity. The rat with the high puromycin dosage showed no increased in TCA soluble DPM.

These results, summarized in Table 29, are generally consistent with the view that the increase in amylase levels during the repletion stage requires the syntheis of new enzyme. No activation of amylase was found during inhibition of protein synthesis.

Isoamylases

The electrophoresis of isolated parotid amylase on acrylamide gel revealed the presence of small amounts of protein other than the major amylase peak (figure 44). The recent reports of several isoamylases in saliva (48-50, 381) suggested that the minor protein bands in figure 44 might be isoamylases. This was investigated in the following experiments.

Disc electrophoresis of both human parotid saliva and rat parotid homogenate was carried out as described in the Analytical Section. Several protein bands were revealed but amylase activity was not well defined as shown in figure 54. The major amylase peak showed very strong amylase activity. Amylase activity was indistinctly smeared in front of and behind that major band.

Isoelectrofocusing in acrylamide gels, however, resulted in very sharp and distinct bands of amylase activity in both human and rat parotid (figure 54). There were six distinct isoamylases in the human parotid saliva and five in the rat parotid homogenate.

The above results indicate the superiority of isoelectrofocusing in studying isoenzymes, at least, in the case of salivary amylase. Other workers have studied the separation of salivary proteins using isoelectrofocusing on gels (383) but did not measure amylase activity.

The separation of isoamylases by disc electrophoresis (48-50, 381) can be due to differences either in the net

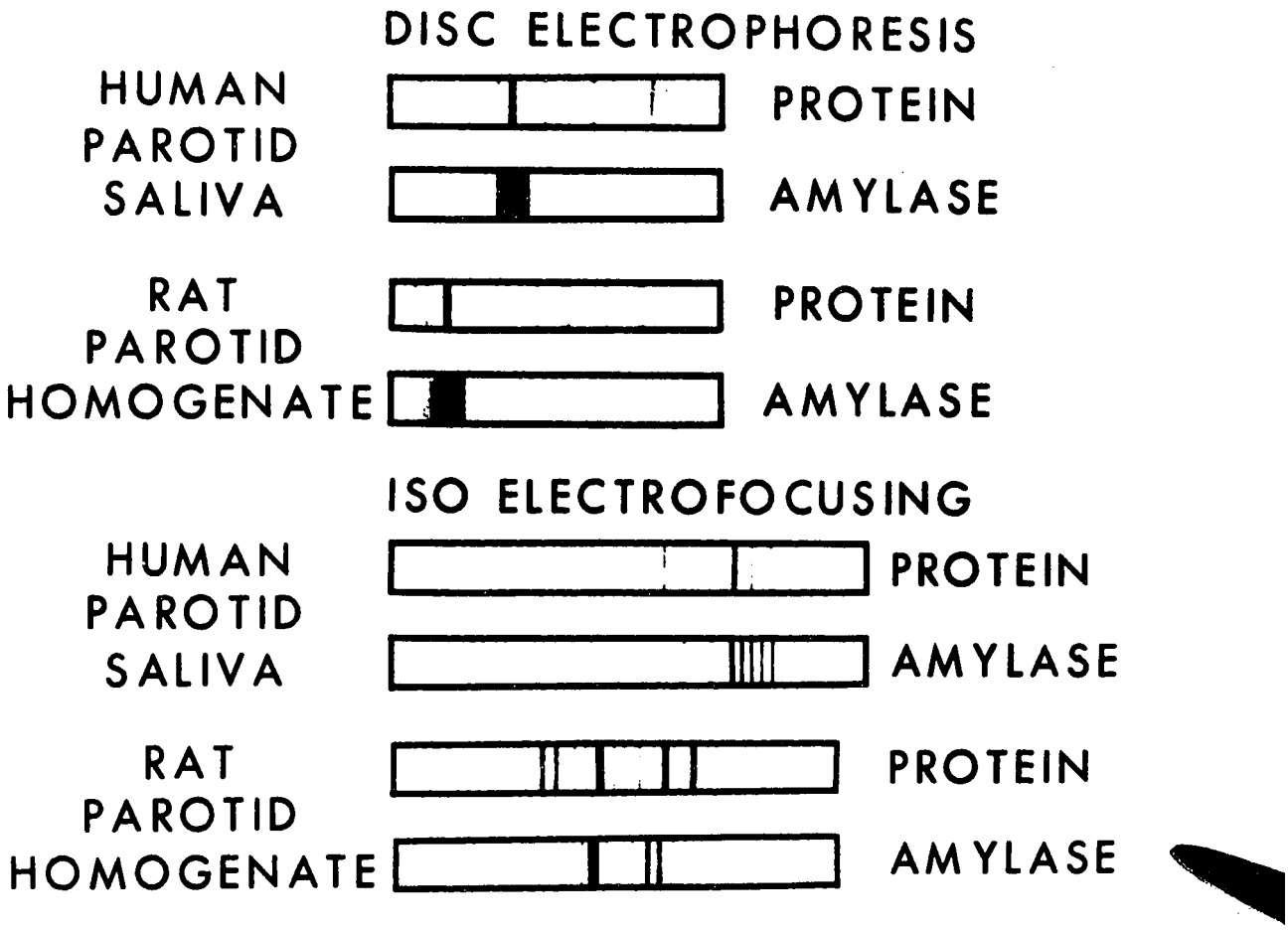


Figure 54 . Rat parotid and human parotid isoamylases : disc electrophoresis and isoelectrofocusing.

charge or in the molecular weight of each isoenzyme. However, the separation of isoamylases by isoelectrofocusing demonstrates that these isoenzymes differ in net charge.

The finding of isoamylases in the parotid gland suggests another possible mechanism of control of amylase activity. Parotid amylases may be similar to LDH in which two subunits of the enzyme form tetramers in all possible combinations with distinct enzymatic constants (382). This possibility of amylase subunits is supported by the work of McGeachin and Brown (58) which indicated that hog pancreatic amylase consisted of three polypeptide chains. It is possible that subunits of amylase may be synthesized separately and then combined to form active amylase at a different time and site in the cell. In this type of mechanism, the inactive subunits could be the "amylase precursor" which was suggested by our data comparing amylase synthesis to amylase repletion (figure 43).

To further investigate the possibility of amylase subunits, several possible experiments could be carried out. The isoenzymes can probably be isolated on a DEAE cellulose column. End group analysis of each isoenzyme would be of interest. Separation of amylase into subunits by EDTA treatment and reduction of disulfide bridges (66) could be followed by electrophoretic separation of subunits. The recombination of subunits by oxidation of sulfhydryl groups in the presence of Ca^{++} (66) may result in different isoenzyme patterns depending upon the proportions of each subunit used. This could be followed by disc electrophoresis or isoelectrofocusing.

Discussion: Models for Repletion of Amylase

Introduction

The unusual kinetics of parotid amylase repletion are shown in figure 36. A number of possible mechanisms for this pattern of repletion have been explored.

1. Increased de novo synthesis: The steadily increasing level of amylase in the parotid gland between 2 and 18 hours after secretion could be a result of an increasing rate of de novo biosynthesis.

2. Amylase secretion: The increase can be the result of a steady rate of biosynthesis with a simultaneous changing rate of secretion.

3. Activation of an inactive precursor: The increase may result from a form of precursor activation, in which the activation of a proenzyme rather than enzyme biosynthesis controls the level of amylase.

4. Changes in degradation and turnover: The changes in amylase levels may be due to changing rates of amylase degradation rather than changing amylase biosynthesis.

5. Stabilization of amylase: Newly synthesized amylase may be stabilized (by association with a cofactor such as Ca^{++} or by translocation to a protected area such as in zymogen granules) thus affecting apparent turnover. Assuming constant turnover of unstabilized amylase, changes in amylase levels may result from changing rates of stabilization.

These mechanisms can be assessed on the basis of the experiments presented in this section as well as on the basis of known cytological and biological changes in mammalian exocrine glands and on the basis of control mechanisms described for other mammalian enzymes.

Increased de novo Biosynthesis

To account for the exponential pattern of amylase repletion, a linearly increasing rate of biosynthesis during the exponential phase is expected. In orther words, the rate of increase of biosynthesis could parallel the incremental increase in amylase levels. Estimates of rates of de novo amylase synthesis were made by determining the extent of ^{14}C-amino acid incorporation during a 30 minute "pulse".

A 3.5-fold increase in amylase DPM occurred between two and eight hours after depletion of the gland (figure 41). The probably more valid estimate of de novo biosynthesis, the ratio of amylase DPM to non-amylase DPM, increased two-fold between two to seven hours (figure 46). The subsequent decrease in the rate of amylase biosynthesis occurred when the increase in amylase levels was greatest. These changes in amylase biosynthesis cannot alone account for the repletion of amylase. The discrepancy between amylase biosynthesis and amylase repletion becomes particularly obvious when amino acid incorporation is compared to the incremental increase in amylase levels (figure 42).

Amylase Secretion

The suggestion was made by Schucher and Hokin (366) that changes in levels of secretory enzymes may be due to constant rates of synthesis with changes in secretion rates superimposed. To account for the exponential pattern of amylase repletion, secretion would have to be substantial in the starved rat, increase immediately after stimulation with isoproterenol, decrease exponentially between two and 18 hours, then immediately increase to the basal (starving) level. Our experiments with anaesthetized rats (figures 47, 48, 49) indicated absolutely no secretion in the unstimulated rat. No secretion was observed three hours after stimulation with isoproterenol. This was supported by other workers (132). Much evidence exists to support the view that amylase is secreted from the parotid glands only in response to specific stimulus (367). Isoproterenol is rapidly metabolized in the rat and none was detected in the parotid gland four hours after injection (217, 294).

Activation of an Inactive Precursor

The exponential increase in amylase is very similar to the increase in enzyme activity during autocatalytic activation of some proteolytic enzymes. Several proteolytic enzymes are stored in pancreatic zymogen granules in the form of inactive proenzymes. Amylase is also stored in parotid and pancreatic zymogen granules, but in an active form. It is tempting to

speculate on the possibility of some form of "pro-amylase", stored in the pancreas and parotid gland. The type of "auto-catalytic" activation found with proteolytic pro-enzymes is not expected with non-proteolytic enzymes. The logarithmic increase in amylase might be explained if "pro-amylase" were closely associated with a "pro-protease" which underwent auto-activation resulting in simultaneous activation of "pro-amylase".

Several experiments were carried out in attempts to activate this elusive precursor. Incubations of detergents, proteolytic enzymes, protein kinase, lipase, phospholipase with parotid homogenates did not increase amylase activity. When inhibitors of protein synthesis were injected into iso-proterenol-stimulated rats, an increase in amylase levels was observed only when amylase and general protein synthesis (incorporation of ^{14}C-amino acids) was uninhibited.

Inactive precursors of proteolytic enzymes are obviously necessary in order to protect cells from autolysis. An inactive form of amylase would only be required where active amylase could disrupt cell integrity or function, such as in the liver or muscle where amylase might interfere with glycogen storage. In fact, latent microsomal amylase is found in the liver and may have some regulatory function in glycogen storage (23).

It is not possible to mathematically relate changes in apparent amylase synthesis (^{14}C-amino acid incorporation) to changes in amylase levels on the basis of precursor activation.

This is because the DPM of isolated amylase is related only indirectly to the rate of synthesis of precursor. Other unknown factors are the rate of activation of precursor and the size of the precursor pool.

Changing Turnover Rates

The importance of changing rates of enzyme degradation in the control enzymes was pointed out by Schimke et al (384). They formulated the following kinetic model:

$$\frac{dE}{dt} = K_s - kE$$

where E is the content of enzyme (units $mass^{-1}$), K_s is the zero order rate constant of synthesis (units $time^{-1}$ $mass^{-1}$) and k is the first order rate constant for degradation ($time^{-1}$). The units of mass can usually be omitted since usually organ mass remains almost constant during most experiments. The rate of degradation of protein is expressed as a first order rate constant because according to Schimke (385); "in all cases studied, except for the red blood cell and its hemoglobin, the degradation of an individual intracellular protein follows first order kinetics".

In the steady state, when $dE/dt = 0$

then $K_s = kE$

In the steady state, the level of an enzyme is a function of both rate of synthesis (K_s) and the rate constant of degradation (k).

This model can be applied to the kinetics of amylase repletion. The steady state is found in the starved, unstimulated rat (time, 0) where no change in amylase levels is observed. Setting E = 100 at time 0, the values of E at various times will be the amylase content expressed as per cent basal level. Estimates of K_s and k can be made as follows: K_s at various times is assumed to be proportional to enzyme synthesis,expressed either as total DPM incorporated into amylase or the ratio of amylase DPM to non-amylase DPM.

The increase in amylase levels is maximum between 16 and 18 hours after isoproterenol stimulation. From the regression line of best fit (figure 40), the maximum increase in amylase was found to be 12.5 per hour (% of basal amylase). If it is assumed that the rate of degradation approaches zero at a time when rate of enzyme increase is maximum, then the turnover time, t, (time required for synthesis of E = 100) was: $t = \frac{100}{12.5} = 8$ hr. In Table 30, the calculations of enzyme kinetics at various times during the repletion of amylase were made with one of three assumptions:

A. that K_s is proportional to incorporation of amino-acids into amylase;
B. that K_s is proportional to the ratio, amylase DPM/non-amylase DPM;
C. that there is no change in K_s during repletion, (changes are due only to changes in degradation, k).

TABLE 30

Enzyme kinetics of amylase repletion in the parotid gland.

Time after Isoproterenol (hours)	E Amylase Content (% of 0 time)	$\frac{dE}{dt}$	KINETIC CONSTANTS *								
			A.			B.			C.		
			K_s	kE	k	K_s	kE	k	K_s	kE	k
0	100	0	5	5	0.05	7.5	7.5	0.075	12.5	12.5	0.125
2	3.12	0	4.85	4.85	1.55	9.0	9.0	33.7	12.5	12.5	4.01
4	4.06	1.3	9.8	8.6	2.15	11.1	9.8	2.6	12.5	11.2	2.76
6	7.80	2.8	14.05	11.2	1.44	14.4	11.6	1.5	12.5	9.3	1.19
8	15.6	4.5	17.55	13.0	0.83	15.0	10.5	0.68	12.5	8.0	0.51
10	26.9	6.3	16.55	10.3	0.39	15.6	9.3	0.33	12.5	5.8	0.22
12	42.5	8.0	15.6	7.6	0.18	15.3	7.3	0.17	12.5	4.5	0.106
14	60.0	9.7	14.65	5.0	0.083	14.7	5.0	0.083	12.5	2.8	0.046
16	81.3	11.5	13.7	2.2	0.027	14.4	2.9	0.035	12.5	1.0	0.012
17	94.0	12.5	13.2	0.7	0.008	13.5	1.0	0.011	12.5	0	0
18	106.3	0	12.75	12.75	0.12	10.1	10.1	0.10	12.5	12.5	0.125
22	100	0	10.85	10.85	0.11	8.1	8.1	0.08	12.5	12.5	0.125

* The kinetic constants are defined in the text.

K_s, kE and k were calculated according to the equation

$\frac{dE}{dt} = K_s - kE$ on the following assumptions about the rate constant for amylase biosynthesis, K_s:

A., K_s is proportional to total amino acid incorporation into parotid amylase (amylase (DPM).
B., K_s is proportional to the ratio, amylase DPM/non-amylase DPM.
C., K_s is constant during repletion.

The variation of kE (the total amylase degraded per hour) with time is shown in figure 55. Figure 56 shows that variation of the first order rate constant for degradation, k, with time. With assumptions A, B and C , there was an initial 40, 50 and 30-fold increase respectively in the values of k. This was followed by an exponential decrease to below the basal level at 17 hours and an immediate return to near basal levels when repletion was complete (18 - 22 hours).

These large changes in the rate of amylase degradation are very difficult to explain. It is difficult to understand why such an unusual mechanism would be required for the function of the parotid gland. After depletion of the gland, there is an apparent 2 or 3-fold increase in the rate of synthesis (K_s) based on increased incorporation of amino acids into amylase. This is understandable since the gland must replace the amylase. The greatly increased rate of degradation (k) at the same time appears to be "self-defeating". Amylase could be repleated much more quickly and efficiently if there were an immediate decrease rather than increase in degradation.

Stabilization of Amylase

The above kinetic analysis was made on the assumption that the complete gland content of amylase was in a single pool with input from synthesis and output from degradation. This assumption may not be valid for several reasons.

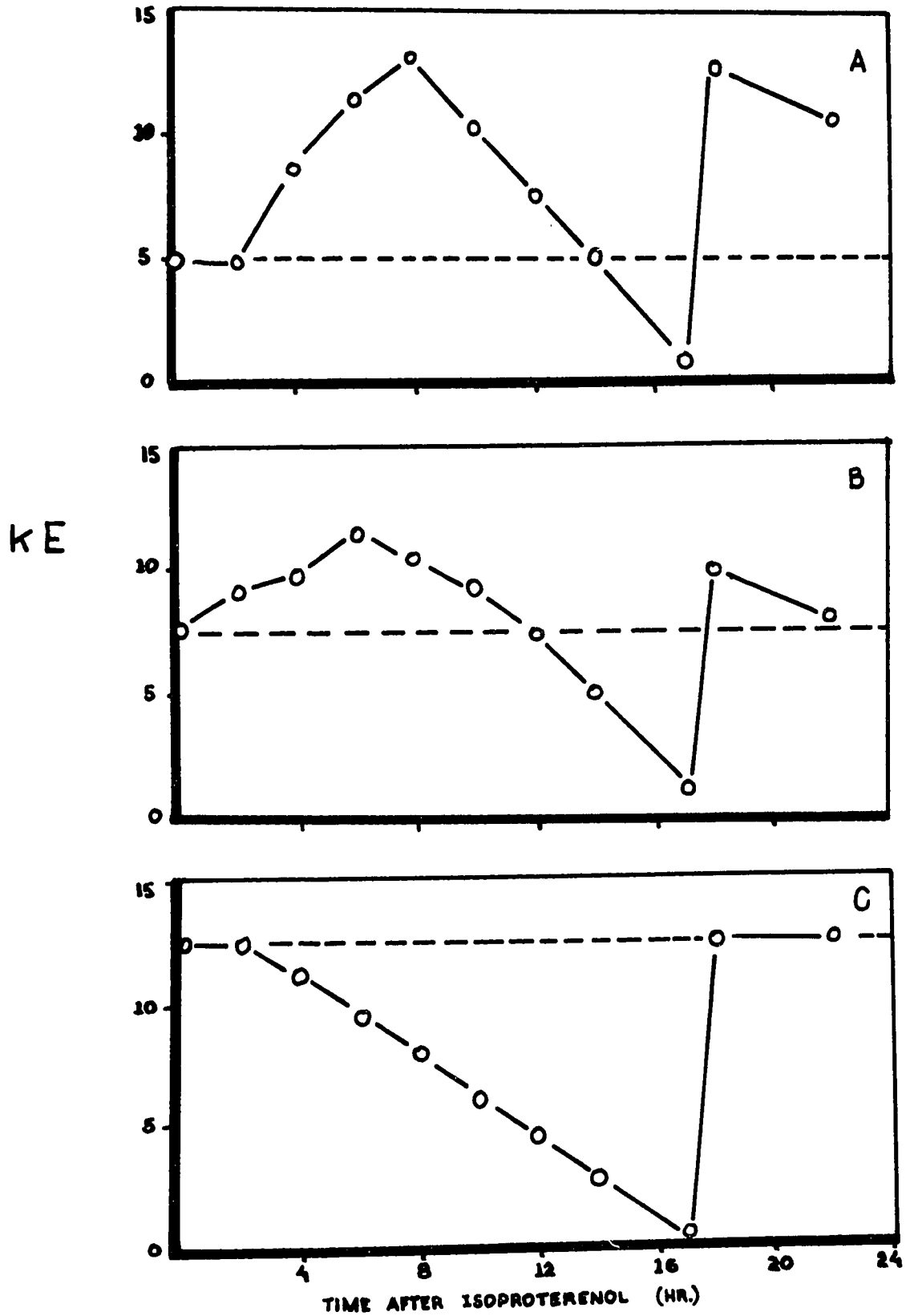


Figure 55 . Changes in total degradation of parotid amylase, kE, during repletion. Curves A, B and C are calculated values of kE based on the assumption that the rate of de novo amylase biosynthesis, K_S : A - is proportional to amylase DPM ; B - is proportional to amylase DPM/non-amylase DPM ; or C - remains constant during repletion.

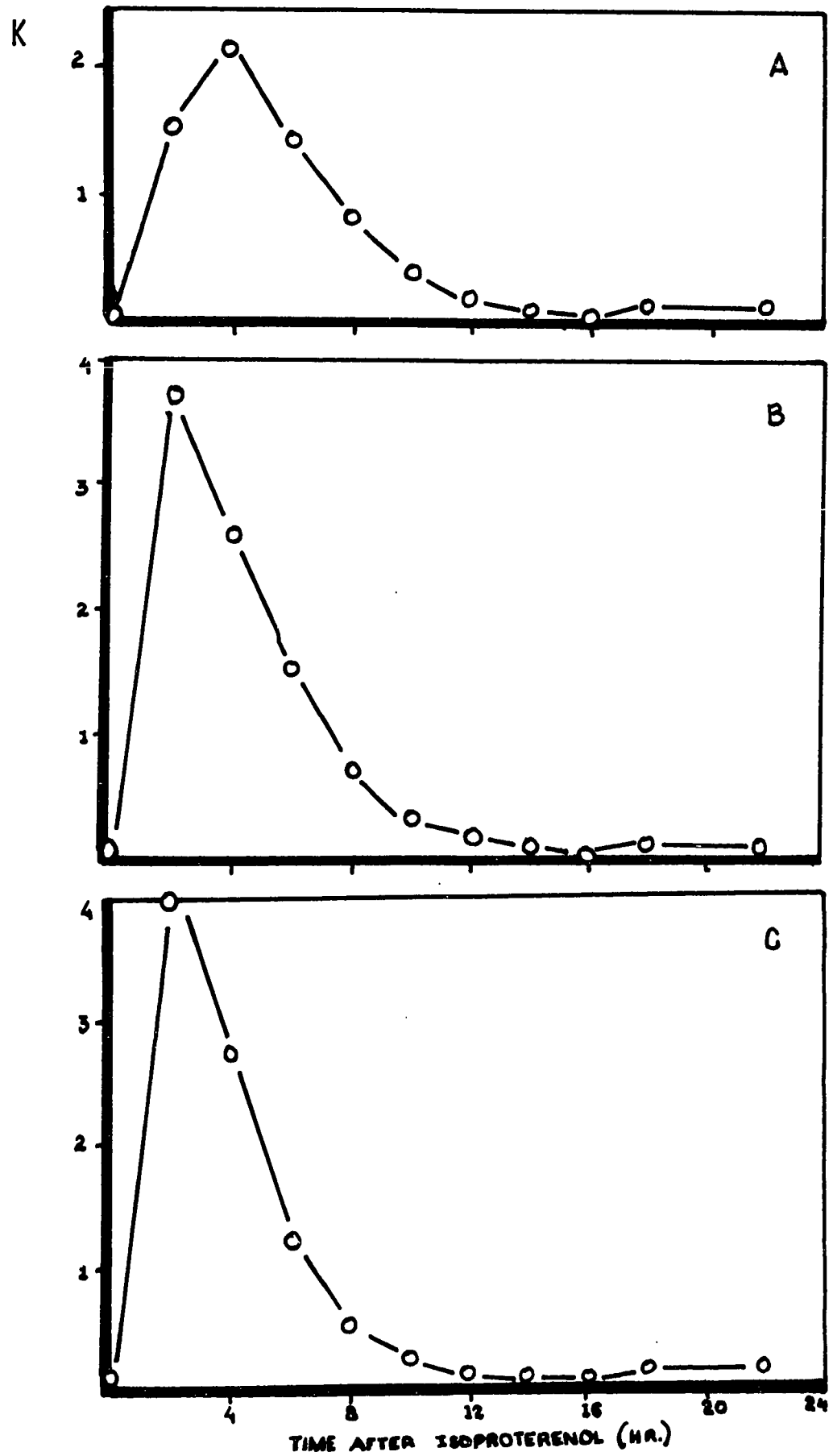


Figure 56 . Changes in k , the first order rate constant of amylase degradation during repletion. A, B and C are based on assumptions defined in figure 55.

Storage of the whole gland overnight or incubation of gland homogenates did not result in any loss of amylase. This indicated the relative stability of the parotid amylase. Gland homogenates were resistant to proteolytic attack, particularly when Ca^{++} was present. Only when EDTA was added did extensive degradation of amylase take place.

Synthesis of amylase takes place on ribosomes in the rough endoplasmic reticulum (142, 144). Cytological studies have indicated that zymogen granules are formed near the Golgi complex (169) in the parotid gland. It has been suggested that newly formed amylase is transferred through the endoplasmic reticulum to the Golgi complex and into the zymogen granules (143, 169). Since the amylase within the zymogen granules seems to be stable (171, 172, 386), amylase stabilization must take place somewhere along the route between the site of amylase synthesis and zymogen formation. This stabilization may be simply removal from contact with degrading enzymes or it may be due to a cofactor such as Ca^{++}. In either case, "vulnerable" or unstabilized amylase may be a small part of the total amylase. The kinetics described above should only apply to unstabilized amylase and thus the size of the pool of unstabilized amylase is very important.

The changes in amylase levels can be explained on the basis of a changing pool of unstabilized amylase, P. If

the rate of synthesis of amylase, K_s, changes as described previously (assumptions A, B or C), then;

$$\frac{dE}{dt} = K_s - kP$$

and the values of kP are the same as the values of kE in Table 30. Assuming no change in the first order rate of degradation, k, then the changes in amylase levels $\left(\frac{dE}{dt}\right)$ can be related to changes in P.

Electron microscopic studies (169) have indicated that stimulation with isoproterenol results in the immediate release of zymogen granule contents into the cell lumen for secretion. Two hours after secretion there were no zymogen granules observed in the cells. At four hours, there was evidence of numerous small condensing vacuoles close to the Golgi complex and later at six hours a few zymogen granules were present in the same area. Condensing vacuoles probably collect amylase from near the Golgi complex and then become zymogen granules. If it is assumed that amylase is stabilized within the zymogen granule but can be degraded outside, then at 2 or 4 hours when no zymogen granules are present, the degradable pool, P, is the same as total amylase E. In Table 31, P was set equal to E at two or four hours after isoproterenol stimulation. On this basis, with k held constant and on the basis of assumptions about amylase biosynthesis, A, B, or C, P was calculated (Table 31).

The initial size of the pool, P, was 2.5 or 3 per cent of the total amylase content. After secretion of amylase, the

TABLE 31

The changing pool of unstabilized amylase in the parotid gland during repletion of amylase.

Time after Isoproterenol (hours)	E Amylase Content (% of Control)	P* Unstabilized Amylase (% of E) A	B	C
0	100	2.36	2.60	3.12
2	3.12	2.29	3.12	3.12
4	4.06	4.06	3.39	2.80
6	7.8	5.29	4.12	2.32
8	15.6	6.13	3.64	2.00
10	26.9	4.86	3.22	1.45
12	42.5	3.59	2.53	1.12
14	60.0	2.36	1.73	0.70
16	81.3	1.04	1.00	0.25
17	94.0	0.33	0.35	0
18	106.3	6.02	3.50	3.12
22	100	5.12	2.81	3.12

* P is the pool of unstabilized amylase calculated on the basis of the assumptions about amylase biosynthesis, A, B and C as described in the text.

size of P increased according to the rate of increase in enzyme synthesis, K_s. There was a subsequent decrease in P during the later stages of repletion. After 18 hours, when repletion was complete, P returned to near control levels.

These changes in the size of the pool, P, can be easily related to the transfer of amylase into the zymogen granules, and the availability of membrane material for formation of granules. On secretion, zymogen granules travel to the cell lumen and discharge their contents. The initial increase in K_s results in increased pool size P. The transfer of amylase from the pool to zymogen granules probably requires greatly increased availability of phospholipids for formation of new zymogen granule membrane. The work of Hokin (170, 174-183) in the field of phospholipid metabolism may be very important in explaining reaccumulation of secretory enzymes after secretion. He proposed a possible mechanism of membrane circulation. The membranes from emptied zymogen granules are rapidly broken down into subunits brought about by the hydrolysis of phosphatidyl inositol. Phosphatidyl inositol is re-synthesized in the intracellular membranes of the endoplasmic reticulum and Golgi apparatus. The membrane subunits may then be re-assembled with phosphatidyl inositol to form new membranes available for the formation of new zymogen granules.

It seems possible that in the parotid gland, the secretion of amylase takes place with simultaneous transfer of zymogen membrane to the cytoplasmic membrane and subsequent break down.

The increased rate of amylase biosynthesis does not initially result in an increased rate of amylase accumulation. This may be because stabilization is limited by the availability of the phospholipids and lipoproteins. These require translocation from the cell wall to the site of amylase synthesis. The logarithmic pattern of amylase repletion may therefore result from a linearly increasing availability of membrane structural units due to increased phospholipid turnover.

The findings of Dreisbach and Taugner (231) that uptake of Ca^{45} in rat parotid and submaxillary acinar cells was greatly reduced two hours after isoproterenol, suggest other possibilities with regard to re-accumulation of amylase. Ca^{++} uptake may be a limiting factor in stabilization of newly synthesized amylase. It may have an effect on the synthesis of membrane available for zymogen granule formation, or it may effect the transport of amylase from the cysternae of the endoplasmic reticulum to the Golgi complex or to condensing vacuoles.

These possibilities are open to a variety of lines of investigation. It would be very interesting to compare the time course of accumulation of P^{32}-inositol into membranes with the accumulation of amylase. If the rate of membrane biosynthesis could be measured by injection of P^{32}-phosphatidyl inositol or a labeled precursor (CTP, phosphatidic acid, inositol) then the availability of new membrane could be compared with the accumulation of amylase. It would also be interesting to isolate the parotid zymogen granules to measure

the labeled phospholipid in the membrane. Autoradiographic studies might be useful in determining whether labeled membranes travel from the endoplasmic reticulum and Golgi apparatus to zymogen granules. The role of calcium might also be explored by similar means, using Ca^{45}.

PART II

CHANGES IN SALIVARY ENZYMES AFTER ISOPROTERENOL STIMULATION

Introduction

In this study, I was particularly interested in the short term changes which take place in salivary glands after isoproterenol treatment. The hypothesis that early changes in salivary gland enzyme activity could be explained by general changes in the levels of subcellular structures, particularly the endoplasmic reticulum was of most interest. It was therefore decided to study the subcellular distribution of enzymes in the salivary glands and the changes which take place after isoproterenol stimulation. The changes in total gland enzyme activities, and the relationship of "marker" enzymes to changes in subcellular particles was also studied.

In these studies, the following enzymes were measured in salivary glands:

1. Cytochrome oxidase: This has been used by many workers as a "marker" for mitochondria (268, 369, 386-389). Mueller et al (271) found no significant increase in rat submaxillary cytochrome oxidase activity after repeated injections of isoproterenol when both gland weight and MAO activity were increased.

2. Monoamine oxidase: In rat liver, MAO was found mainly in the mitochondrial fraction although a small portion was separated with the microsomal fraction (388). A large proportion of MAO was found in the microsomal fractions of heart, salivary glands, vas deferens and spleen (268, 275) and it has been suggested that it may be associated with the norepinephrine storage particles (268) or with the endoplasmic reticulum (271) of these tissues. An alternative suggestion (275) is that microsomal MAO originates in the outer membrane of mitochondria (390) which may be released as small vesicles during homogenization of the tissue.

3. Acid phosphatase: This enzyme has been well established as a lysosomal marker (369, 387, 389, 391) in cell fractionation studies. It has been reported that after chronic administration of isoproterenol to rats, the level of acid phosphatase activity in acinar cells was greatly increased (392).

4. Lactate dehydrogenase: This is a soluble enzyme (369). After administration of large doses of isoproterenol, increased serum LDH has been reported (393). This increase was associated with decrease in heart LDH, as well as hypertrophy and lesions of the heart.

5. Catechol-O-methyl transferase: COMT is a soluble enzyme which is involved in the metabolism of the drug, isoproterenol and other catecholamines (349).

6. Glucose-6-phosphatase: This has been used as a microsomal marker in liver, kidney and intestine (369,394).

7. UDP-Glucuronyl transferase: This enzyme, which is present in many tissues and acts with varying degrees of specificity on many phenolic substrates, has been found only in microsomal preparations (334).

8. Cytochrome-P-450: This pigment is found largely in the microsomes (277). It is part of a drug hydroxylating complex and is increased after exposure to many aromatic compounds (277). It has also been found in mitochondria and may be involved with steroid hydroxylation (278).

9. Oxidative demethylation: The enzyme which catalyzes the oxidative demethylation of aminopyrene is very closely associated with P-450 and may be part of a drug metabolizing complex (277). It is also increased after exposure to some drugs and is usually associated with microsomes (277).

Sucrose Density Gradient Centrifugation

Experimental Design

Two male rats were killed and immediately the livers and the salivary glands were removed and placed in 0.25 M sucrose at 0°. Within 30 minutes, the tissues were homogenized with a cooled Potter-Elvejhem homogenizer equipped with a teflon pestle. The liver was homogenized in 4 volumes of 0.25 M sucrose.

Linear sucrose gradients were prepared and stored on ice for two hours before centrifugation. In the plastic tubes which fit the Spinco S.W.-56 rotor, sucrose gradients of between

0.50 and 2.0 M in 3.0 ml volume were prepared by continuous mixing of equal volumes of 0.5 and 2.0 M sucrose in a Beckman plastic gradient apparatus. Over the gradients were layered 1.0 ml of each homogenate. The tubes were completely filled by the addition of 0.25 M sucrose and centrifuged at 56,000 RPM for 2.5 hours at 4° in a Spinco S.W.-56 rotor.

After centrifugation, each tube was observed and the separated bands were noted. Six fractions were collected from each tube according to the observed bands. This was done by careful removal of each fraction with a Pasteur pipette. The volume of each fraction was determined and then each fraction was diluted with 0.03 M phosphate buffer, pH 7.0 containing 0.2% Triton X-100. Aliquots were taken for the determination of protein concentration and the activity of cytochrome oxidase, MAO, acid phosphatase and amylase.

Results

Figures 57, 58 and 59 are histograms of protein concentration and enzyme activity in the fractions separated by sucrose gradient centrifugation of liver submaxillary and parotid homogenates respectively. The bands are shown and the volume of each fraction is indicated. Activities are shown in terms of units/ml. Per cent distribution is also shown.

The separation of a liver homogenate in figure 57 is similar to that found by other workers. Highest activity of cytochrome oxidase, MAO and highest protein concentration corresponded to a heavy band at a sucrose concentration

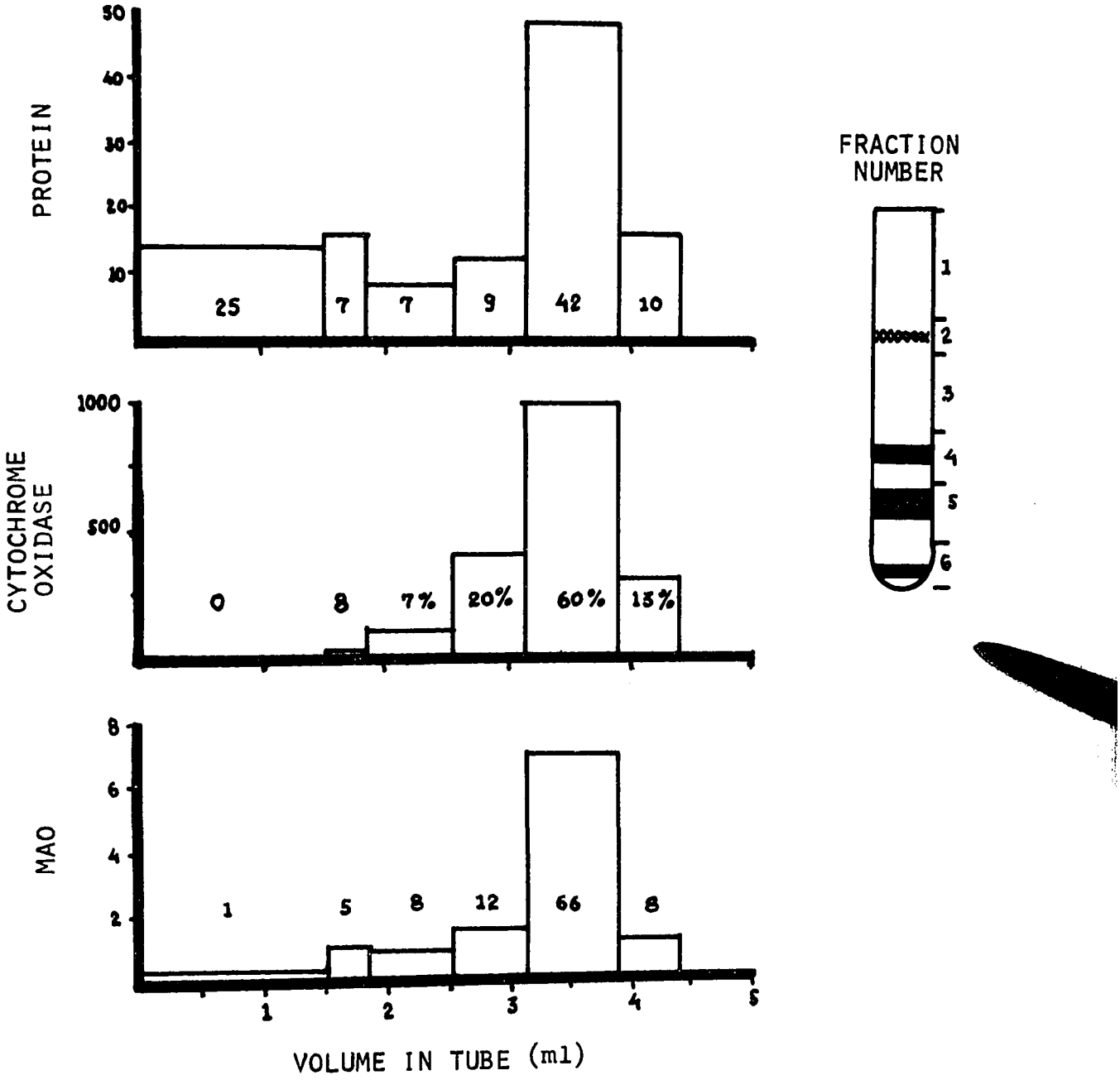


Figure 57 . Sucrose density gradient centrifugation of a rat liver homogenate. Numbers in each bar indicate % distribution. Units are given in terms of concentration: protein - mg/ml ; cytochrome oxidase - units/ml ; MAO - units/ml x 10^6.

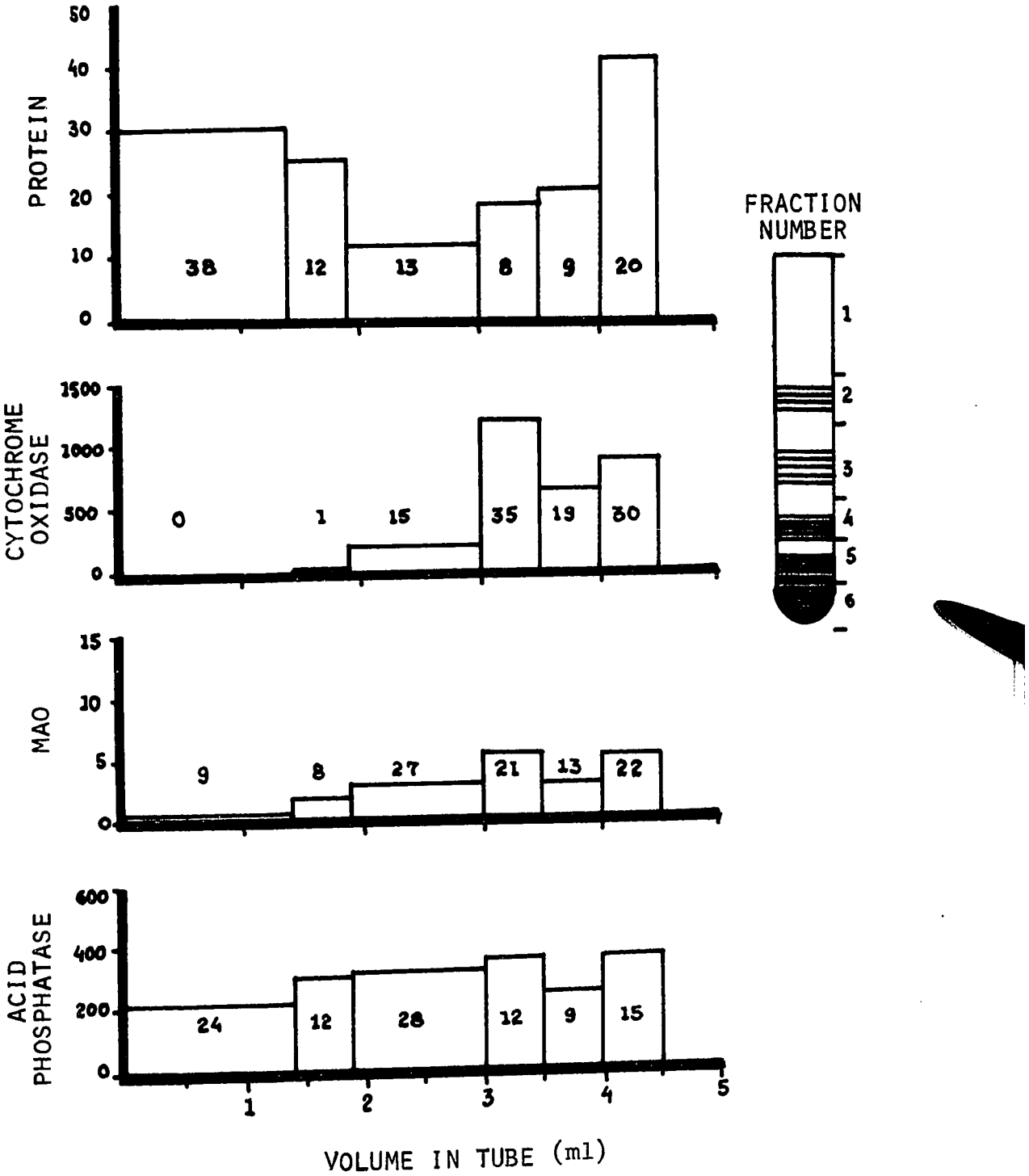


Figure 58 . Sucrose density gradient centrifugation of a rat submaxillary gland homogenate. Numbers in each bar indicate % distribution. Units are given in terms of concentration : protein - mg/ml ; cytochrome oxidase - units/ml ; MAO - units/ml x 10^6 ; acid phosphatase - units/ml.

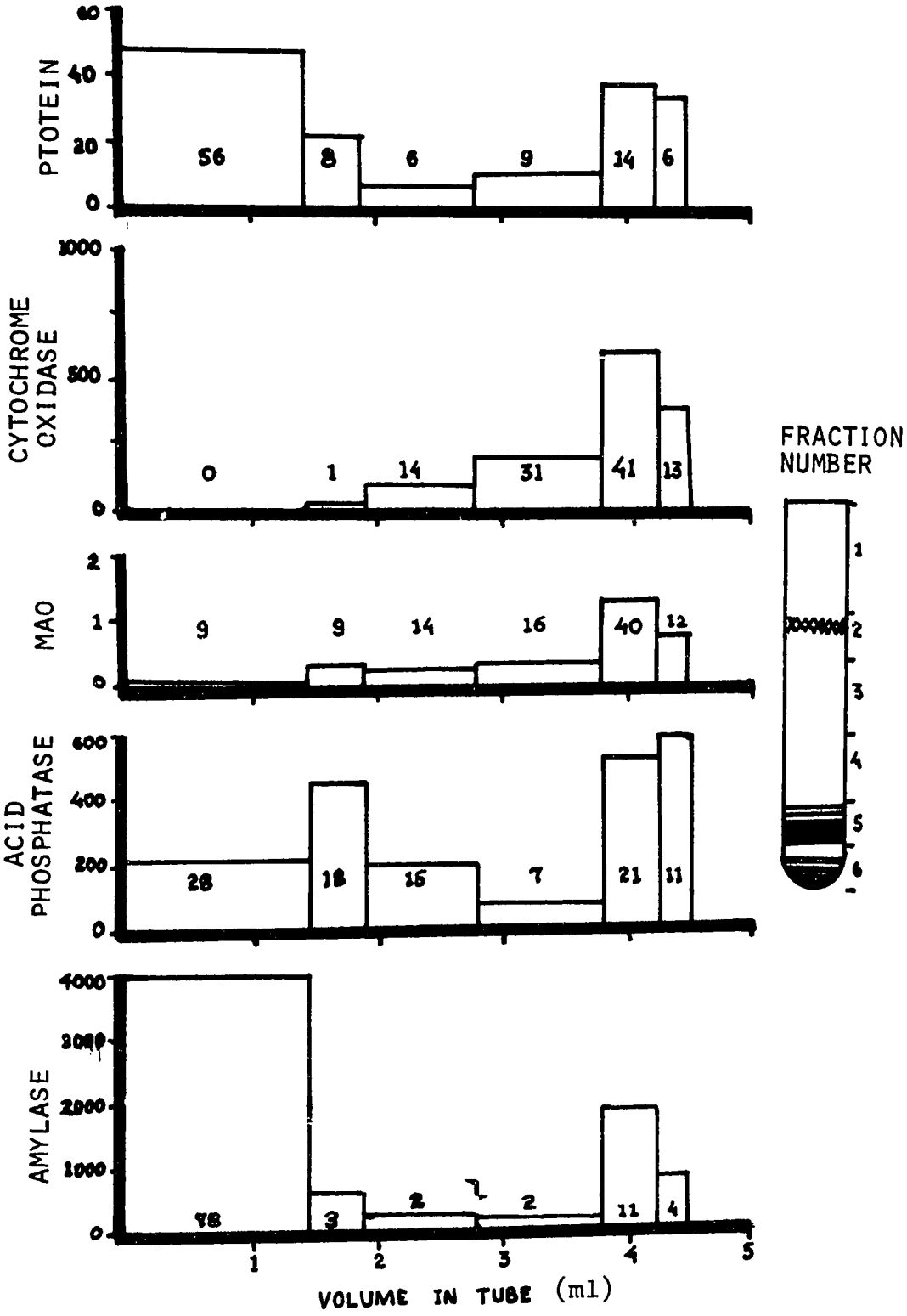


Figure 59 . Sucrose density gradient centrifugation of a rat parotid gland homogenate. Numbers in each bar indicate % distribution. Units are given in terms of concentration : Protein - mg/ml ; Cytochrome oxidase - units/ml ; MAO - units/ml x 10^6 ; Acid Phosphatase - units/ml ; Amylase - units/ml.

about 1.5 M. De Duve (387) observed that liver mitochondria reached equilibrium at a density of 1.45 M sucrose and microsomes at 0.9 M sucrose. Our results were similar to those of de Champlain et al (268). Most MAO was found in the mitochondria but the distribution of MAO and cytochrome oxidase were not completely parallel. The distribution indicated a small amount of MAO activity in the microsomal fraction.

The separation of submaxillary homogenate was much more complex, with several observable bands (figure 58). The strongest bands were at about 1.4 and 1.8 M sucrose. The band at 1.4 M was mainly mitochondrial since cytochrome oxidase was most concentrated in that band. Although this band showed MAO activity, most MAO activity was behind it associated with the microsomal and soluble fractions. Acid phosphatase activity was not concentrated into any single band, being spread almost equally among all fractions including the soluble fraction. Protein was most concentrated in both the soluble (lightest) fraction and in the most dense.

It is of interest that the most dense fraction (1.8 - 2.0 M) contained a peak of activity for all three enzymes. Whole, unbroken cells would normally be expected in this fraction. If this were the case, the same percentage of each of the enzymes should be found. The fact that acid phosphatase and MAO activities were lower than cytochrome oxidase activity can be explained as being due to interference by mucous in the

cell. (homogenization of the cells breaks open the cell membrane, allowing the escape of soluble and particulate components. Some of the particles may be trapped in the heavy, viscous mucous, and spun down in association with the fragmented cell membrane and the nucleus.) The possibility of this type of interference was further explored in some experiments which will be described below.

Disregarding the most dense fraction, it becomes apparent that a large part of MAO activity was associated with the microsomes of submaxillary gland. The MAO in the most dense fraction may have been of mitochondrial or microsomal origin or both.

The work of de Champlain et al (268) also indicated that a large portion of MAO activity of rat salivary gland is microsomal. The pattern after sucrose gradient centrifugation indicated very low MAO activity in the mitochondrial fraction. As a preliminary to sucrose density gradient centrifugation, however, he removed a fraction at 600 x g for ten minutes. It is possible that this step removed a large proportion of the submaxillary mitochondria due to entrapment by mucous. The predominance of microsomal and soluble MAO which they observed may have been due to a loss of mitochondria.

The density gradient centrifugation of a parotid homogenate resulted in a separation similar to that of the submaxillary (figure 59). This may have also been due to interference by mucous. Only two distinct bands were observed;

a weak one at a concentration of 0.6 M sucrose and a heavy band at 1.6 M sucrose. The light band (close to 0.6 M sucrose) may be composed of altered or partly damaged lysosomes since it shows a peak of acid phosphatase activity. It has been reported (395) that lysosomes treated in vivo with detergent have an equilibrium density peak at 0.8 M sucrose.

The heavy band at 1.6M sucrose contains peaks of amylase, acid phosphatase, cytochrome oxidase and MAO activity as well as high protein concentration. For this reason, it is probably not a pure mitochondrial fraction. Mitochondria are expected to band close to 1.45 M sucrose (386). It cannot be due to unbroken cells since only 10% of amylase activity was found there, 80% being found in the soluble fraction. The amylase in this band may be due to surviving zymogen granules, although they cannot usually be separated by density gradient centrifugation because of their sensitivity to osmotic shock (396). Alternatively, it may be membrane-bound, or microsomal amylase.

The peak of acid phosphatase activity is likely due to lysosomes which have been reported to band at 1.65 M sucrose (387). The mixture of enzymes in this band strongly suggested a type of interference similar to that in the submaxillary gland.

The clumping of subcellular particles by mucous cannot only explain the band at 1.6 M sucrose but also the "smearing" of cytochrome oxidase activity into the microsomal fraction.

Disregarding the last two fractions, the distributions of cytochrome oxidase and MAO are very dissimilar. Their relative patterns indicate that some MAO activity is microsomal.

Differential Centrifugation

Preliminary Experiments

The separation of subcellular components by differential centrifugation has been described for many tissues (387, 389, 391, 397). The following experiments using these methods of differential centrifugation were hindered because salivary mucins tended to entrap subcellular particles.

Fractionation A: Parotid and submaxillary glands from two unstarved rats were removed. The pooled parotids and pooled submaxillaries were homogenized as described in the previous section except that 0.25 M sucrose containing 0.005 M phosphate pH 6.9 was used and each homogenate had a final volume of 10 ml.

The nuclear fraction was obtained by centrifugation at 600 x g for ten minutes. The precipitate was resuspended in about 3 ml of the homogenizing medium and recentrifuged at 600 x g for ten minutes.

The mitochondrial fraction was obtained by centrifuging the combined nuclear supernatants at 25,000 x g for ten minutes. The precipitate was resuspended in about 2 ml homogenizing medium and recentrifuged at 25,000 x g for ten minutes.

The microsomal fraction was obtained by centrifuging the combined mitochondrial supernatants at 100,000 x g for sixty minutes. The microsomal supernatant was the soluble fraction. All fractions were resuspended in isotonic sucrose and stored on ice. Aliquots were removed for the determination of MAO (Method A), cytochrome oxidase (Method A), amylase, LDH, and protein concentration. The results are shown in Table 32 and all values are expressed as per cent of the sum of the fraction values.

Fractionation B: This was essentially the same as Fractionation A except that the final tissue homogenates were more concentrated, (5 ml final volume). Each fraction was isolated by centrifugation as described for Fractionation A except that precipitates were not resuspended and recentrifuged. Instead, homogenates and supernatants were layered over 0.32 M sucrose before centrifugation. Fractions were assayed for LDH and cytochrome oxidase activity. Results are also shown in Table 32.

Fractionation C: The nuclear fractions from Fractionation B were rehomogenized in 6 ml of 0.25 M sucrose and centrifuged at 500 x g for ten minutes to precipitate the nuclear fractions. The supernatants were centrifuged at 25,000 x g for ten minutes to precipitate the mitochondrial fractions. Cytochrome oxidase was determined. The results are shown in Table 32 expressed as per cent of the activity originally present in the crude

TABLE 32

Per cent distribution* of enzymes among subcellular fractions obtained by differential centrifugation of parotid and submaxillary homogenates.

	** FRACTIONATION A					FRACTIONATION B		FRACTIONATION C
	Cytochrome Oxidase	MAO	LDH	Amylase	Protein	Cytochrome Oxidase	LDH	Cytochrome Oxidase
Parotid								
Total	59	158	88.5	131	116	-	-	100
Nuclear	39	33	4.2	19	23	86	4	60
Mitochondrial	53	32	2.2	10	21	8	0	11
Microsomal	7	19	.7	0.3	5	4	1	-
Soluble	0	16	92.9	71	51	2	96	-
Submaxillary								
Total	56	155	116	-	103	-	-	100
Nuclear	95	82	21	-	42	85	7	45
Mitochondrial	4	12	4	-	7	9	2	26
Microsomal	1	5	2	-	5	3	1	-
Soluble	0	1	73	-	46	3	90	-

* Distribution is expressed as % of the sum of subcellular enzyme content.

** The fractionation by differential centrifugation is described in the text.

nuclear fractions from Fractionation B.

It can be seen in Table 32 that a high proportion of cytochrome oxidase and MAO activity was found in the nuclear fractions of both parotid and submaxillary glands by each method of fractionation. It was unlikely that this high nuclear MAO and cytochrome oxidase activity was due to unbroken cells, since LDH which is a soluble enzyme was almost completely released into the soluble fraction. These results suggested entrapment of mitochondria and probably microsomal particles in the nuclear fraction. Re-homogenization of the nuclear fraction (Fractionation C) released only part of the cytochrome oxidase from entrapment.

The observed high viscosity of the nuclear fractions indicated the presence of salivary mucins. It was thought that this viscous material was probably responsible for entrapment of mitochondria.

Solubilization of Interfering Salivary Mucins

The nuclear fractions of salivary glands were very viscous. Under the light microscope, a wet smear of these preparations showed very few whole cells. A large number of small particles were seen to move in large clumps along with nuclei. These were identified as mitochondria by their staining with Janus Green. Staining of these smears with a periodic acid-Schiff (PAS) stain for carbohydrate indicated the presence of large amounts of mucin. A study of smears of total cell homogenate and the nuclear fraction indicated the presence of only a few

whole cells. There were large clumps of PAS staining material containing many nuclei and small particles but without any cell walls.

The suggestions of Blobel et al (398) and Chauveau et al (399) for the preparation of "clean" nuclei were considered as possible means of overcoming entrapment due to mucins. Salivary glands were homogenized in two volumes of isotonic sucrose (0.25 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$, 0.05 M tris pH 7.5), thoroughly mixed with two volumes of 2.3 M sucrose, layered over one volume of 2.3 M sucrose and centrifuged at 124,000 x g for 30 minutes. The nuclear precipitate was resuspended in 0.25 M sucrose. It was very viscous. Under the microscope, a mixture of nuclei and many small particles was observed.

In order to find a "mucin solubilizer", human saliva was treated with a variety of agents (400). Change in viscosity was assessed by visual inspection. Agents which were able to decrease viscosity were used to treat salivary homogenates. After centrifugation, cytochrome oxidase was determined. Several of these agents interfered in the determination of cytochrome oxidase. This is indicated in Table 33.

Treatment of salivary gland homogenates with NaCl, KCl and neuraminidase did not interfere with the cytochrome oxidase assay. The effect of these agents upon the fractionation of subcellular particles was investigated. Parotid and submaxillary

TABLE 3.3

Effect of various agents upon the viscosity of salivary mucins and on the determination of cytochrome oxidase activity.

Treatment*	Decrease in Viscosity of Saliva†	Effect on Determination of Cytochrome Oxidase
a. Ascorbate + $CuSO_4$	+++	Interference
b. Ascorbate + H_2O_2	++++	"
c. Mercaptoethanol	++	"
d. $NaBH_4$	++++	"
e. KCl	+++	No Interference
f. NaCl	++	"
g. Neuraminidase	+	"

*Treatments: to one volume of saliva or homogenate was added one volume of the solubilizing solution,

a. 0.06 M Ascorbate, 0.12 M $CuSO_4$ pH 6.0
b. 0.06 M Ascorbate, 0.25 M H_2O_2 pH 6.0
c. 0.25 M B Mercaptoethanol pH 8.0
d. 0.25 M $NaBH_4$ pH 7.6
e. 0.15 M KCl
f. 0.15 M NaCl
g. 1 mg/ml Neuraminidase (Cl. Perfringens)

†Visual assessment

gland homogenates (in 0.25 M sucrose, 0.02 tris pH 6.0, 0.003 M $MgCl_2$) were incubated for 20 minutes at 37° with neuraminidase. The incubation mixtures were readjusted to pH 7.4, subcellular fractionation was carried out, and cytochrome oxidase was determined. The results, shown in Table 34, indicate that the neuraminidase was not efficient in the release of mitochondria from the "mucin clot".

To determine the effect of KCl on cell fractionation, the following experiment was carried out. Parotid and submaxillary homogenates were prepared in 0.25 M sucrose. An aliquot of each was rehomogenized with an equal volume of 0.15 M KCl or 0.25 M sucrose for the control. They were layered over 0.32 M sucrose and centrifuged at 600x g for ten minutes. The residues were resuspended in 0.15 M KCl or 0.25 M sucrose and recentrifuged at 600x g for ten minutes. The residue was the nuclear fraction. The mitochondrial fraction was isolated by centrifuging the combined supernatants, resuspension and recentrifugation at 18,000x g for ten minutes. The supernatant contained both the microsomal and soluble fractions which were not separated. Cytochrome oxidase and protein was assayed in each fraction.

The results are shown in Table 35. In the control, most cytochrome oxidase activity is in the nuclear fraction. Treatment with KCl, on the other hand, resulted in the appearance of over 80% of the cytochrome oxidase activity in the mitochondrial fraction. The release of entrapped particles by KCl was further indicated by the increase of protein in the mitochondrial fraction, and the soluble fraction (which included the microsomes).

TABLE 34

Effect of preincubation of salivary gland homogenates with neuraminidase on the distribution of cytochrome oxidase in subcellular fractions.

	Cytochrome Oxidase Activity*	
	Control	Neuraminidase
Parotid		
Nuclear	65	69
Mitochondrial	28	23
Microsomal	7	8
Soluble	0	0
Submaxillary		
Nuclear	80	76
Mitochondrial	17	20
Microsomal	3	4
Soluble	0	0

* Per cent distribution among the subcellular fractions.

TABLE 35

Effect of KCl upon the distribution of cytochrome oxidase among subcellular fractions after differential centrifugation.

	PER CENT DISTRIBUTION*			
	Control		KCl	
	Cytochrome Oxidase	Protein	Cytochrome Oxidase	Protein
Parotid				
Nuclear	75	24	13	13
Mitochondrial	21	8	80	15
Soluble†	4	68	7	72
Submaxillary				
Nuclear	52	20	10	10
Mitochondrial	44	11	84	15
Soluble†	3	69	6	75

* Activity in each fraction expressed as a per cent of the sum of activities in all fractions.

† Mitochondrial supernatant: Contains microsomal and soluble fractions.

Effect of Starving and Isoproterenol on Subcellular Fractionation

On the basis of the reduction of entrapment of mitochondria in the nuclear fractions after treatment of salivary homogenates with KCl, the following experiments were performed.

Rats were starved overnight. In the morning, rats were injected with 5 mg isoproterenol. Other rats received no isoproterenol. The rats were killed. Those receiving isoproterenol were killed 1½ to 4 hours after the injection. Parotid and submaxillary glands were immediately removed, transferred to cold isotonic KCl and then homogenized.

Homogenization was in a sucrose-KCl medium (0.25 M sucrose, 0.154 M KCl, 0.03 M $MgCl_2$ and 0.02 M tris, pH 7.6), with a final volume of 5 ml for each pair of glands. The homogenate was layered over 2 ml of 0.32 M sucrose, and centrifuged at 600 x g for ten minutes. The precipitate was resuspended in 4 ml of the homogenizing medium, and recentrifuged at 600 x g for ten minutes, yielding the nuclear precipitate. The combined nuclear supernatants were layered over 1 ml of 0.32 M sucrose and centrifuged at 22,000 x g for ten minutes. The precipitate was resuspended in 4 ml homogenizing medium and recentrifuged at 22,000 x g for ten minutes, yielding the mitochondrial precipitate. The combined mitochondrial supernatants were centifuged at 105,000 x g for one hour, yielding the microsomal precipitate and the soluble supernatant. The nuclear mitochondrial and microsomal precipitate were resuspended in 1 or

2 ml of 0.25 M sucrose. Cytochrome oxidase (Method A), MAO (Method B) and protein were assayed in each fraction.

Table 36 shows the mean percentage distributions of cytochrome oxidase, MAO and protein among subcellular fractions from several experiments. The effect of starving and treatment with isoproterenol (undepleted and depleted) are compared. This indicates that entrapment of mitochondria in the nuclear fraction was not completely overcome by the use of KCl in the homogenizing medium. Little cytochrome oxidase or MAO activity was found in parotid nuclei of starved rats, but when treated with isoproterenol, one quarter of total MAO and cytochrome oxidase activity was found in the nuclear fraction. The low value for total cytochrome oxidase activity compared to the sum of activities in the fractions may have been due to varying concentrations of inhibitors in each fraction. Inhibition of cytochrome oxidase is discussed in the Analytical Section.

In starved rats, almost half of the MAO and cytochrome oxidase activity was found in the nuclear fraction indicating continued interference from mucins. The decrease in submaxillary nuclear cytochrome oxidase after isoproterenol can be explained by the secretion of mucins. Changes in protein distribution mainly reflect the loss of soluble proteins due to isoproterenol induced secretion, particularly in the parotid gland.

These experiments indicate that while the inclusion of KCl in homogenizing media can partially overcome entrapment of particles in the nuclear fraction, the results are quite variable. The solubilization of mucins under these conditions,

TABLE 36

Effect of starving and isoproterenol on the distribution of cytochrome oxidase, MAO and protein among subcellular fractions of salivary glands. Homogenization and centrifugation were carried out in a sucrose KCl medium.

	PER CENT DISTRIBUTION*					
	Starved**			Isoproterenol†		
	Cytochrome Oxidase	MAO	Protein	Cytochrome Oxidase	MAO	Protein
Parotid						
Total	85	90	104	61	115	100
Nuclear	13	11	14	25	25	18
Mitochondrial	69	52	15	67	57	18
Microsomal	18	27	11	8	14	15
Soluble	0	10	60	0	4	49
Submaxillary						
Total	73	92	101	62	139	99
Nuclear	45	43	24	33	36	23
Mitochondrial	45	34	13	58	40	14
Microsomal	10	17	12	9	20	14
Soluble	0	4	51	0	4	49

* Activity in each fraction is expressed as % of the sum of activities in all fractions.

** Two rats were starved overnight and killed in the morning.

† Six rats were starved overnight, injected with 5 mg isoproterenol in the morning and killed between 1½ and 4 hours later.

may depend upon the concentration or composition of mucins in the gland. This probably depends, in turn, upon the physiological state of the gland (such as the depleted or repleted state). In any case, the variability in the fractionation makes it of little value in the study of subcellular changes in the salivary glands.

It is surprising that interference from mucins in the subcellular fractionation of salivary glands has not been generally observed. In a recent study of bovine submaxillary glycoproteins (49), the precipitation of most of a microsomal protein in a viscous mass was described but not explained. It is possible that in the experiments described by some authors, the salivary mucins may have interfered and may even have led to erroneous conclusions.

The Use of Marker Enzymes

Introduction

The previous section dealt with attempts to study subcellular changes in salivary glands induced by isoproterenol treatment. The interference of salivary mucins in the separation of subcellular particles of differential centrifugation or sucrose gradient centrifugation prevented the use of these methods. As an alternative, marker enzymes can be used to indicate changes taking place in subcellular structures. There are several enzymes which are known to be associated

with specific subcellular particles. Several of these have been found in the salivary glands.

We did not study any nuclear markers but other workers have followed changes in DNA, RNA, DNA synthesis, RNA synthesis and chromosome number in the salivary glands (241, 246, 248, 250, 253). Of the enzymes studied in the present work: cytochrome oxidase has been found in parotid and submaxillary mitochondria; acid phosphatase was also found in salivary glands and it is a lysosomal enzyme; amylase, in the parotid gland, is a secretory enzyme stored in zymogen granules; LDH, a soluble enzyme, was also found in salivary glands.

There were no known microsomal enzymes reported in the salivary glands. The increase in incorporation of amino acids into a specific protein, amylase, was reported in the previous section. While protein synthesis takes place on ribosomes attached to the endoplasmic reticulum, the increased rate of synthesis of a specific protein does not necessarily indicate a general increase in microsomal activity. Attempts were made to measure the activity of known microsomal enzymes, glucose-6-phosphatase, UDP-glucuronyl transferase, P-450 and oxidative demethylation, in the salivary glands.

Microsomal Markers

Glucose-6-phosphatase: The salivary glands and liver from two rats which had been starved overnight were homogenized in sucrose-KCl medium (0.25 M sucrose containing 0.15 M KCl, 0.03 M $MgCl_2$ and 0.02 M tris, pH 7.6), and

centrifuged at 17,500 x g for ten minutes to remove heavy particles. Microsomes were obtained by centrifuging the supernatant at 105,000 x g for one hour. The precipitate was resuspended in a small amount of isotonic sucrose. Glucose-6-phosphatase activity and protein concentration were determined in each microsomal preparation.

The results in Table 37 are expressed in μM of phosphate liberated/mg microsomal protein in 15 minutes for rat liver, parotid and submaxillary. The activity in the salivary glands was very low. The sample absorbance was only very slightly greater than blank absorbance for the salivary glands. Salivary gland glucose-6-phosphatase activity was less than 2% of that in liver. It is apparent that the salivary glands contain very low or no glucose-6-phosphatase activity, since the activity measurement was too close to the blank value for experimental error to be excluded as the source of the difference.

UDP-glucuronyl transferase: Salivary glands and liver from an unstarved rat were homogenized in 0.15 M KCl, and centrifuged at 17,500 x g for ten minutes. The supernatants were centrifuged at 105,000 x g for one hour, and the microsomes were resuspended in isotonic KCl. UDP-glucuronyl transferase activity was determined with about 2 mg microsomal protein for each sample.

TABLE 37

Glucose-6-phosphatase activity in the microsomes of rat liver and salivary glands.

	Glucose-6-phosphatase activity*	
	Units	% of liver Activity
Liver	0.218	100
Parotid	.001	0.46
Submaxillary	.004	1.83

* Moles phosphate liberated/mg microsomal protein during incubation at 37° for 15 minutes.

TABLE 38

UDP-glucuronyl transferase activity in rat parotid and submaxillary microsomes

	CPM		
	Blank	Sample	Formation* of Glucuronide
Liver	20154	1503	19,451
Parotid	20308	20791	-483
Submaxillary	20775	20264	+511

Two mg of microsomal protein from each tissue was incubated with UDP-glucuronic acid and ^{3}H-17 α-estradiol for 30 minutes at 37°.

* Formation of glucuonide is the difference between sample and blank.

The crude results in Table 38 indicate that salivary glands contained negligible UDP-glucuronyl transferase activity, at least with 17 α-estradiol as acceptor.

Cytochrome-P-450: Three rats were starved overnight and the liver, parotid and submaxillary glands were removed. Each tissue was homogenized in 6 volumes of 0.15 M KCl. The homogenates were centrifuged at 600 x g for ten minutes and the precipitate discarded. The mitochondria were sedimented by centrifuging the mitochondrial supernatant at 105,000 x g for one hour. The mitochondrial and microsomal precipitates were resuspended in a small volume of isotonic KCl. Protein and cytochrome-P-450 activities were determined in each sample. For P-450 determination, the samples were diluted with 0.15 M KCl to the maximum concentration at which the spectra could be measured. (This varied according to sample turbidity.)

In Table 39, P-450 measured in the usual way (uncorrected) was readily detected in liver microsomes. The lower level of P-450 in liver mitochondria was measured near the limits of detection. No activity could be detected in the microsomes or mitochondria of salivary glands.

The presence of hemoglobin in all fractions was indicated by sharp absorption peaks at 434 mμ (reduction difference spectra) and 422 mμ (CO difference spectra). Hemoglobin interferes in the measurement of P-450 due to a reduction of absorbance at 450 mμ (402). With tissues high in P-450,

TABLE 39

Cytochrome P-450 in subcellular fractions of rat liver and salivary glands.

		P-450 Activity (mμM/mg Protein)		
	Protein in assay (mg/ml)	Uncorrected	Correction A*	Correction B**
Liver				
Microsomes	2	0.330	0.400	0.385
	3	0.330	0.400	0.350
Mitochondrial	3.5	0.063	0.089	0.076
Soluble	5	-0.21	0	0
Parotid				
Microsomal	1.5	0	0.033	0.033
Mitochondrial	1	-.045	-	-0.01
Submaxillary				
Microsomal	2	-0.05	-0.006	-0.011
Mitochondrial	2.5	-0.06	-0.013	-0.022

* Correction A - correction for interference from hemoglobin based on the reduction difference spectrum peak of hemoglobin at 434 mμ.

** Correction B - correction for interference from hemoglobin based on the CO difference spectrum peak of hemoglobin at 422 mμ.

hemoglobin interference may be relatively low. With low P-450 concentration, hemoglobin may interfere substantially, and may even completely obscure P-450 absorbance. It was thought this might be the case with salivary glands, i.e. that the apparent lack of P-450 as measured by the usual method might be due to low P-450 masked by hemoglobin. Calculations were therefore made to correct for hemoglobin activity.

The corrections were based on the absorption spectra of hemoglobin. The soluble fraction from a rat liver homogenate which contained a fairly high concentration of hemoglobin and no P-450, was used to estimate the interference from hemoglobin. Hemoglobin had two very strong peaks; a reduction difference spectrum peak at 434 mμ and a CO difference spectrum peak at 422 mμ. From the liver soluble fraction, the following spectral measurements were made:

$E_{450} - E_{490}$ (CO difference) = -0.11
$E_{434} - E_{490}$ (Reduction difference) = +0.58
$E_{422} - E_{490}$ (CO difference) = +0.61

Correction A:

Correction factor $A = \frac{E_{450} - E_{490}}{E_{434} - E_{490}} = -0.19$

Therefore; P-450 $= \frac{(E_{450} - E_{490}) + 0.19\ (E_{434} - E_{490})}{91} \times 10^3 \text{mμM}$

Correction B:

$$\text{Correction factor B} = \frac{E_{450} - E_{490}}{E_{422} - E_{490}} = -0.18$$

$$\text{Therefore; P-450} = \frac{(E_{450} - E_{490}) + 0.18\ (E_{422} - E_{490})}{91} \times 10^3 \text{m}\mu\text{M}$$

Correction B is probably accurate only when P-450 values are low since P-450 reduces absorption slightly at 422 mμ.

Table 39 indicates that there was no P-450 present in the microsomes of submaxillary gland or the mitochondria of parotid or submaxillary gland. By the usual method, P-450 was not detected in the parotid microsomes. By using the correction factors, a very slight P-450 activity was estimated in parotid microsomes which was lower than that found in liver mitochondria.

The concentration of parotid microsomal P-450, even using the correction factors, was at the limits of detectability. We have not established the reliability of our correction factors, and the presence of low levels of P-450 in rat parotid microsomes requires further evidence.

Oxidative Demethylation

This enzyme is probably the same as, or closely associated with, P-450 (277). Its determination was much more sensitive, particularly when the modification utilizing fluorimetric detection (Method B) was used.

In two experiments, the oxidative demethylation activity of microsomes from liver, parotid and submaxillary was determined. The main differences between experiments 1 and 2 is the method of enzyme assay.

Experiment 1: A male rat was starved overnight, the liver and salivary glands were removed and homogenized in 0.15 M KCl. Microsomes were prepared (the same way as in the experiment on P-450), and resuspended in .15 M KCl. Between 2 and 4 mg of microsomal protein was used in each oxidative demethylation assay (Method A). In this experiment, no sample blank correction was made. Results are expressed in Table 40 as mμmole formaldehyde formed/min/mg protein.

Experiment 2: Liver and salivary gland microsomes from two unstarved rats were prepared in the same way as described in Experiment 1. Oxidative demethylation activity was determined on about 2 mg microsomal protein by Method B. These results are also shown in Table 40.

In Experiment 1, the activity of oxidative demethylation salivary gland microsomes was very low (1 to 2% of liver activity). The sample values were only slightly above the reagent blank. In Experiment 2, where sensitivity was increased due to lowered reagent blank (Method B), and where the slight interference from the sample itself was subtracted, it is apparent that salivary gland oxidative demethylation was completely absent (less than 0.4% of liver activity).

The Effect of Isoproterenol on Cytochrome Oxidase, MAO, Acid Phosphatase, LDH and COMT Activities in Parotid and Submaxillary Glands

In several experiments, male rats weighing between 155

TABLE 40

Oxidative demethylation in microsomes isolated from rat liver and salivary glands.

	OXIDATIVE DEMETHYLATION ACTIVITY			
	Experiment 1*		Experiment 2*	
	Units/mg Protein†	% of liver Activity	Units/mg Protein†	% of liver Activity
Liver	2.81	100	1.8	100
Parotid	.030	1.06	.004	0.2
Submaxillary	.057	2.03	.007	0.35

* In Experiment 1, Method A was used and no correction was made for fluorescence due to the sample. In Experiment 2, Method B was used and a sample blank was subtracted.

† Units are mμM formaldehyde formed/min.

and 185 g were starved overnight and injected with 5 mg isoproterenol in the morning. They were killed 4 or 8 hours after injection. Some were killed in the morning without injection (0 hours). The salivary glands were immediately removed and chilled in cold isotonic KCl. Each pair of glands was homogenized in buffered isotonic KCl, (0.15 M KCl, 0.003 M $MgCl_2$, 0.02 M tris, pH 7.6), and made to a final volume of 9 ml. A small aliquot was removed for protein and acid phosphatase assays. The homogenates were centrifuged at 105,000 x g for ten minutes. The precipitate was called the total particulate fraction (containing nuclei, mitochondria and microsomes) and the supernatant was the soluble fraction. The precipitate was resuspended in the homogenizing medium and made to a final volume of 2.0 ml. MAO and cytochrome oxidase (Method B) activities were measured in the total particulate fraction. COMT and LDH activities were determined in the soluble fraction. There were at least six animals at each time (0, 4 or 8 hours after isoproterenol) for each enzyme assay.

The response of parotid enzymes to isoproterenol is summarized in Table 41. There were no significant changes in cytochrome oxidase, MAO, acid phosphatase or LDH total activity, 4 or 8 hours after isoproterenol. COMT was increased about 70%, 4 hours after injection and returned almost to the basal level 8 hours after injection. The increase between 0 and 4 hours was significant ($P<0.05$).

TABLE 41

Changes in the total activity of enzymes in the parotid gland after treatment with isoproterenol. Units of enzyme activity are defined in the text. Mean values of six assay with standard error of the mean are given.

Time after Isoproterenol (hours)	TOTAL ENZYME ACTIVITY PER PAIR OF PAROTID GLANDS*				
	Cytochrome Oxidase	Monoamine Oxidase	Acid Phosphatase	LDH	COMT
0	1820±210	174±19	125±10	1640±240	29±6
4	1750±240	160±23	121± 8	1910±300	59±5†
8	1760± 31	165±21	127± 7	1760±330	37±8

* Enzyme units are defined in the text.

† $P<0.05$

TABLE 42

Changes in the total activity of enzymes in the submaxillary gland after treatment with isoproterenol. Mean values of six assays are given with the standard error of the mean. Units of enzyme activity are defined in the text

T Time after Isoproterenol (hours)	TOTAL ENZYME ACTIVITY PER PAIR OF SUBMAXILLARY GLANDS				
	Cytochrome Oxidase	MAO	Acid Phosphatase	LDH	COMT
0	3010±240	260±34	165±14	4250±440	163±14
4	2850±280	279±41	178±13	4480±370	293±11*
8	2880±190	293±32	175±16	3820±490	201±27

* $P<0.01$

The response of submaxillary enzymes to isoproterenol, which is summarized in Table 42, paralleled the response in the parotid gland. No changes were found in cytochrome oxidase, MAO, acid phosphatase, or LDH levels in the submaxillary gland. The increase in COMT was almost two-fold four hours after injection of isoproterenol. This change was highly significant ($P<.01$). The level of COMT eight hours after isoproterenol stimulation was higher than the basal level, but the difference was not significant.

Discussion

The results in general for Part II of the Experimental Section can be summarized as follows: Very few changes in enzyme activities were found in salivary glands in the repletion stage of the secretory cycle following isoproterenol stimulation. The moderate increase in *de novo* amylase biosynthesis (described in Part I of the Experimental Section) suggested a possible stimulation of microsomal enzyme activity. However, a number of typical microsomal enzymes (glucose-6-phosphatase, cytochrome-P-450, oxidative demethylase and UDP-glucuronyl transferase) were not found in either the parotid or submaxillary glands of rats. Furthermore, the interference of salivary mucins in subcellular fractionations prevented the isolation of microsomes for the study of changes following isoproterenol.

Other subcellular "marker" enzymes were measured, and no changes were found in the activities of mitochondrial cytochrome oxidase, lysozomal acid phosphatase or soluble LDH. The catecholamine metabolizing enzymes were also measured. MAO, which has been found in both mitochondrial and microsomal fractions of rat salivary glands (268) was reported by Mueller et al (271) to be increased approximately 30% three hours after isoproterenol injection. The paper did not make clear, however, whether these results were obtained with pooled parotid and submaxillary glands, or submaxillary alone. The results in the present study conflict with the findings of Mueller et al, because no change in MAO activity was found in either submaxillary or parotid. One possible explanation is the following:

Mucins in the homogenate bind the subcellular particles in a mucin "clot" which can rapidly precipitate these particles out of suspension. If a preliminary low speed centrifugation to remove cell debris and nuclei were carried out (268), or if care were not taken to keep the particles suspended while removing an aliquot for MAO determination, some MAO activity would be lost. The salivary mucin concentration varies according to the secretion and repletion cycle. This could, therefore, result in apparent changes in MAO activity.

Another possible explanation is related to the choice of substrate for MAO determination. Mueller et al (271) measured MAO with tryptamine as substrate. In the present study, kynuramine was used. Tryptamine as well as serotonin, dopamine and tyramine have been described as having similar properties as a MAO substrate, being much more active than compounds such as epinephrine, norepinephrine, benzylamine and kynuramine (403). Sierens (280) has shown that in rat liver there are two separable MAO isoenzymes which display distinctly different kinetics. Serotonin is a good substrate for one isoenzyme, whereas benzylamine is a good substrate for the second. More recent studies in this laboratory have shown that kynuramine is also a substrate for the second isoenzyme (404). Axelrod and Vesell (292) have also indicated the presence of two isoenzymes of MAO activity. The pair of enzymes are the same in all the tissues of each species. It is possible that the discrepancy between the present work and that of Mueller et al (271) may be due to the measurement of two different MAO enzymes. It would therefore be interesting to measure the changes in the serotonin (tryptamine)-specific, and benzylamine (kynuramine)-specific enzymes separately.

The increase in COMT activity in both parotid and submaxillary glands four hours after isoproterenol stimulation which was described in the Experimental Section may be simply

a response of the gland to metabolize high glandular concentrations of the drug. The fact that COMT activity subsequently returned to normal (at 8 hours) after all parotid isoproterenol had been metabolized, is consistent with this view. The report of Barka (254) that salivary COMT activity was greatly decreased 24 hours after isoproterenol stimulation is very difficult to explain, either in terms of drug metabolism, or as an expected response to increased cell growth or cell division. The dosage of isoproterenol used by Barka (254) however, was many times greater than that used in the present work. It is possible that this may have something to do with the decreased COMT levels at 24 hours. In order to clarify this issue, COMT activities could be measured over the complete range of time from 0 to 24 hours after injection of varying doses of isoproterenol. It has recently been suggested by Jarrott (289) and Broch et al (290) that COMT activity in salivary glands is located in both acinar cells and neuronal cells. The changes in salivary COMT activity following isoproterenol may therefore reflect a change in one of the two localized COMT enzymes. The changes in COMT at 4 hours and at 24 hours may even have a different localization. This possibility might be investigated by using sympathectomized rats, and rats pretreated by salivary duct ligation.

SUMMARY AND CONCLUSION

A summary of the experimental work described in the Analytical and Experimental Sections is listed below with conclusions drawn from this work.

Analytical

The following analytical procedures and modifications of methods were developed in order to carry out the studies in this thesis.

1. An automated amylase method was developed for the routine assay of amylase activity in parotid homogenates, and isolated amylase preparations. It was based on the saccharogenic method of Bernfeld (3) and utilized the ordinary modules of the Technicon AutoAnalyzer. The method retained the speed and sensitivity of the manual method with the added advantage of greater convenience and precision.

2. An adaptation of the automated amylase method allowed the continuous monitoring of parotid amylase secretion in anaesthetized rats.

3. For the routine determination of protein in isolated amylase preparations, the method of Lowry et al (306) was adapted to the Technicon AutoAnalyzer. This automated modification measured precisely and rapidly protein

concentrations between 25 and 500 µg/ml in sample volumes as small as 0.4 ml.

4. Inhibition of cytochrome oxidase activity by normal cell components in salivary gland homogenates was overcome by the suitable choice of pH and ionic strength as well as addition of RNA to the incubation medium.

5. The measurement of very low levels of oxidative demethylase activity in the salivary glands required greater sensitivity than was afforded by the method of Orrenius (227). Increased sensitivity was achieved by solvent extraction and fluorometric determination.

Experimental

Part I

The effect of isoproterenol induced secretion on rat parotid amylase was investigated. This work can be summarized as follows:

1. Previous workers have shown that injection of isoproterenol results in depletion of rat parotid amylase within two hours, followed by repletion between 2 and 18 hours (132). The repletion followed exponential kinetics. In the present study, similar effects were found with a smaller dose of isoproterenol. The kinetics of repletion fitted a quadratic equation. This indicated the complexity of the repletion. Changes in protein concentration were much smaller but were

parallel to the changes in amylase levels.

2. De novo synthesis of amylase was estimated on the basis of the rate of incorporation of amino acids into amylase, or on the ratio of amylase incorporation to non-amylase incorporation. By the first method of estimation, de novo synthesis was increased by a factor of 3.5 during the first 8 hours after isoproterenol treatment, then returned almost to the initial rate after 24 hours. By the second estimation, the changes were less dramatic, but the rate was doubled at six hours and returned to the basal level at 24 hours. Neither of these estimations can alone account for the exponential or quadratic kinetics of amylase repletion.

3. The observed pattern of amylase repletion cannot result from changing rates of secretion. Coninuous monitoring of amylase secretion indicated the complete cessation of parotid amylase secretion within three hours of the isoproternol injection.

4. A possibility was investigated that the activation of an inactive precursor of amylase might be responsible for the unusual kinetics of amylase repletion. Incubation of parotid homogenates with a variety of hydrolytic enzymes, detergents, cyclic AMP, or protein kinase in an attempt to activate "proamylase" resulted in no increase in amylase activity. The effect of specific inhibitors of protein synthesis on the in vivo repletion of amylase was investigated.

In general, when amino acid incorporation into protein was inhibited, amylase repletion was also inhibited. When no inhibition of amino acid incorporation was observed, amylase repletion was normal. This indicated the close dependence of repletion on protein synthesis and failed to show any evidence of precursor activation.

5. Several other possible mechanisms were discussed. These included changing rates of amylase degradation due to changing activity of degradative enzymes or changing stabilization of newly synthesized amylase. The large changes in degradative activity which would be required to account for the repletion of amylase could not be explained on the basis of known biochemical or cytological changes. The repletion kinetics could, however, be caused by a changing pool of unstabilized amylase. The changes in pool size which could account for the unusual repletion kinetics were discussed in relation to known cytological changes such as zymogen granule formation, phospholipid turnover and changes in Ca^{++} transport and concentration.

6. Salivary isoamylases have been previously demonstrated by disc electrophoresis. Their existence was confirmed in the present study by the use of isoelectrofocusing on polyacrylamide gels. A superior separation of the isoamylases into distinct bands was achieved by this method. Their separation by isoelectrofocusing indicates that

isoamylases differ in ionic charge rather than molecular weight.

Part II

General changes in salivary gland enzymes following the injection of isoproterenol were investigated in Part II. The findings are summarized as follows:

1. Subcellular fractionation of salivary glands by differential or by sucrose gradient centrifugation was unsuccessful because of interference from salivary mucins. This was due to the entrapment of mitochondria and possibly other subcellular particles in a "mucin clot".

2. The interference by mucins was partially overcome by the addition of KCl to the homogenizing medium. The effectiveness of KCl varied with the time after isoproterenol injection, and therefore, subcellular fractionations could not be used with confidence in studying changes during the repletion cycle. It is also possible that experiments of some other workers may have been affected by salivary mucins.

3. Four microsomal markers, glucose-6-phosphatase, UDP-glucuronal transferase, cytochrome P-450 and oxidative demethylation were measured. No significant activity of any of these enzymes could be detected in either the parotid or the submaxillary gland.

4. No significant changes were found in cytochrome oxidase, acid phosphatase and lactate dehydrogenase activities of salivary glands following isoproterenol induced secretion.

5. No change in MAO activity was found in salivary glands following isoproterenol induced secretion. This contradicted the work of Mueller et al (271) who reported a 30% increase in salivary gland MAO 3 hours following isoproterenol stimulation. Possible reasons for this discrepancy have been discussed.

6. COMT activity in both parotid and submaxillary glands was almost doubled, 4 hours after isoproterenol injection, and returned almost to the basal level 8 hours after isoproterenol injection. These changes may be the specific response of a degradative enzyme to high levels of substrate (isoproterenol) followed by a return to normal levels after complete metabolism of the substrate.

REFERENCES

1. E.H. Fischer and E.A. Stein: The Enzymes 4 313 (1961) ed. P.D. Boyer, H. Lardy and K.Myrbäck, Academic Press, N.Y.

2. P. Bernfeld: Advances in Enzymology 12 379 (1951) ed.F.F. Nord, Interscience Publishers, N.Y.

3. P. Bernfeld: Methods in Enzymology 1 149 (1955) ed. Collowick and Kaplan, Academic Press, N.Y.

4. K. Myrbäck and G. Neumüller: The Enzymes 1 653 (1950)

5. G.C.S. Kirchhoff: Mem.Acad.Imp.Sci. St. Petersbourg 4 27 (1811) cited in (1)

6. E.F. Leuchs: Poggendorff's Ann.Phys.Chem. 22 623 (1831) cited in (1)

7. A. Payen and J. Persoz: Ann.Chim.Phys. 63 73 (1933) cited in (1)

8. M. Magendie: Compt.Rend.Acad.Sci. 23 189 (1846) cited in (1)

9. R. W. Atkinson: Proc.Roy.Soc. 32 299 (1881) cited in (1)

10. R. Kuhn: Ber. 57 1965 (1924)

11. C.T. Greenwood, A.W. MacGregor and E.A. Milne: Arch. Biochem.Biophys. 459 466 (1965)

12. S.Schwimmer and A.K.Balls: J.Biol.Chem. 179 1063 (1949)

13. T.Fukushi, A.Imanishi, T.Isemura: J.Biochm. 63 409 (1968)

14. E.H.Fischer and R.de Montmollin: Nature 168 606 (1951)

15. M.Müller, P.Baudhuin and C.de Duve: J.Cell.Physiol. 68 165 (1966)

16. W.W.Doane: Amer.Zool. 5 697 (1965)

17. R.L.McGeachin: in Screebny and Meyer, 1964 (17a), p.325

17a. L.M.Sreebny and J.Meyer: ed. Salivary Glands and their Secretions, Macmillan, N.Y. (1964)

18. R.L.McGeachin: Ann.N.Y.Acad.Sci. 151 208 (1968)

19. L.C.U.Junqueira: in Schneyer and Schneyer, 1967 (19a) p.286

19a. L.H.Schneyer and C.A.Schneyer: ed. Secretory Mechanisms of Salivary Glands, Academic Press, N.Y. (1967)

20. R.L.McGeachin, J.R.Gleason and M.R.Adams: Arch.Biochem. 75 403 (1958)

21. M.Arnold and W.J.Rutter: J.Biol.Chem. 238 2760 (1963)

22. R.J.McGeachin and J.P.Lewis: J.Biol.Chem. 234 795 (1959)

23. J.Mordoh, C.R.Krisman, A.J.Parodi and L.F.Leloir: Arch.Biochem.Biophys. 127 193 (1968)

24. W.J.Rutter, M.L.Arnold, R.W.Brosemer and J.A.Miller: J.Biol.Chem. 236 1259 (1961)

25. M.R.Robinovitch and L.M.Sreebny: Arch.Oral Biol. 14 935 (1969)

26. L.H.Schneyer and C.A.Schneyer: Ann.N.Y.Acad.Sci. 85 189 (1960)

27. D.Afonsky: Saliva and its Relation to Oral Health, U. of Alabama Press (1961)

28. J.Muus: Compt.Ren.Trav.Lab. Carlsberg, Ser.Chim. 28 317 (1953) cited in (6)

29. K.H.Meyer, E.H.Fischer, A.Staub and P.Bernfeld: Helv.Chim.Acta 31 2158 (1948)

30. E.H.Fischer, F.Duckert and P.Bernfeld: Helv.Chim.Acta 33 1060 (1950)

31. K.H.Meyer, E.H.Fischer and P.Bernfeld: Helv.Chim.Acta 30 64 (1947)

32. K.H.Meyer, M.Fuld and P.Bernfeld: Experientia 3 411 (1947)

33. S.Akabori, T.Ikenaka and B.Hagihara: J.Biochem. 41 577 (1954)

34. B.M.Pogell: Methods in Enzymology, 9 9 (1966) ed. S.P.Collowick and N.O.Kaplan, Academic Press, N.Y.

35. E.Starkenstein: Biochem.Z. 24 210 (1910), cited in (1)

36. F.B.Straub: Acta Physiol.Acad.Sci.Hung. 12 295 (1957)

37. A.Loyter and M.Schramm: Biochim.Biophys.Acta 65 200 (1962)

38. A.Levitzki, J.Heller and M.Schramm: Biochem.Biophys. Acta 84 101 (1964)

39. B.Gelotte: Acta Chem.Scand. 18 1283 (1964)

40. R.Shainkin and Y.Birk: Biochim.Biophys.Acta 122 153 (1966)

41. H.Mutzbauer and G.V.Schulz: Biochim.Biophys.Acta 102 526 (1965)

42. P.Wilding: Clin.Chim.Acta 8 918 (1963)

43. G.Marchis-Mouren and L.Paséro: Biochim.Biophys.Acta 140 336 (1967)

43a. P.Cozzone, L.Paséro and G.Marchis-Mouren: Biochim. Biophys.Acta 200 590 (1970)

44. R.W.Brosemer and W.J.Rutter: J.Biol.Chem. 236 1253 (1961)

45. D.L.Kauffman, N.I.Zager, E.Cohen and P.J.Keller: Arch.Biochem.Biophys. 137 325 (1970)

46. W.W.Doane: Proc. 12th Intl.Cong.Ent., London (1964)

47. W.W.Doane: Dros.Inf.Serv., Cited in Canalco Abstract #356

48. J.Muus and J.M.Vnenchak: Nature 204 283 (1964)

49. R.O.Wolf and L.L.Taylor: Nature 213 1128 (1967)

50. B.Boettcher and F.A.de la Lande: Austral.J.Exp.Biol.Med. Sci. 47 97 (1969)

51. G.M.Malacinski and W.J.Rutter: Biochemistry, 8 4382 (1969)

52. J.A.Thoma, C.Brothers and J.Spradlin: Biochemistry 9 1768 (1970)

53. H.Heller and R.G.Kulka: Biochim.Biophys.Acta 165 393 (1968)

54. J.Muus: J. Am. Chem.Soc. 76 5163 (1954)

55. M.L.Caldwell, E.S.Dickey, V.M.Hanrahan, H.C.Kung, J.T.Kung and M.Miski: J. Am. Chem.Soc. 76 143 (1954)

56. E.A.Stein, J.M.Junge and E.H.Fischer: J.Biol.Chem. 235 371 (1960)

57. C.E.Danielsson: Nature 160 899 (1947)

58. R.L.McGeachin and J.H.Brown: Arch.Biochem.Biophys. 110 303 (1965)

59. S.Akabori and T.Ikenaka: J.Biochem. 42 603 (1956)

60. T.Ikenaka: J.Biochem. 43 255 (1956)

61. K.Sugae: J.Biochem. 47 170 (1960)

62. L.L.Campbell and P.D.Cleveland: J.Biol.Chem. 236 2966 (1961)

63. G.Marchis-Mouren, L.Paséro and P.Nesnuelle: Biochem. Biophys.Res.Commun. 13 262 (1963)

64. M.L.Caldwell: Abstr.Am.Chem.Soc.Meeting, p.66C (1960) Cited in (40, 138)

65. G.A.Malodova, I.D.Ivanov and G.M.Nikolaev: Izv.Akad. Nauk.SSSR, Ser.Biol. 30 359 (1965), Cited in Chem. Abstr. 63 4601f (1965)

66. J.Friedman and C.J.Epstein: J.Biol.Chem. 242 5131 (1967)

67. I.Kato, H.Koda and K.Narita: J.Biochem. 63 479 (1968)

68. I.Kato, H.Koda and K.Narita: J.Biochem. 63 487 (1968)

69. T.Fukushi, A.Imanishi and T.Isemura: J.Biochem. 63 409 (1968)

70. E.A.Stein and E.H.Fischer: J.Biol.Chem. 232 867 (1958)

70a. N.E.Welker and L.L.Campbell: Biochemistry 6 3681 (1967)

71. M.Schramm and L.Loyter: Methods of Enzymology 8 533 (1965) ed. Collowick and Kaplan, Academic Press, N.Y.

72. R.J.Henry and N.Chiamari: Clin.Chem. 6 434 (1960)

73. J.A.Thoma: J.Theoret.Biol. 19 297 (1968)

74. I.Radichevitch, M.M.Becker, M.Eitingon, V.H.Gettler, G.C.Toralballa and M.L.Caldwell: J.Am.Chem.Soc. 81 2845 (1959)

75. J.Wakim, M.Robinson and J.A.Thoma: Carbohydrate Research 10 487 (1969)

76. W.C.Davidson: Bull.John Hopkins Hosp. 37 281 (1925) Cited in 76a

76a. R.L. Searcy, P.Wilding and J.E.Berk: Clin.Chim.Acta 15 189 (1967)

77. R.Elman and J.M.McCaughan: Arch.Intl.Med. 40 58 (1927)

78. L.C.Chesley: J.Biol.Chem. 92 171 (1931)

79. V.C.Meyers and E.Reid: J.Biol.Chem. 99 595 (1933)

80. G.Gomori: Am.J.Clin.Pathol. 27 714 (1957)

81. P.Rona and C.van Eweyk: Biochem.Z. 149 174 (1924); Cited in 76a

82. J.M.Waldon: J.Lab.Clin.Med. 38 148 (1951)

83. P.H.Guth, S.A.Komarov and H.Shay: Am.J.Physiol. 173 461 (1953)

84. L.Jacobson and H.Hansen: Scand.J.Clin.Lab.Invest. 4 134 (1952)

85. P.H.Guth: Am.J.Gastroenterol. 33 139 (1960)

86. F.L.Bates, D.French and R.E.Rundle: J.Am.Chem.Sec. 65 142 (1943)

87. D.L.Mould and R.L.M.Synge: Biochem.J. 58 585 (1954)

88. T.J.Schoch: Proc.Ann.Meet.Amer.Soc.Brewing Chemists 83 (1961); Cited in 76a

89. J.Wohlgemuth: Biochem.Z. 9 1 (1908); Cited in 76a

90. M.Somogyi: J.Biol.Chem. 125 399 (1938)

91. C.Huggins and P.S.Russell: Ann.Surg. 128 668 (1948)

92. B.W.Smith and J.H.Roe: J.Biol.Chem. 179 53 (1949)

93. H.V.Street and J.R.Close: Clin.Chim.Acta 1 256 (1956)

94. A.Dahlqvist: Scand.J.Clin.Lab.Invest. 14 145 (1962)

95. R.L.Searcy, J.Ujihira, S.Hayashi, J.E.Berk: Clin.Chim. Acta 9 505 (1964)

96. N.R.Pimstone: Clin.Chem. 10 891 (1964)

97. K.Moeckel and F.Rost: Z.Physiol.Chem. 67 433 (1910); Cited in 76a

98. N.Nelson: J.Biol.Chem. 153 375 (1944)

99. S.Z.Sorkin: J.Clin.Invest. 22 329 (1943)

100. B.Fingerhut, R.Ferzola, A.Poock and W.H.Marsh: Clin. Chem. 9 862 (1965)

101. A.C.Kilbrick, H.E.Rogers and S.Skupp: J.Biol.Chem. 190 107 (1951)

102. K.Lorentz: Ärztl.Forsch. 19 578 (1965); Cited in 76a

103. K.Lorentz and D.Oltmans: Clin.Chem. 16 300 (1970)

104. V.C.Myers and J.A.Killian: J.Biol.Chem. 29 179 (1917)

105. V.C.Myers, A.J.Free and E.E.Rosinski: J.Biol.Chem. 154 39 (1944)

106. F.Hosteller, E.Borel and H.Devel: Helv.Chim.Acta 34 2132 (1951)

107. J.B.Sumner: J.Biol.Chem. 47 5 (1921)

108. J.B.Sumner: J.Biol.Chem. 108 51 (1935)

109. G.Noelting and P.Bernfeld: Helv.Chim.Acta 31 286 (1948)

110. K.H.Meyer, E.H.Fischer and P.Bernfeld: Helv.Chim.Acta 38 64 (1947)

111. R.L.Searcy, S.Hayashi and J.E.Berk: Am.J.Clin.Path. 36 582 (1966)

112. A.D.Jamieson, K.M.Pruitt and R.C.Caldwell: J.Dent.Res. 48 483 (1969)

113. S.Akabori, K.Usihara, Y.Shimazu and K.Nakanishi: J.Chem.Soc.Japan 61 1046 (1943)

114. A.P.Jansen and P.G.A.B.Wydeveld: Nature 182 525 (1958)

115. H.Rinderknecht, P.Wilding and B.J.Haverback: Experientia 23 805 (1967)

116. J.A.Hathaway, C.R.Berrett and D.T.Hunter: Clin.Chem. 15 781 (1969)

117. A.L.Babson, S.A.Tenney and R.E.Megraw: Clin.Chem. 16 39 (1970)

118. M.Ceska, E.Hultman and B.G.A.Ingelman: Experientia 25 555 (1969)

119. M.Ceska, K.Birath and B.Brown: Clin.Chim.Acta 26 437 (1969)

120. B.Klein, J.A.Foreman and R.L.Searcy: Analyt.Biochem. 31 412 (1969)

121. S.Take, J.E.Berk and L.Fridhandler: Clin.Chim.Acta 26 533 (1969)

122. H.Rinderknecht and E.P.Marbach: Clin.Chim.Acta 29 107 (1970)

123. L.T.Skeggs: Am.J.Clin.Pathol. 28 311 (1957)

124. R.A.Scheidt: Technicon Symp.I. (L.T.Skeggs, Jr., et al Eds.) Medioc New York (1964)

125. J.A.Lott and J.E.Mercier: Clin.Chem. 16 390 (1970)

126. J.Fukumoto: J.Agr.Chem.Soc.Japan 19 489, 634, 689, 853 (1943); 20 23, 121, 309 (1944); Cited in (1)

127. L.E.Hokin: Biochem.J. 48 320 (1951)

128. G.W.Rapp: J.Dent.Res. 40 1226 (1961)

129. R.L.McGeachin and B.A.Potter: Adv.Enz.Reg. 3 137 (1965)

130. M.M.Daly and A.E.Mirsky: J.Gen.Physiol. 36 243 (1952)

131. A.K.Laird and A.D.Barton: Biochim.Biophys.Acta 27 12 (1958)

132. P.Byrt: Nature 212 1212 (1966)

133. I.Nordfelt: in Schneyer and Schneyer, 1967 (19a) p.142

134. M.Nomura and H.Hoshikawa: Biochim.Biophys.Acta 31 125 (1959)

135. M.Nomura: J.Biochem. 44 87 (1957)

136. A.Ullmann, T.Garzo and F.B.Straub: Acta Physiol.Hung. 13 179 (1958)

137. A.Ullmann and F.B.Straub: Acta Physiol.Hung. 11 31 (1957)

138. A.K.Laird and A.D.Barton: Biochim.Biophys.Acta 25 56 (1957)

139. T.A.Douglas and H.N.Munro: Exp.Cell Res. 16 148 (1959)

140. P.Siekevitz and G.E.Palade:
a) J.Biochem.Biophys.Cytol. 7 619 (1960)
b) J.Biochem.Biophys.Cytol. 7 631 (1960)

141. L.E.Caro and G.E.Palade: J.Cell Biol. 20 473 (1964)

142. G.M.Redman, P.Siekevitz and G.E.Palade: J.Biol.Chem. 241 1150 (1966)

143. J.D.Jamieson and G.E.Palade: Proc.Nat.Acad.Sci. 55 424 (1966)

144. P.Siekevitz and G.E.Palade: J.Cell Biol. 30 519 (1966)

145. J.D.Jamieson and G.E.Palade: J.Cell.Biol. 34 577 (1967)

146. M.Schramm: Ann.Rev.Biochem. 36 307 (1967)

147. L.M.Sreebny and D.A.Johnson: in Schneyer and Schneyer 1967 (19a) p.275

148. Z.Gromet-Elhanan and T.Winnick: Biochim.Biophys.Acta 69 85 (1963)

149. M.Schramm and A.Bdolah: Arch.Biochem.Biophys. 104 67 (1964)

150. R.J.Grand and P.R.Gross: J.Biol.Chem. 244 5608 (1969)

151. R.J.Grand and P.R.Gross: Proc.Nat.Acad.Sci. 65 1081 (1970)

152. R.J.Grand: Biochim.Biophys.Acta 195 252 (1969)

153. M.R.Robinovitch, E.A.Smuckler and L.M.Sreebny: J.Biol.Chem. 244 5361 (1969)

154. R.L.Tapp: Exptl.Cell Res. 47 536 (1967)

155. N.Jacobsen: Arch.Oral Biol. 14 679 (1969)

156. J.Hurliman and C.Zuber: Immunology 14 819 (1968)

157. C.F.Chignell: Biochem.Pharmacol. 17 2225 (1968)

158. P.Filner and J.E.Varner: Proc.Nat.Acad.Sci. 58 1520 (1967)

159. A.S.V.Burgen and N.G.Emmelin: Physiology of the Salivary Glands, Edward Arnold, London (1961)

160. R.de Graaf: (1677), cited by Burgen and Emmelin (159)

161. J.Müller: (1839), cited by Burgen and Emmelin (159)

162. C.Bernard: (1856), cited by Burgen and Emmelin (159)

163. I.P.Pavlov: (1897), cited by Burgen and Emmelin (159)

164. W.Andrew: in Sreebny and Meyer, 1964 (17a) p.13

165. R.P.Suddick and R.J.Dowd: Arch.Oral Biol. 14 567 (1969)

166. B.L.Scott and D.C.Pease: in Sreebny and Meyer, 1964 (17a) p.13

167. G.Siefert: in Schneyer and Schneyer, 1967 (19a) p.191

168. Takahama and Barka: J.Ultra.Res. 17 452 (1967)

169. A.Amsterdam, I.Ohad and M.Schramm: J.Cell Biol. 41 753 (1969)

170. L.E.Hokin: Internat.Rev.Cytol. 23 187 (1968)

171. L.E.Hokin: Biochim.Biophys.Acta 18 379 (1955)

172. M.Schramm and D.Danon: Biochim.Biophys.Acta 50 102 (1961)

173. M.Schramm: in Sreebny and Meyer, 1964 (17a) p.315

174. L.E.Hokin: Ann.N.Y.Acad.Sci. 165 693 (1969)

175. L.E.Hokin and M.R.Hokin: J.Biol.Chem. 233 805 (1958)

176. M.R.Hokin, L.E.Hokin, M.Saffran, A.V.Schally and B.U.Zimmerman: J.Biol.Chem. 233 811 (1958)

177. M.R.Hokin, B.G.Benfey and L.E.Hokin: J.Biol.Chem. 233 814 (1958)

178. M.R.Hokin: Ann.N.Y.Acad.Sci. 165 695 (1969)

179. L.D.Eggman and L.E.Hokin: J.Biol.Chem. 235 2569 (1960)

180. L.E.Hokin and A.L.Sherwin: J.Physiol. 135 18 (1957)

181. C.M.Redman and L.E.Hokin: J.Biophys.Biochem.Cytol. 6 207 (1959)

182. L.E.Hokin and D.Huebner: J.Cell Biol. 33 521 (1967)

183. P.Bernhard: Scientific Res. 3 #22 34 (1968)

184. L.M.Sreebny and D.A.Johnson: Arch.Oral.Biol. 14 397 (1969)

185. C.Ludwig: (1951), cited by Junqueira (19) and Burgen (159)

186. S.C.Wang: in Sreebny and Meyer, 1964 (17a) p.145

187. N.Emmelin: in Schneyer and Schneyer, 1967 (19a) p.127

188. J.Raynaud: in Sreebny and Meyer, 1964 (17a) p.47

189. B.L.Baker, H.W.Clapp,Jr. and J.A.Light: ibid. p.63

190. L.M.Sreebny and J.Meyer: ibid. p.83

191. C.A.Schneyer and H.D.Hall: in Schneyer and Schneyer 1967 (19a) p.155

192. P.Photo: J.Oral.Ther.Pharmacol. 4 467 (1968)

193. B.C.R.Stromblad and M.Nickerson: J.Pharmacol.Exp.Therap. 134 154 (1961)

194. L.L.Iversen: Nature 214 8 (1967)

195. J.H.Burn: Recent Adv.Pharmacol. p.155 (1968) ed. J.M.Robson and R.S.Stacey; Little, Brown, Boston

196. I.Yamamoto, R.Inoki, A.Tsujimoto, S.Kojima: Eur.J.Pharmacol. 3 117 (1968)

197. R.P.Alquist: Am.J.Physiol. 153 586 (1948)

198. G.A.Bray: Endocrinology 79 554 (1966)

199. S.Kojima: Nippon Yakurigaku Zasshi 63 169 (1967); cited in Chem.Abstr. 69 4267g (1968)

200. G.A.Robison, R.W.Butcher and E.W.Sutherland: Ann.N.Y.Acad.Sci. 139 703 (1967)

201. G.A.Robison and E.W.Sutherland: Circ.Res.Suppl. 21 147 (1970)

202. E.W.Sutherland and G.A.Robison: Pharm.Rev. 18 145 (1966)

203. E.W.Sutherland, I.Øye and R.W.Butcher: Recent Prog. in Horm.Res. 21 623 (1965) ed. G.Pincus, Academic Press,N.Y.

204. E.W.Sutherland: J.Am.Med.Assoc. 214 128 (1970)

205. L.Triner, Y.Vulliemoz, M.Verosky and G.G.Nahas: Life Sci. 9 707 (1970)

206. M.Johnson, H.S.A.Sherratt, R.M.Case and T.Scratcherd: J.Biol.Chem.Collq.on Cyclic AMP and Adenyl Cyclase, #3, Nov.1970

207. D.Malamud: Biochem.Biophys.Res.Comm. 35 754 (1969)

208. M.Schramm and E.Naim: J.Biol.Chem. 245 3225 (1970)

209. A.Bdolah and M.Schramm: Biochem.Biophys.Res.Comm. 18 452 (1965)

210. H.Babad, R.Ben-Zvi, A.Bdolah and M.Schramm: Eur.J.Biochem. 1 96 (1967)

211. R.G.Kulka and E.Sternlicht: Proc.Nat.Acad.Sci. 61 1123 (1968)

212. T.Posternak, E.W.Sutherland and W.F.Henion: Biochim.Biophys.Acta 65 558 (1962)

213. S.Batzri, A.Amsterdam, Z.Selinger, I.Ohad and M.Schramm: Proc.Nat.Acad.Sci. 68 121 (1971)

214. D.M.Horwood: Personal Communication

215. D.M.Horwood, A.P.Gaunce and A.D'Iorio: Proc.Can.Fed. Biol.Sci. 14 141 (1971)

216. Y.Salomon and M.Schramm: Biochem.Biophys.Res.Comm. 38 106 (1970)

217. R.Baserga, T.Sasaki and J.P.Whitlock,Jr.: Biochemistry of Cell Division p.77, ed. R.Baserga: C.C.Thomas, Springfield, Ill. (1969)

218. T.A.Langan and L.K.Smith: Fed.Proc. 26 603 (1967)

219. W.D.Riley, J.De Lange, G.E.Bratvold and E.G.Krebs: J.Biol.Chem. 243 2209 (1968)

220. D.A.Walsh, J.B.Perkins and E.G.Krebs: J.Biol.Chem. 243 3763 (1968)

221. E.G.Krebs: Abstr.8th Int.Congr.Biochem.,Switz., p.234 (1970)

222. T.A.Langan: Science 162 579 (1968)

223. J.D.Corbin and E.G.Krebs: Biochem.Biophys.Res.Comm. 36 328 (1969)

224. K.Marushige, V.Ling, B.Jergil and G.H.Dixon: Fed.Proc. 27 336 (1968)

225. E.Miyamoto, J.K.Kuo and P.Greengard: Science 165 63 (1969)

226. E.Miyamoto, J.F.Kuo and P.Greengard: J.Biol.Chem. 244 6396 (1969)

227. H.Naeno, E.M.Johnson and P.Greengard: J.Biol.Chem. 246 134 (1971)

228. L.E.Hokin: Biochim.Biophys.Acta 115 219 (1966)

229. S.Heisler and A.Tenenhouse: Proc.Can.Fed.Biol.Sci. 14 62 (1971)

230. A.Bdolah, R.Ben-Zvi and M.Schramm: Arch.Biochem.Biophys. 104 58 (1969)

231. R.H.Dreisbach and R.Taugner: Arch.Oral.Biol. 14 1395 (1969)

232. H.Feinstein and M.Scramm: Eur.J.Biochem. 13 158 (1970)

233. Z.Selinger and E.Naim: Biochim.Biophys.Acta 203 335 (1970)

234. Z.Selinger, E.Naim and M.Lasser: Biochim.Biophys.Acta 203 326 (1970)

235. H.Rasmussen and A.Tenenhouse: Proc.Nat.Acad.Sci. 59 1364 (1968)

236. H.Selye, R.Veilleux and M.Cantin: Science 133 44 (1961)

237. T.Barka: Exp.Cell Res. 37 662 (1965)

238. W.C.Chan: J.Path.Bact. 88 563 (1964)

239. H.Wells: in Schneyer and Schneyer, 1967 (19a) p.178

240. C.A.Schneyer: Am.J.Physiol. 203 232 (1962)

241. C.A.Schneyer, W.H.Finley and S.C.Finley: Proc.Soc. Exp. Biol.Med. 125 723 (1967)

242. H.D.Hall and C.A.Schneyer: Proc.Soc.Exp. Biol.Med. 117 789 (1964)

243. C.A.Schneyer and H.D.Hall: Am.J.Physiol. 211 943 (1966)

244. M.Fukuda: Jap.J.Pharmacol. 18 185 (1968)

245. H.Wells: Am.J.Physiol. 212 1293 (1967)

246. T. Barka: Exp.Cell Res. 41 573 (1966)

247. T. Barka: Lab.Invest. 18 38 (1968)

248. R. Baserga and S.Heffler: Exp.Cell Res. 46 571 (1967)

249. D.Malamud and R.Baserga: Biochim.Biophys.Acta. 195 258 (1969)

250. D.Malamud and R.Baserga: Sci. 162 373 (1968)

251. E.D.Mayfield, Jr., J.J.Ghidoni, E.Bresnick and H.C.Stanton: Proc.Soc.Exp.Biol.Med. 129 91 (1969)

252. D.Malamud and P.Keenan: Fed.Proc. 26 467 (1968)

253. T.Barka: Exp.Cell Res. 39 355 (1965)

254. T.Barka: Lab.Invest. 22 73 (1970)

255. R.Baserga: Life Sci. 5 2033 (1966)

256. J.Radley: Aust.J.Exp.Biol.Med. 46 795 (1968)

257. D.Malamud and R.Baserga: Life Sci. 6 1765 (1967)

258. J.P.Whitlock, Jr., R.Kaufman and R.Baserga: Cancer Res. 28 2211 (1968)

259. L.Pegoraro and R.Baserga: Lab.Invest. 22 266 (1970)

260. D.Malamud and R.Baserga: Exp.Cell Res. 50 581 (1968)

261. T.Sasaki, G.Litiwack and R.Baserga: J.Biol.Chem. 244 4831 (1969)

262. G.S.Stein and R.Baserga: J.Biol.Chem. 245 6097 (1970)

263. T.Ekfors and T.Barka: Lab.Invest. 24 197 (1971)

264. K.C.Kirby,Jr., D.Severn and R.Baserga: Molec.Pharmacol. 55 72 (1969)

265. J.Himms-Hagen: Pharmacol.Rev. 19 367 (1967)

266. E.Quevedo: Doctoral Thesis, U.of Ottawa (1970)

267. T.Barka and H.Van der Noen: Lab.Invest. 20 377 (1969)

268. J.de Champlain, R.A.Mueller and J.Axelrod: J.Pharmacol. Exp.Ther. 166 339 (1969)

269. M.Takahama and T.Barka: J.Ultrastruct.Res. 17 457 (1967)

270. B.L.Scott and D.C.Pease: Am.J.Anat. 104 115 (1959)

271. R.A.Mueller, J.de Champlain and J.Axelrod: Biochem. Pharmacol. 17 2455 (1968)

272. P.Ohlin: J.Oral Ther.Pharmacol. 3 190 (1966)

273. J.de Champlain, L.R.Krakoff and J.Axelrod: Circ. Res. 23 361 (1968)

274. A.Horita: Nature 215 411 (1967)

275. L.Stjärne, R.H.Roth and N.J.Giarman: Biochem. Pharmacol. 17 2008 (1968)

276. A.P.Martin and R.Baserga: Proc.Soc.Exp.Biol.Med. 131 1022 (1969)

277. S.Orrenius: J.Cell Biol. 26 213 (1965)

278. R.A.Miegs and K.J.Ryan: Biochim.Biophys.Acta 165 476 (1968)

279. P.J.Anderson: Ph.D. Thesis, U.of Ottawa (1967)

280. L.Sierens: Ph.D. Thesis, U.of Ottawa (1969)

281. J.Axelrod: Pharmacol.Rev. 18 95 (1966)

282. I.S. Kopin: Pharmacol.Rev. 16 179 (1964)

283. V.Z.Gorkin: Pharmacol.Rev. 18 115 (1966)

284. G.Almgren, N.E.Andén, J.Jonason, K.A.Norberg and L.Olson: Acta.Physiol.Scand. 67 21 (1966)

285. G.I.Klingman: Biochem.Pharmacol. 15 1729 (1966)

286. J.Jonason: Acta.Pharmacol.Tox. 27 60 (1969)

287. J.Jonason: Acta.Physiol.Scand. 76 299 (1969)

288. J.Axelrod, W.Albers and C.D.Clemente: J.Neurochem. 5 68 (1959)

289. B.Jarrott: J.Neuronchem. 18 17 (1971)

290. O.J.Broch,Jr., H.G.Guldberg and C.A.Marsden: Brit.J.Pharmacol. 41 393 (1971)

291. P.J.Anderson and A.D'Iorio: Biochem.Pharmacol. 17 1943 (1968)

292. J.Axelrod and E.S.Vesell: Mol.Pharmacol. 6 78 (1970)

293. J.Jonason: J.Pharm.Pharmacol. 22 93 (1970)

294. G.Hertting: Biochem.Pharmacol. 13 1119 (1964)

295. C.D.Morgan, C.R.J.Ruthven and M.Sandler: Clin.Chim. Acta 26 381 (1969)

296. T.J.Schoch: Adv.Carbohyd.Chem. 1 247 (1945) ed. W.W.Pigman, M.L.Wolfrom, Academic Press, N.Y.

297. D.H.Strumeyer: Analyt.Biochem. 19 61 (1967)

298. L.G.Miller: Analyt.Chem. 31 426 (1959)

299. M.Saffran, P.Ford, E.K.Matthews, M.Kraml and L.Garbaczowska: Can.J.Biochem. 45 1901 (1967)

300. C.A.Schneyer and L.H.Schneyer: in Sreebny and Meyer, 1964 (17a) p.309

301. E.C.Greene: Anatomy of the Rat, Hafner, N.Y. (1955)

302. R.Holtzer, J.L.Van Lancker and H.Swift: Arch.Biochem. Biophys. 101 439 (1963)

303. J.L.Van Lancker and R.L.Holtzer: J.Biol.Chem. 234 2359 (1959)

304. A.Loyter and M.Schramm: Biochim.Biophys.Acta 65 200 (1962)

305. E.A.Lane and C.Maurides: Analyt.Biochem. 27 363 (1969)

306. O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall: J.Biol.Chem. 193 265 (1951)

307. H.Schuel and R.Schuel: Analyt.Biochem. 20 86 (1967)

308. B.Zak and J.Cohen: Clin.Chim.Acta 6 665 (1961)

309. W.T.Roubal and A.L.Tappel: Analyt.Biochem. 9 211 (1964)

310. P.A.Anastassiadis and R.H.Common: Analyt.Biochem. 22 409 (1968)

311. R.E.Thiers, R.R.Cole and W.J.Kirsch: Clin.Chem. 13 451 (1967)

312. A.P.Gaunce and A.D'Iorio: Analyt.Biochem. 37 204 (1970)

313. M.A.Peters and J.P.Fouts: Analyt.Biochem. 30 299 (1969)

314. J.Eichberg and L.C.Mokrasch: Analyt.Biochem. 30 386 (1969)

315. J.Bonitati, W.B.Elliott and P.G.Miles: Analyt.Biochem. 31 399 (1969)

316. C.W.Tabor, H.Tabor and S.M.Rosenthal: J.Biol.Chem. 208 645 (1954)

317. H.Weissback, T.E.Smith, J.W.Daly, B.Witkop and S.Undenfriend: J.Biol.Chem. 235 1160 (1960)

318. S.Otsuka and Y.Kobayashi: Biochem.Pharmacol. 13 995 (1964)

319. J.Southgate and G.G.S.Collins: Biochem.Pharmacol. 18 2285 (1969)

320. M.Kraml: Biochem.Pharmacol. 14 1684 (1965)

321. B.D.Drujan and J.M.Diaz Borges: cited in M.Roth. ed. Methods of Biochemical Analysis, 17 189 (1969)

322. B.Century and K.Rupp: Biochem.Pharmacol. 17 2012 (1968)

323. S.J.Cooperstein and A.Lazarow: J.Biol.Chem. 189 665 (1951)

324. F.G.Smith and E.Stotz: J.Biol.Chem. 179 891 (1949)

325. L.Smith: Methods in Enzymology, 2 732 (1955) ed. S.P.Collowick and N.O.Kaplan,Academic Press, N.Y.

326. L.Smith and H.Conrad: in Haematin Enzymes, I.U.B.Symp. 19 260 (1961), ed. J.E.Falk, R.Lemberg and R.K.Morton, Pergamon Press, Oxford

327. L.Smith and H.Conrad: Arch.Biochem.Biophys. 63 403 (1956)

328. P.Person and A.S.Fine: Arch.Biochem.Biophys. 94 392 (1961)

329. Y.Orii and K.Okunuki: J.Biochem. 58 561 (1965)

330. L.Smith and N.Newton: Oxidases and Related Redox Systems, 2 745 (1965), ed. T.E.King, H.S.Mason, M.Morrison; J.Wiley and Sons Inc., New York

331. W.W.Wainio, B.Eichel and A.Gould: J.Biol.Chem. 235 1521 (1960)

332. P.Person, P.T.Mora and A.S.Fine: J.Biol.Chem. 238 4103 (1963)

333. A.Kornber: Methods of Enzymology, 1 441 (1955) ed. S.P.Collowick and N.O.Kaplan, Academic Press, N.Y.

334. H.U.Bergmeyer, E.Bernt and B.Hess: Oxidases and Related Redox Systems, 1 323 (1965), ed. T.E.King,H.S.Mason, M.Morrison; J.Wiley & Sons Inc., N.Y.

335. M.K.Schwartz and O.Bodansky: Methods of Enzymology, 1 294 (1955), ed. S.P.Collowick and N.O.Kaplan, Academic Press, N.Y.

336. W.J.Reeves,Jr., and G.M. Fimognari: Methods of Enzymology, 1 288 (1955), ed. S.P.Collowick and N.O.Kaplan, Academic Press, N.Y.

337. E.Amador, L.E.Dorfman and W.E.C.Wacker: Clin.Chem. 9 391 (1963)

338. J.B.Neilands: Methods of Enzymology, 1 449 (1955) ed. S.P.Collowick and N.O.Kaplan, Academic Press, N.Y.

339. G.Hübscher and G.C.West: Nature 205 799 (1965)

340. J.B.Sumner: Science 100 413 (1944)

341. G.J.Dutton: Glucoronic Acid, Free and Combined, Academic Press Inc., N.Y. (1966)

342. D.C.Collins, H.Jirku and D.S.Layne: J.Biol.Chem. 243 2928 (1968)

343. L.Ernster, P.Siekevitz and G.E.Palade: J.Cell Biol. 15 541 (1962)

344. T.Nash: Biochem.J. 55 416 (1953)

345. S.Belman: Analyt.Chim.Acta 29 120 (1963)

346. C.A.C.Haley and P.Maitland: J.Chem.Soc. 3155 (1951)

347. T.Omura and R.Sato: J.Biol.Chem. 239 2370 (1964)

348. T.Omura and R.Sato: J.Biol.Chem. 239 2379 (1964)

349. R.E.McCaman: Life Sci. 4 2353 (1965)

350. L.Ornstein: Ann.N.Y.Acad.Sci. 121 321 (1964)

351. B.J.Davis: Ann.N.Y.Acad.Sci. 121 404 (1964)

352. G.Dale and A.L.Latner: Lancet 294 847 (1968)

353. C.Wrigley: Sci.Tools 15 17 (1968)

354. A.Chrambach, R.A.Reisfeld, M.Wyckoff and J.Zaccari: Analyt.Biochem. 20 150 (1967)

355. G.R.Revis: Proc.Soc.Exp.Biol.Med. 128 1110 (1968)

356. P.V.Tishler and C.T.Epstein: Analyt.Biochem. 22 89 (1968)

357. A.Chrambach: Analyt.Biochem. 15 544 (1966)

358. R.R.Sokal and F.J.Rohlf: Biometry W.H.Freeman San Francisco (1969)

359. Olivetti-Underwood Corp: Statistical Analysis, Program #2.19, ed. J.B.Williams (1968)

360. Olivetti-Underwood Corp: Statistical Analysis, Program #6.10 (corrected), ed. J.B.Williams (1968)

361. Olivetti-Underwood Corp: Statistical Analysis, Program #2.51 (modified), ed. J.B.Williams (1968)

362. S.Goldstein and W.J.Reddy: Biochim.Biophys.Acta 141 310 (1967)

363. S.Goldstein and W.J.Reddy: Biochim.Biophys.Acta 150 733 (1968)

364. O.Kridakorn: Alabama J.Med.Sci. 2 194 (1965)

365. O.Kridakorn: J.Oral Therap.Pharmacol. 2 362 (1966)

366. R.Schucher and L.E.Hokin: J.Biol.Chem. 210 551 (1954)

367. L.H.Schneyer, W.Pigman, L.Hanahan and R.W.Gilmore: J.Dent.Res. 35 109 (1956)

368. H.Horitsu and M.Tomoeda: Nippon Nogei Kagaku Kaishi 40 387 (1966), cited in Chem.Abstr. 66 82601w (1967)

369. H.R.Mahler and E.H.Cordes: Biological Chemistry, Harper and Row, N.Y. (1966)

370. R.J.DeLange, R.G.Kemp, W.D.Riley, R.A.Cooper and E.G.Krebs: J.Biol.Chem. 243 2200 (1968)

371. E.G.Krebs, D.S.Love, G.E.Bratvold, K.A.Trayser, W.L.Meyer and E.H.Fischer: Biochemistry 3 1022 (1964)

372. E.S.Fiale and F.F.Davis: Biochem.Biophys.Res.Commun. 18 115 (1965)

373. C.Mavrides and E.A.Lane: Science 156 1376 (1967)

374. B.E.H.Maden, R.R.Traut and R.E.Monro: J.Mol.Biol. 35 333 (1968)

375. A.M.Nemeth and G.de la Haba: J.Biol.Chem. 237 1190 (1962)

376. T.Sasaki, G.Litwack and P.Baserga: J.Biol.Chem. 244 4831 (1969)

377. M.V.Simpson, E.Farber and H.Tarver: J.Biol.Chem. 182 81 (1950)

378. E.Farber and M.S.Corban: J.Biol.Chem. 233 625 (1958)

379. H.Sidransky and E.Farber: J.Biol.Chem. 219 231 (1956)

380. W.E.Loring and L.J.Hartley: Am.J.Path. 31 521 (1955)

381. B.L.Lamberts, T.S.Meyer and F.L Losee: Fed.Proc. 24 441 1736 (1965)

382. C.L.Markert: Ann.N.Y.Acad.Sci. 151 14 (1968)

383. J.A.Beeley: Arch.Oral Biol. 14 559 (1969)

384. R.T.Shimke, E.W.Sweeney and C.M.Berlin: Biochem.Biophys. Res.Comm. 15 214 (1964)

385. R.T.Shimke and D.Doyle: Ann.Rev.Biochem. 39 929 (1970)

386. R.L.Holtzer and J.L.Van Lancker: Arch.Biochem.Biophys. 101 439 (1963)

387. C.de Duve: Harvey Lectures 59 49 (1963)

388. P.Baudhuin, H.Beaufay, H.Rahman-Li, O.Z.Sellinger, R.Wattiaux, P.Jacques and C.de Duve: Biochem.J. 92 179 (1964)

389. A.P.Mathias: Br.Med.Bull. 22 146 (1966)

390. C.Schnaitman, V.G.Erwin and J.W.Greenawalt: J.Cell Biol. 32 719 (1967)

391. J.L.Van Lancker and R.L.Holtzer: J.Biol.Chem. 234 2359 (1959)

392. G.Seifert: Beitr.Pathol.Anat.Allgem.Pathol. 130 294 (1964), cited in (195)

393. P.G.Wenzel and J.P.Lyon: Toxicol.Appl.Pharmacol. 11 215 (1967)

394. J.K.Grant: ed. Methods of Separation of Subcellular Components, Biochem.Soc.Symp. 23 (1963)

395. H.Beaufay, P.Jacques, P.Baudhuin, O.Z.Sellinger, J.Berthet and C.de Duve: Biochem.J. 92 184 (1964)

396. R.Ben-Zvi: Doctoral Thesis, Hebrew University, Jerusalem (1965), cited by M.Schramm (146)

397. W.C.Schneider and G.H.Hogeboom: J.Biol.Chem. 195 161 (1952)

398. G.Blobel and V.R.Potter: Science 154 1662 (1966)

399. J.Chauveau, Y.Moulé and C.Rouiller: Exp.Cell Res. 11 317 (1956)

400. Y.Ericsson and L.Stjernström: O.Surg., O.Med., O.Path. 4 1465 (1951), cited in Afonsky (27) p.33

401. A.Hagopian and E.H.Eylar: Arch.Biochem.Biophys. 129 515 (1969)

402. K.Paigen: Biochim.Biophys.Acta 19 297 (1956)

403. E.Werle: in Hoppe-Seyler/Thierfelder's Handbuch der Physiologisch- und Pathologisch-chemischen Analyse 10. Auflage, Band 6, Teil A, p. 653. Springer Verlag, Berlin (1964), cited by L.Sierens (280)

404. J.M.Diaz Borges and A.D'Iorio: Adv.Biochem.Psychopharmacology, Vol 5 (in Press)