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I am greatly indebted to Dr. W. K. Paik  
for advice and direction during the  
course of these studies.



FURTHER STUDIES ON  $\epsilon$ -LYSINE ACYLASE

H. Por-ton - Coral

THESIS

Submitted to the Faculty of Medicine in  
partial fulfilment of the requirements  
for the degree of Doctor of Philosophy.

Department of Biochemistry

University of Ottawa

May 1965



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SECTION I INTRODUCTION AND REVIEW OF LITERATURE

Lysine was one of the earliest amino acids to be recognized as essential (1). Later, nutrition studies also showed that the minimum daily requirement of lysine, for nitrogen balance, is higher than that for most other essential amino acids for animals and birds (2). It is surprising, therefore, that little was known about the mechanisms of its utilization and function in animal tissues until quite recently. In fact, the exact steps in its breakdown are still not completely known (3), and indications of the role which the  $\epsilon$ -amino group of lysine may play in protein structure and function have only begun to emerge in the last three or four years.

One reason for the slow development of knowledge concerning lysine was, undoubtedly, the fact that lysine did not appear to be acted upon by the enzymes which were important in the metabolism of most other amino acids - the transaminases and the amino acid oxidases. For this reason, the intensive investigations of amino acid metabolism during the 1930's and 1940's contributed little to our knowledge of lysine metabolism. However, one important discovery which was made during this period was that when the epsilon amino group of lysine was blocked (eg. by an acetyl group) the alpha amino group became more reactive (4).

The discovery, in 1957, of an enzyme which specifically deacylates  $\epsilon$ -N-acyl derivatives of lysine was, therefore, of particular importance (5). Several questions were raised:

was the distribution of this enzyme universal?, was its function connected with the utilization, conservation, or the degradation of lysine?, what was the specificity of the enzyme? was its action reversible? Investigations of these questions have been undertaken mainly by two groups (6,7,8,9) and the present studies were undertaken to expand some aspects of the earlier investigations.

Since most of the earlier work had been on animals and bacteria, the main emphasis in the present studies has been placed on avian tissues. The experimental results to be reported here have been divided into three parts: I) A survey of the occurrence of the enzyme in various bird and animal tissues, including some investigations into its subcellular distribution and pH optima. II) A series of attempts to purify the enzyme from chicken mitochondria. III) A comparison of the properties of the enzyme derived from chicken mitochondria with that derived from other sources.

## REVIEW OF LITERATURE

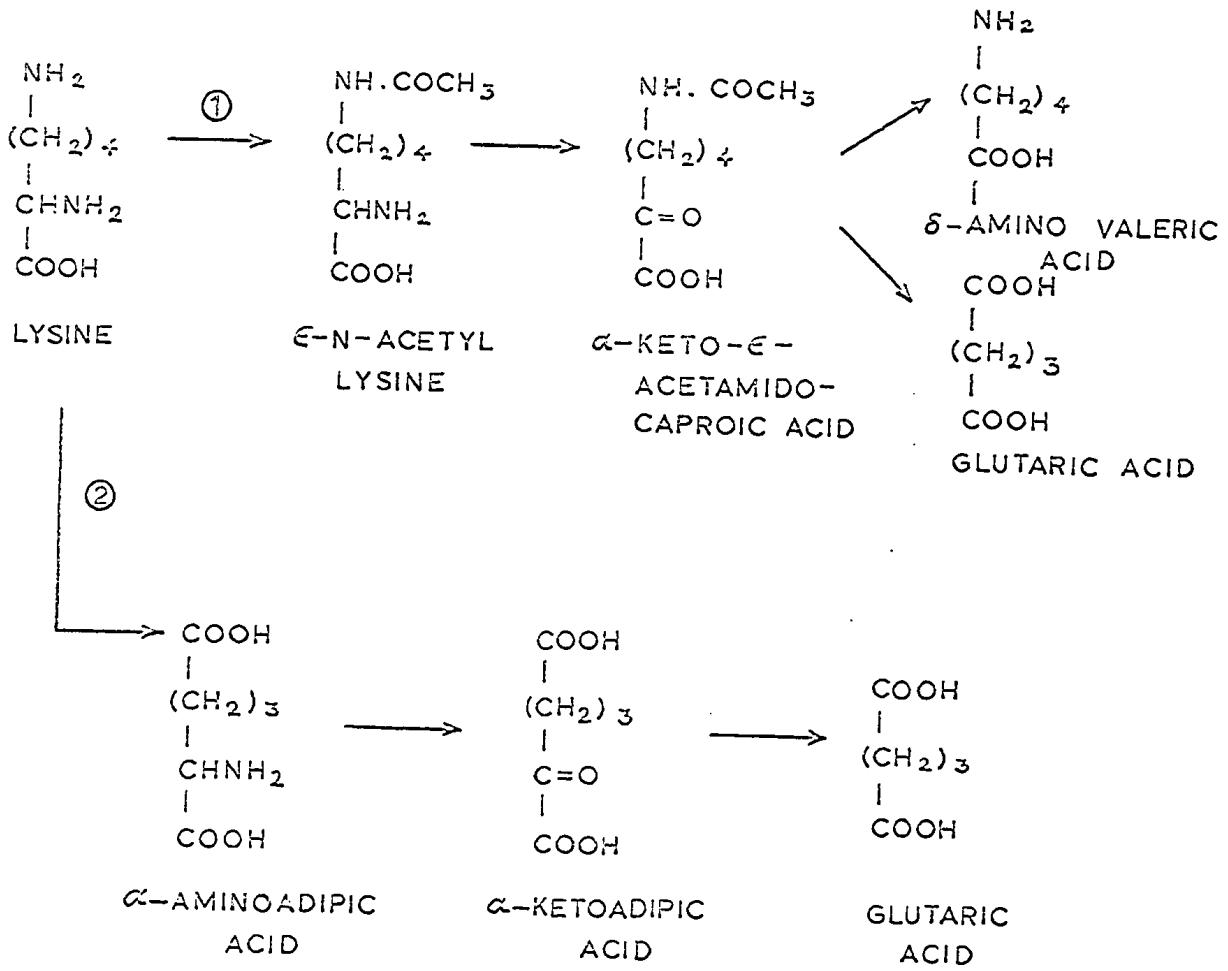
### A) Lysine metabolism in animal tissues.

In 1913 Ringer, Frankel and Jonas (10) suggested that glutaric acid was an intermediate in lysine metabolism. Concrete proof of this fact was not however, forthcoming until 1954 when Rothstein and Miller (11), showed that  $C^{14}$ , administered to rats as lysine, was subsequently found in glutaric acid.

In the intervening years, much of the information accumulated on lysine metabolism was purely negative. For example: lysine was not attacked by D-amino acid oxidase (12); did not enter into transamination (13,14); was not attacked by L-amino acid oxidase (Green - quoted in (4)); could not be substituted for by its  $\alpha$ -hydroxy analogue, nor by its di- or mono- $\alpha$ -methyl analogues (15).

The first positive result was the demonstration by Neuberger and Sanger in 1944 (4), that lysine acetylated in the epsilon position was susceptible to oxidation by both D- and L-amino acid oxidase (when the corresponding isomers were used as substrates). Based on this finding, Neuberger and Sanger proposed the first complete scheme for lysine degradation (Fig. 1). They suggested two alternate pathways - both of which required that the epsilon amino group must be either blocked or removed before oxidation of the alpha amino group. Both pathways satisfy the requirement that the end product of lysine metabolism must be neither ketogenic nor glucogenic (16,17,18), since the products are either  $\delta$ -aminovaleric acid or glutaric acid.

FIG. 1.



From Neuberger A., and Sarger F., *Biochem J.* 38, 119, 1944

In 1948 Borsook et al. (19), showed the formation of  $C^{14}$   $\alpha$ -aminoadipic acid ( $\alpha$  AAA) from  $C^{14}$  lysine by guineapig liver slices. These workers also showed the deamination of  $\alpha$  AAA to  $\alpha$ -ketoadipic acid (20) and the rapid decarboxylation of this acid to glutaric acid by guineapig liver homogenates. The overall chain of reactions was also confirmed in vivo by the work of Rothstein and Miller in 1954 (II). Many questions concerning the individual steps in the chain remained to be answered at that time and only a few of them have been settled even at the present time.

Before proceeding to a discussion of the individual steps in this pathway, one further comment should be made on the action of L-amino acid oxidase on lysine. The original report that L-amino acid oxidase did not act on lysine (4) was made in the same year that this enzyme was first isolated. Subsequent work has modified this view. The activity of L-amino acid oxidase on lysine appears to depend on the source of the enzyme. For example, it has been shown that enzyme from *Neurospora crassa* (21), some snake venoms (22), turkey liver (23), and marine invertebrates (24), have varying, but appreciable, activities on lysine. Boulanger (25), has made the interesting correlation that L-amino acid oxidase, active on dibasic amino acids, has been isolated only from aminotelic and uricotelic species; never from ureotelic species.

One recent paper also suggests that the whole question of

L-amino acid oxidase specificity may have to be reinvestigated. Paik and Kim (26), have shown that the pH optimum, and the shape of the pH curve, is dependent not only on the source of the enzyme, but also on the substrate used. Although lysine remains an unsusceptible substrate with the enzyme from rat kidney or liver at a wide range of pH values, this may not be true with all enzyme preparations which are currently believed not to attack lysine.

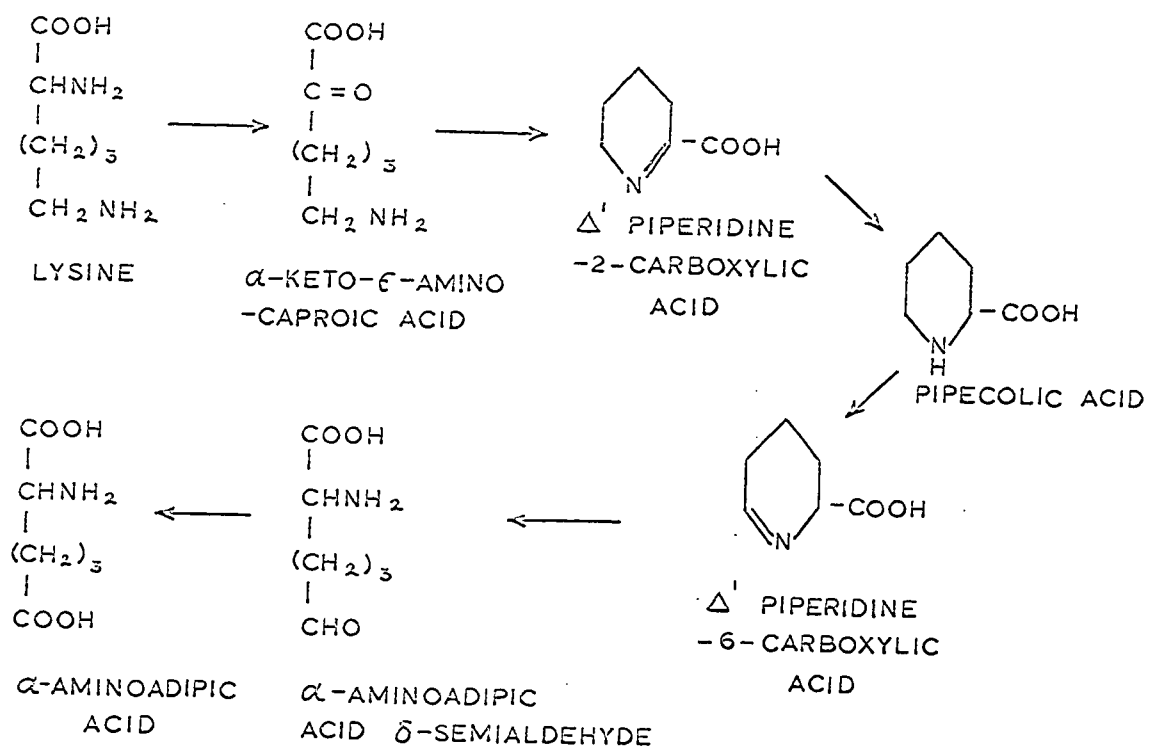
Recognition of the role of cyclic compounds.

One fact which delayed the recognition of some parts of the degradative pathway of lysine was the very rapid, spontaneous formation of a cyclic product. The cyclic compound, which, on catalytic hydrogenation yielded pipercolic acid, was demonstrated in turkey liver by Boulanger and Gsteux in 1952 (27,28) and in *Neurospora crassa* by Lowy, Holden and Schweet in the same year (29). Subsequently, Rothstein and Miller (30) incorporated these cyclic compounds into a scheme of lysine degradation, placing them between lysine and  $\alpha$ -AAA (Fig. 2).

Boulanger challenged this scheme and, in a review in 1959, described the proven and postulated pathways of lysine degradation in animals, plants, and *Neurospora* (25). He suggested that a uniform scheme, which would apply to all cells, would be one which placed the cyclic compounds in alternate pathways rather than on the main route of lysine catabolism (Fig. 3).

The controversy between these two groups has continued to the present time, each side presenting evidence that the other

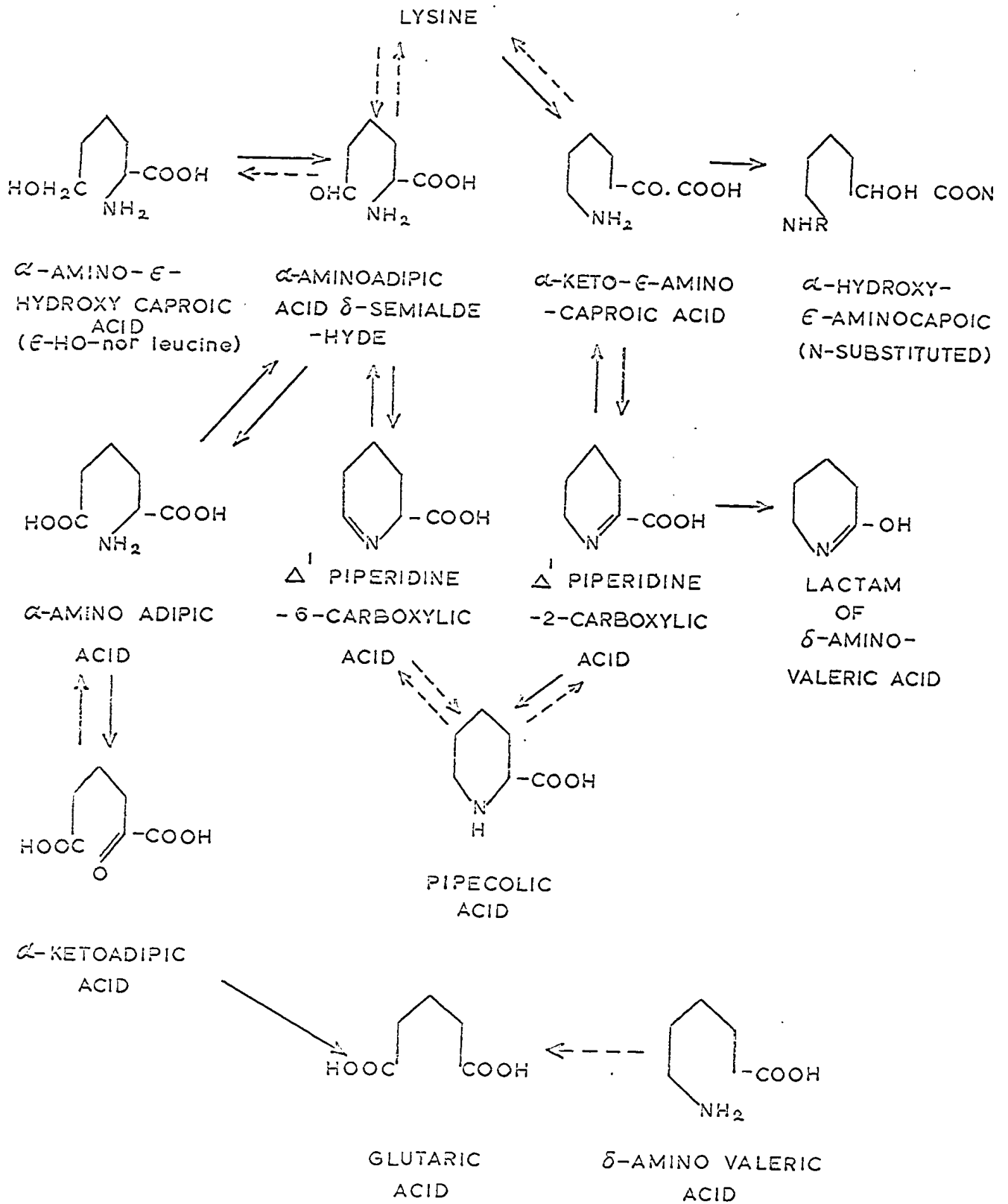
FIG.2.



From Rothstein M., and Miller L.L. J. Biol. Chem. 211, 851, 1954.

Note that the original  $\epsilon\text{NH}_2$  group becomes the final  $\alpha\text{NH}_2$  group.

FIG. 3.



From Boulanger P. in *Biochimie Comparée des Acides aminés basiques*. (Pub. C.N.R.S., Paris, 1960) See p. 135.

has not entirely refuted. Boulanger's claim is that with rat liver homogenates he was unable to show the formation of  $\alpha$ -AAA from pipercolic acid (31) whereas Borsook et al. had shown  $\alpha$ -AAA formation from lysine in the same system. Boulanger also points out that the conditions used by Rothstein's group (involving the use of high doses of unlabeled amino acid simultaneously with small amounts of labeled "marker" amino acids) produced an unnatural or overloaded system. From his discussion (25) one might postulate that the excess amino acid could not be degraded rapidly enough by the normal route and, as a result, some was converted to pipercolic acid.

There is one error in this argument which should be mentioned before discussing Rothstein's view. This is the fact that, on examination of the original papers, it can be seen that Borsook's group used guineapig liver (20,32) and rat kidney (33) - not rat liver. This difference might be of major importance in view of the fact that Rothstein has reported (34) that, whereas rat liver mitochondria were active, whole rat liver homogenates were unsatisfactory as enzyme preparations for this reaction. Unfortunately Rothstein does not elaborate on this statement but it implies that Boulanger based part of his argument on negative results in a tissue preparation different from those used by other workers.

Rothstein and his co-workers have shown the conversion of  $C^{14}$  pipercolic acid to  $\alpha$ -AAA by rat liver mitochondria (34). They have substantiated their claims by identification of the

products with chromatographic and electrophoretic separation. However, they find only 10% of the externally added pipecolic acid is converted to  $\alpha$ -AAA. They explain this as a possible difference between the location of internally produced and externally added substrate. They postulate that the pipecolic acid formed from internal sources may be formed near enzymic sites and so have a preferential position for further oxidation - compared to that coming from outside. Considering the fact that recent figures on the amino acid content of mitochondria give a value of about 5.5  $\mu\text{m/gm}$  mitochondrial nitrogen, and that Rothstein et al. used large amounts of substrate and a 2 hour incubation period, one would expect that any such preferential state would be eliminated early in the incubation. Such speculation does not eliminate the conclusion, made by Boulanger and Osteux, that pipecolic acid is either an end product of lysine degradation or a very unreactive metabolite (25).

At present, therefore, the weight of evidence would appear to support the contention, by Rothstein's group, that the cyclic compounds lie on the main pathway of lysine degradation; but Boulanger's claim, that they are a side mechanism, seen only when the system is overloaded, is not completely disproved. The definitive answer to such questions probably will only be obtained when all the enzymes involved in the pathway have been isolated, purified, and studied under controlled conditions. Considerable progress has been made in this direction with a few of the enzymes concerned with lysine metabolism in micro-

organisms (as will be discussed below). With enzymes obtained from animal tissues it has been shown that  $\epsilon$ -lysine acylase from hog kidney catalyzes the conversion of  $\alpha$ -keto- $\epsilon$ -acetamidocaproic acid to  $\alpha$ -keto- $\epsilon$ -aminocaproic acid, which cyclizes rapidly and spontaneously to form  $\Delta^1$ -piperidine-2-carboxylic acid (35). It has also been shown that an enzyme from beef liver can catalyze the formation of  $\epsilon$ -N-acetyl-L-lysine using acetyl phosphate as the acetate donor (35). This latter enzyme may be ornithine transcarbamylase (36). The role of  $\epsilon$ -N-acetyl-L-lysine in lysine catabolism is, therefore, once more a critical question. The possible metabolic pathway indicated by these recent publications is outlined in Fig. 4. It is obvious from these studies that  $\epsilon$ -lysine acylase may be one of the enzymes involved in lysine catabolism in animals and further studies on this enzyme may produce some of the answers needed.

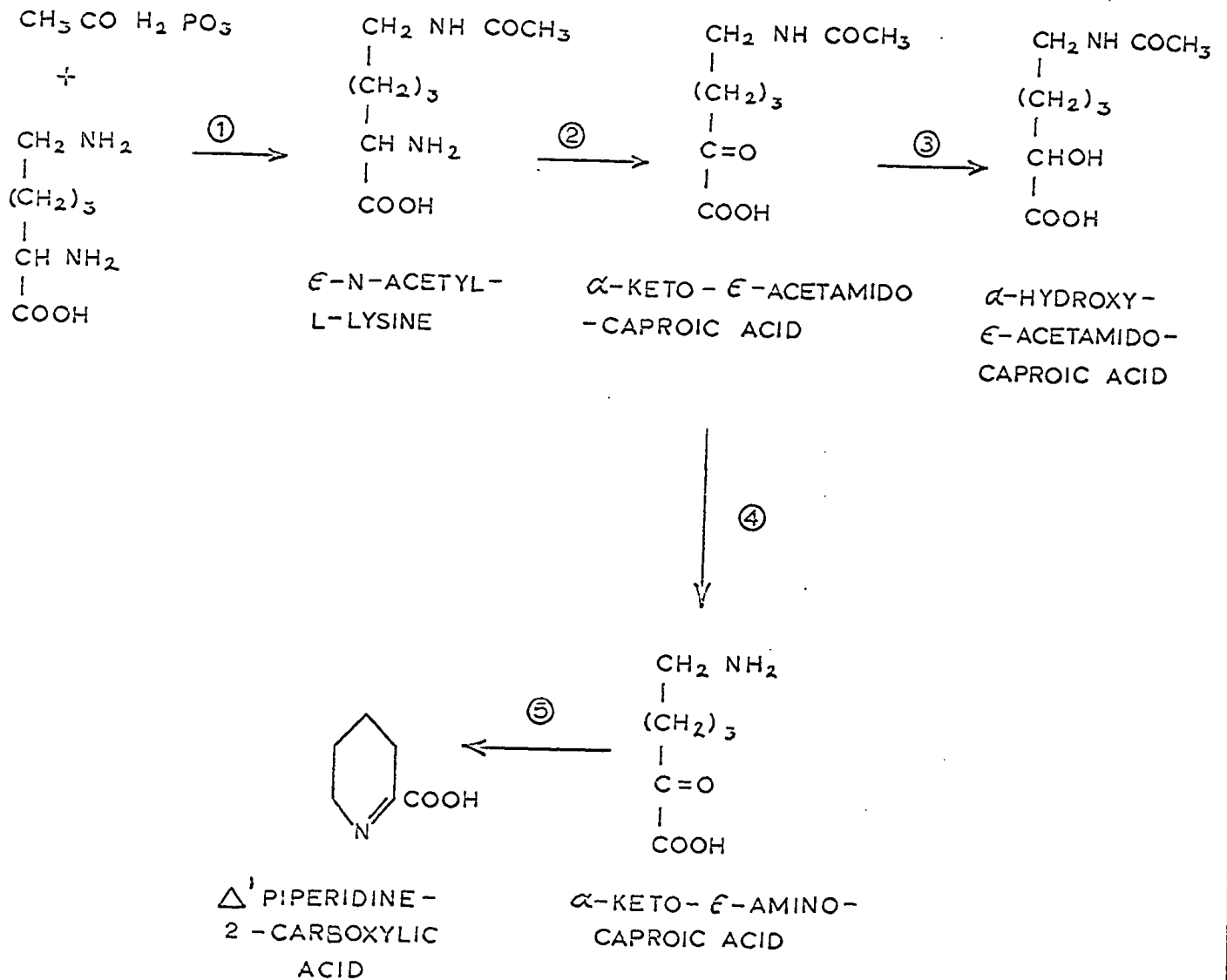
### 3) Lysine metabolism in lower orders.

#### 1) Biosynthetic pathways.

Considerable information on the individual enzymes concerned with lysine metabolism has been obtained from studies on lysine biosynthesis in bacteria, yeasts and molds. Since the same, or similar enzymes are probably involved in lysine catabolism in animals and birds (37), it seems pertinent to review the present knowledge of this topic.

Three main pathways of lysine biosynthesis are known in plants and lower animal forms. The first, the diamminopimelic

FIG. 4.



① From Paik W.K., Kim S., Arch. Biochem. Biophys. 108, 221, 1964

②-⑤ From Paik W.K., Biochem. Biophys. acta 65, 518, 1962.

② L-AMINO ACID OXIDAZE OR TRANSAMINAZE

③  $\alpha$  DEHYDROGENAZE ?

④  $\epsilon$ -LYSINE ACYLAZE

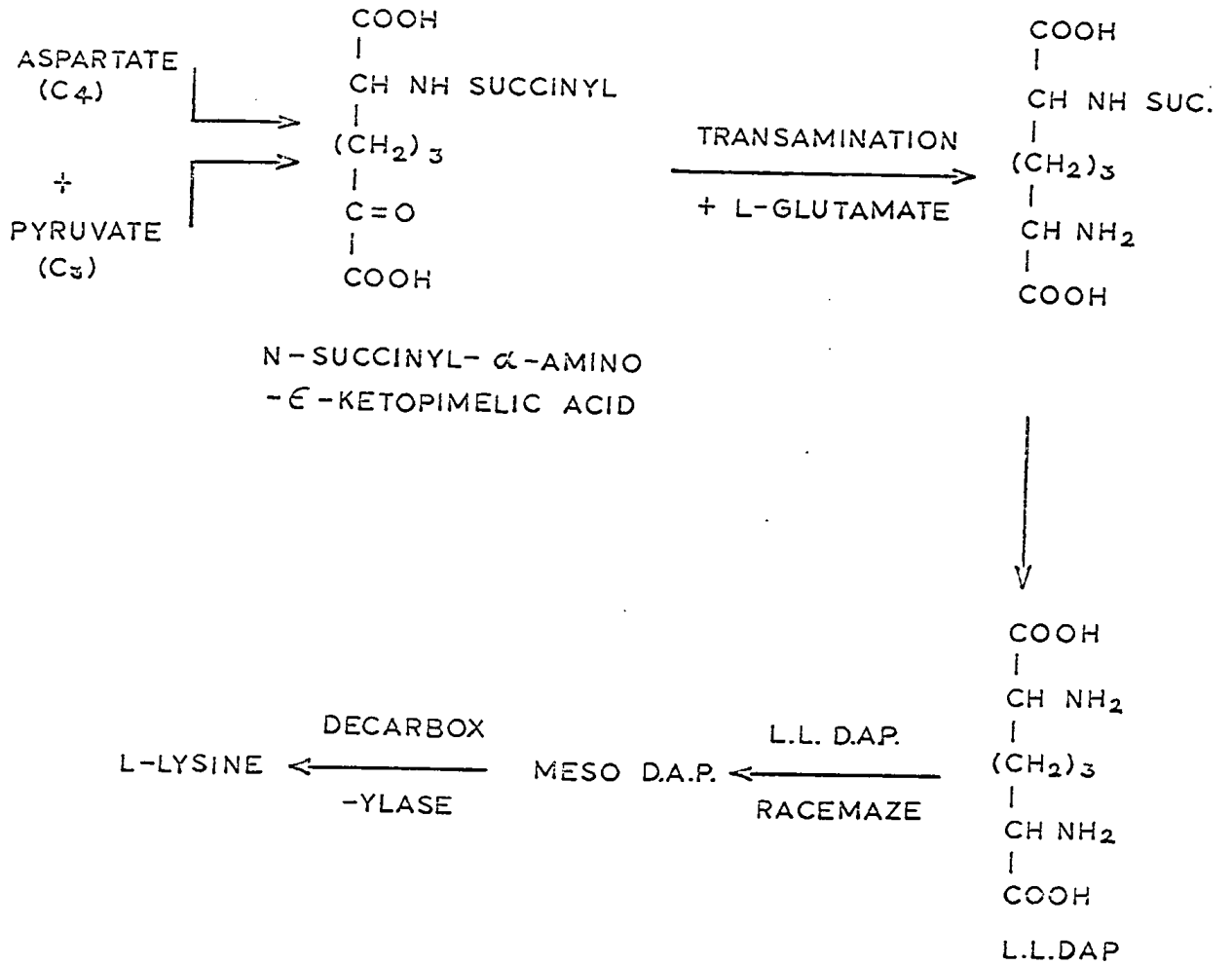
⑤ SPONTANEOUS

acid path, occurs in many bacteria and higher plants (38), and there is not yet any indication of a similar pathway in higher species. This pathway has been thoroughly investigated since the first isolation of diaminopimelic acid by Work in 1950, 1951 (39,40 and Gilvarg (41)). The accepted pathway is outlined in Fig. 5.

The second pathway has been found in yeast. It too has no apparent parallel in animal systems. This pathway was first noticed when an unknown compound "Compound B" was isolated from growing yeast (42). This compound was then identified by Kjaer and Larson (43), as  $\epsilon$ -N-(L-glutaryl-2-)-L-lysine or "saccharopine". It has been shown that  $\alpha$ -AAA- $\delta$ -semialdehyde condenses with L-glutamate to form saccharopine in a system consisting of a cell-free yeast extract and reduced nicotinamide-adenine dinucleotide (NADH) (44). If whole yeast cells are used, saccharopine is converted to free lysine (44).

The third pathway, observed in yeasts and molds, and involving  $\alpha$ -AAA, still contains many uncertainties. This pathway is essentially the same as that believed to operate in lysine degradation in animals. In fact, the earliest work on microorganisms (45) arose, in part from the studies of Borsook and co-workers with mammalian tissues (29,32,33). In 1948, Mitchell and Houlahan showed that a neurospora mutant utilized  $\alpha$ -AAA, but not the corresponding keto acid, as a precursor of lysine (45).

FIG.5.



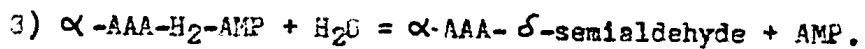
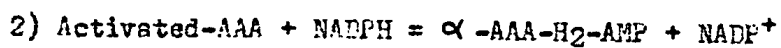
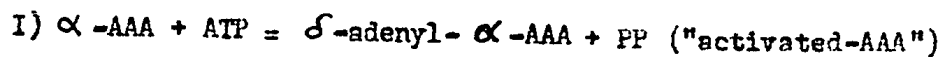
From E. Work in Biochimie Comparée. des Acides aminés  
 basiques. (Pub. C.N.R.S., Paris, 1960) see p.147.

Within the next few years it was shown that  $\alpha$ -keto-adipic acid could support the growth of certain lysine-requiring mutant ophiostoma (46); that  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid promoted the growth of some neurospora mutants (47); and that radioactivity from  $\alpha$ -AAA was found only in lysine when  $\alpha$ -AAA was the sole carbon source - thus demonstrating that the route from this compound to lysine was direct and not via any general pool of metabolites (48). Thus it became apparent that  $\alpha$ -AAA was a key compound in lysine biosynthesis.

Studies on other amino acids during the next few years suggested similarities in lysine metabolism. For example, the discovery that proline was converted to glutamic- $\delta$ -semialdehyde (49,50), in conjunction with studies on the cyclization of this compound (51), led to intensive investigation of the role of  $\alpha$ -AAA- $\delta$ -semialdehyde. It was already known that  $\alpha$ -AAA- $\delta$ -semialdehyde could replace lysine for growth in an E. coli mutant (52), and was a precursor of pipercolic acid in *Aspergillus nidulans* (53).

In 1960 Sagisaka and Shimura published the first of a series of papers on the conversion of  $\alpha$ -AAA to its semialdehyde in an isolated preparation in vitro. They used an enzyme preparation from yeast to demonstrate that the overall reaction required adenosine triphosphate (ATP), reduced glutathione, reduced nicotinamide-adenine trinucleotide (NADPH), and magnesium ions. They called the enzyme "AAA-reductase" and showed that it was

activated by reduced glutathione (54). In a further publication in 1962 (55), these authors reported the properties of this enzyme in detail and were able to show the two steps of activation and reduction separately. The mechanism is as follows:



Step 1), the activation step, was revealed by adding KF to repress pyrophosphatase action and allow accumulation of PP. This step requires magnesium but is not activated by reduced glutathione. The activation by glutathione is seen only with the whole reaction. It must therefore, be concerned with the actual reduction. Sagisaka and Shimura claim that the reaction is between the  $\omega$ -COOH group and adenylic acid in the formation of "activated-AAA". This makes it an entirely different type of compound from that involved in protein synthesis - where the linkage is with the  $\alpha$ -COOH of the activated amino acid.

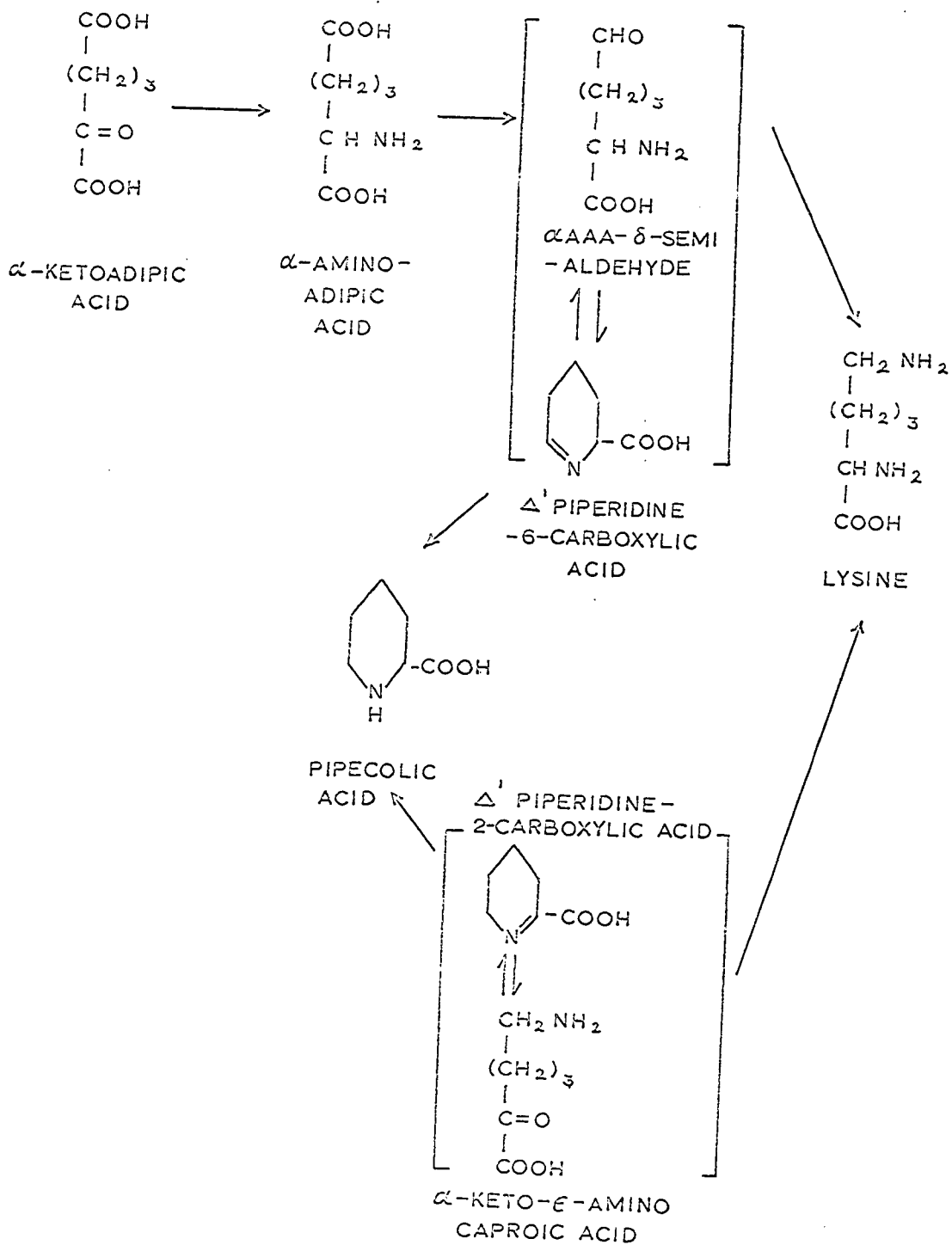
Although the cyclic compounds have been implicated in lysine biosynthesis in microorganisms in early reviews (30,56), it is only recently that detailed studies have been carried out to assess their role. In 1962 Rao, Rodwell and Basso (57,58) identified  $\alpha$ -aminoadipic- $\delta$ -semialdehyde and  $\Delta^1$ -piperidine-6-carboxylic acid as produced by a strain of *Pseudomonas* growing on pipercolic acid. These compounds were not isolated separately, but were identified in the following manner: further metabolic reactions were blocked by the addition of bisulfite, and the

material which accumulated was separated chromatographically and electrophoretically from other metabolites. An extensive series of chemical and physical reactions showed that it was an equilibrium mixture of these two compounds. Although this confirms the close relationship between  $\alpha$ -AAA-semialdehyde and the cyclic compounds, this does not indicate whether the cyclic compounds are on the direct pathway under normal circumstances. The situation is, therefore, similar to that in animal tissues.

In microorganisms, the question does not seem to be whether the cyclic compounds are on the pathway, as much as which cyclic compounds are involved. In 1963, Larson et. al. (59) were studying the step from  $\alpha$ -AAA-semialdehyde to  $\Delta^1$ piperidine-6-carboxylic acid. Their findings on the utilization of  $\alpha$ -keto- $\epsilon$ -aminocaproic acid and pipercolic acid, by *Saccharomyces cerevisiae*, were in conflict with earlier work (47 and 52 respectively). To explain this fact they summarized the present knowledge of lysine biosynthesis in microorganisms in a scheme which permits alternate paths in different organisms (Fig. 6).

This figure also illustrates the fact that most attention has been given to the central reactions in this pathway. The origin of the adipic acid derivatives is still not elucidated. One reason for this is the lack of lysine-requiring yeast mutants. Two such mutants have been found and it has been shown that they were able to utilize hexahomoserine ( $\alpha$ -amino- $\epsilon$ -hydroxy-caproic acid) (47). An enzyme has also been discovered which oxidizes

FIG. 6.



From Larson R.L., Sandine W.D., Broquist H.P.,  
 J. Biol. Chem. 238, 275, 1963.

this compound to  $\alpha$ -AAA- $\delta$ -semialdehyde. The steps prior to this are completely unknown. If labeled ( $C^{14}$ ) acetate is used as the carbon source for yeast it is found that the carboxyl carbon is the source of  $C_1$  and  $C_5$  of lysine and the methyl carbon is the source of the other carbons (60). From these results it has been postulated that lysine synthesis commences with the condensation of a two-carbon fragment and a four-carbon fragment (51). The four-carbon fragment cannot be succinate because of the unsymmetrical labelling in carbons 2 and 6 (60). Alpha keto-glutarate has been suggested by Strassman and Weinhouse (51), but it has been shown that there is far more  $C^{14}$  (from methyl-labeled acetate) in lysine than in glutamate (62). This would seem to exclude  $\alpha$ -keto-glutarate with its active role in transamination. Mattoon and Haight (63) have shown that one lysine-requiring yeast mutant accumulates glutaric acid and suggest a possible pathway involving glutaryl-CoA (ie. analagous to fatty acid synthesis).

Thus, the present knowledge of lysine biosynthesis via  $\alpha$ -AAA can be summarized as follows:

- 1) Condensation of a two-carbon and a four-carbon fragment.
- 2) Formation of  $\alpha$ -AAA and its semialdehyde.
- 3) Formation of cyclic compounds either as part of the route or as side-effects.
- 4) Amination of straight chain compound to form lysine.
- 5)  $\alpha$ -keto-  $\epsilon$ -amino-caproic and  $\alpha$ -amino-  $\epsilon$ -hydroxy caproic acids may be either intermediates in these steps, or alternates to  $\alpha$ -AAA in some organisms.

Lysine catabolism in lower orders

Two pathways of lysine degradation have been identified in bacteria. One is via  $\alpha$ -AAA and appears to involve the same mechanisms as lysine degradation in animals, and biosynthesis by this route in molds and yeast. In fact, much of the experimental evidence can be viewed from either direction. For example, the work of Sagisaka and Shimura (54,55), on conversion of  $\alpha$ -AAA to its semialdehyde, has shown that the reaction is reversible. Since most microorganisms are synthesizing lysine rather than degrading it, there has been little attempt to investigate this pathway as a purely degradative system.

The second pathway of lysine catabolism in bacteria involves features which are unique. This pathway was first indicated by the discovery by Stadtman, in 1962, (54) that lysine was converted to fatty acids and ammonia by a mechanism requiring a cobamide coenzyme. In a further publication (55) Stadtman has shown that the simultaneous action of two strains of *E. coli* is required for this reaction. Other bacteria are able to carry out the entire reaction. The steps have not been elucidated yet, but, since as many as six coenzymes are required by some bacterial strains, it is obviously a complex series of reactions. One point which is of interest in the present discussion is, that of many substrates tested, only  $\alpha$ -acetyl-L-lysine would replace lysine. ( $\epsilon$ -N-acetyl-L-lysine was negative). The preparation was free of acylase I since many substrates of this enzyme were tested and found to be negative. When  $\alpha$ -acetyl-L-

lysine is the substrate it must be deacetylated to lysine first since a mixture of  $C^{14}$  lysine and unlabelled  $\alpha$ -acetyl lysine did not yield any labeled acetyl lysine with this enzyme system, Pipecolic acid,  $\alpha$ -AAA and  $\epsilon$ -amino caproic acid also failed to become labeled under these conditions. Neither do threonine nor diaminopimelic acid react. It would appear therefore, that the formation of fatty acids from lysine involves neither acylase I nor  $\epsilon$ -Lysine acylase, and proceeds by a route not involving the major components of the  $\alpha$ -AAA route or the diaminopimelic acid route of lysine metabolism.

C) The possible function of  $\epsilon$ -N-acetyl lysine in animal metabolism

In 1929 Kimura found that many tissues contained enzymes which deacetylated  $\alpha$ -N-acetyl amino acids (66). This was extended by Greenstein et al. (67,68). However, as with so many other reactions, lysine proved to be an exception. Subsequently many  $\alpha$ -N-acetyl amino acids (but not  $\alpha$ -N-acetyl lysine) were shown to be able to replace their respective amino acids in test diets (69,70,71). Following the discovery by Neuberger and Sanger that  $\epsilon$ -N-acetyl-DL-lysine would replace lysine almost quantitatively for the growth of young rats (72), it became of interest to discover whether an enzyme existed for the deacetylation of lysine in the epsilon position. It was not until 1957 that such an enzyme was demonstrated in rat kidney by Paik et al. (5). Since then, similar enzymes have been shown to be present in many animal tissues and in fowl

kidney (8); in bacteria (6,73,74); and in molds (7). Despite the widespread occurrence of the enzyme which deacetylates lysine at the epsilon position, free  $\epsilon$ -acetyl lysine has never yet been found in vivo (35). However, many other acetylated and methylated amino acids have been discovered in trace amounts in recent years (75), and it is possible that the failure to find  $\epsilon$ -N-acetyl lysine may be due to problems of technique. Perhaps the methods used have not been sufficiently sensitive or the compound is rapidly converted to its products and some form of trapping agent will be necessary to block the reaction before  $\epsilon$ -N-acetyl lysine can be identified.

There is also the possibility that lysine is acetylated only after incorporation into protein. There are many suggestions in the recent literature that  $\epsilon$ -N-acetyl-L-lysine may play an important functional role in histones and other proteins (76, 77,78,79). There is also one recent work which implies that acetylation of lysine at the epsilon position may be an essential step in the formation of the stable acyl-enzyme intermediate in the glyceraldehyde-3-phosphate dehydrogenase reaction (80).

Many reports (81,82,83) have shown that the N-terminal amino acid of several proteins are acetylated. This acetylation involves many amino acids, not just lysine, but it indicates that the difference between a free and a substituted amino group is a critical recognition system in metabolism. Thus, although  $\epsilon$ -N-acetyl- and  $\epsilon$ -N-methyl-lysine have not been incontestably identified in many proteins, they have been

clearly identified in two cases (84,85) and there is strong support for the idea that blockage of the  $\epsilon$ -amino group may have an important biological function in some proteins. It is because of this concept that the recent studies of Phillips (79) and Allfrey et al. (77,86,87) on nuclear histones have assumed great importance.

In 1963, Phillips (79) investigated the low yield of N-terminal amino acids from the lysine-rich fraction of calf thymus histones. Many possible N-substituents were tested for, and eliminated, but acetate was detected. Therefore, in common with earlier reports on other proteins (81-83), Phillips assumed that the N-terminal amino acids were acetylated. Furthermore, since these acetyl residues were not released by acylase I (which deacetylates  $\alpha$ -N-acyl amino acids (67,68)), he offered a qualified suggestion that the acetyl groups might be  $\epsilon$ -N-substituents.

In the same year it was shown that histones inhibit RNA polymerases in several systems (87,88), that removal of histones from the nucleus causes an increased rate of "messenger" RNA synthesis (69), and that DNA-histone complexes are unable to serve as "primers" for RNA synthesis in vitro (90). Thus, the original idea, postulated by Stedman and Stedman more than ten years earlier (91), that histones had an inhibitory role in chromosomal function, at last was supported by some biochemical evidence. In 1964 Allfrey et al. (77) extracted histones from calf thymus nuclei and acetylated them chemically. They found that histone fractions differed in their

rate of acetylation and that those with the highest total acetate content did not necessarily have the highest specific activity when the acetylation was carried out with  $C^{14}$  acetate. Although these authors do not state it, one would conclude that the histone fractions with high total acetate but low specific activity were acetylated in vivo, before isolation and  $C^{14}$  acetylation. It is interesting that these were the lysine-rich histones. These authors found isolated arginine-rich histones (which are obviously poorly acetylated in vivo since they acquire a low total acetate but a high specific activity when acetylated in vitro) lose much of their ability to inhibit DNA-dependent RNA polymerases after partial acetylation in vitro, although they can still combine with DNA.

From this evidence Allfrey and his co-workers postulate that acetylation and methylation of histones may offer the means of switching-on or -off RNA synthesis at different loci along the chromosome. Since a puromycin level which inhibits both non-histone protein, by 90% and 70% respectively, does not affect methylation of histone protein, it would appear probable that methylation (and by analogy, acetylation) occurs after protein formation and is potentially reversible in vivo.

In contrast to this last result it must also be pointed out that some acetylated amino acids enter protein synthesis at a very early stage (as measured by the promotion of  $P_i$ -ATP exchange and incorporation into s-RNA) while still retaining their acetyl groups (92). Presumably these are the amino acids which form the N-terminal amino acids of some proteins. While

this paper is very interesting, there is one technical criticism which must be made and which might modify the results if it was corrected. Nowhere in the paper do the authors indicate that they carried out any purification of the commercial samples of amino acids which they used. Thus, their acetylated amino acids may have contained traces of free amino acids. It has been found by Paik and Kim (personal communication), that even very small traces of free lysine gave false positive results when the incorporation of N-acetyl-L-lysine into s-RNA was being studied in a similar system. Therefore, unless the experiments of Pearlman and Bloch (82) were carried out with absolutely pure amino acids, their results are open to question on these grounds.

The question of acetylated or methylated amino groups acting as biological controllers or markers is, therefore, a very important field of biochemical investigation at the present time. Acetylated lysine is of particular interest in this connection for two reasons. First, as a dibasic amino acid, it can enter into N-acetylation reactions while bound in a normal peptide linkage. Secondly, one of the characteristic fractions of nuclear histones is exceptionally rich in lysine compared to other proteins. Thus, any enzyme capable of acetylating or deacetylating lysine is also of particular interest. The studies on  $\epsilon$ -lysine acylase to date have all been conducted on the free amino acid or small peptides. It would be of great interest to know whether this enzyme would also act on the acetylated histones of Allfrey et al. (77).

D) Distribution of  $\epsilon$ -lysine acylase.

Although the possibilities which arise from the work of Allfrey et al. are fascinating, they are difficult to reconcile with the known distribution of the enzyme under investigation in the present study. In the first place, the organ distribution of  $\epsilon$ -lysine acylase suggests a function related to excretion since, in animals, the highest concentration is found in the kidney (8), and in birds, it is found in the kidney or not at all. Secondly, the subcellular distribution of the enzyme indicates that it occurs as a soluble enzyme in animals (5,9), and as a mitochondrial enzyme in birds (8). If its prime role concerned genetic control, the enzyme would be expected to be found primarily in the most rapidly multiplying tissues and primarily in the nucleus. The fact that it is not found primarily in rapidly multiplying tissue is shown by the comparison of chick embryo and adult chicken in the present work (see p 40 ). Against these facts is the possibility that the enzyme has two roles, and that the present crude methods of identification may not be sufficiently sensitive to show enzyme activity in the nucleus. Presumably the deacetylation of histones would require very little enzyme protein.

E) Possible roles of  $\epsilon$ -lysine acylase in the kidney

Even if this enzyme does occur in the nucleus, and does have a genetic role related to histones, the question remains as to the role of this enzyme in the kidney. There is no positive answer to this question but some suppositions may be made.

I) Since lysine is an essential amino acid, it is possible that

the enzyme is involved in some mechanism for conserving lysine.

€-N-acetyl-L-lysine is readily attacked by amino acid oxidase but free lysine is not (4). Thus deacetylation would protect this amino acid from attack by this enzyme - but - in this case, free €-N-acetyl-L-lysine would be expected to be found in the blood and it is not. This, then, cannot be the function.

II) It has been shown that uptake of amino acids by the intestine depends very largely on the position of amino groups relative to the carboxyl group (93). It may be that blockage of the epsilon amino group is necessary for resorption of lysine in the kidney and that the enzyme allows the excretion of excess lysine by freeing the €-amino group and preventing resorption of the amino acid. The same objection applies as in case I. €-N-acetyl-L-lysine has not been found as a free amino acid *in vivo*.

III) Christensen (94), using Ehrlich ascites cells, and Rosenberg et al. (95), using kidney slices, have shown that complex inter-relationships exist between different types of amino acids competing for transport systems. For example, Christensen has shown that neutral amino acids inhibit the entry of lysine into Ehrlich cells but that lysine can eliminate only a fixed part of the uptake of neutral amino acids (94). Rosenberg has shown that the basic amino acids compete among themselves for a common transport system in the kidney (95). This provides an explanation for the well established fact that infusion of any basic amino acid into the kidney in vivo causes increased excretion of other dibasic amino acids (96,97).

From these studies it is possible to imagine that the presence of  $\epsilon$ -lysine acylase in the kidney may be related to its ability to convert lysine from a basic to a neutral amino acid and so influence its resorption by different amino acid transport systems. Two facts which would be required for such a system are, however, not yet proved: 1) free acetyl lysine would have to exist in the kidney under some nutritional conditions. This is not impossible because, while free acetyl lysine has not yet been reported in vivo, neither are there any reports of experiments designed to look for it under conditions of either extreme lysine deficiency or excess. 2)  $\epsilon$ -lysine acylase or some other enzyme in the kidney would be required to acylate lysine. Attempts to use  $\epsilon$ -lysine acylase to acetylate lysine have not so far been successful but acetylation of lysine by a preparation from beef liver has been shown (36).

F) The present knowledge of  $\epsilon$ -lysine acylase.

A recent listing of known enzymes according to the Enzyme Commission classification (98) shows one enzyme - 3.5.1.e,  $\epsilon$ -N-acyl-L-lysine aminohydrolase - with  $\epsilon$ -lysine acylase activity. Since this list was prepared the same, or similar, enzymes have been isolated from several sources (5,6,7,8,9). These enzymes differ markedly in their relative substrate specificity, their pH optima and the degree of purification obtained to date. The fact which links these enzymes is their ability to deacylate lysine substituted at the epsilon position. It was discovered in the earliest investigations that the best substrates for the

enzyme(s) prepared from animal sources were the  $\epsilon$ -N-acetyl, formyl, and chloracetyl derivatives of -L-lysine (5,9), whereas in microorganisms the best substrate was  $\epsilon$ -N-benzoyl-L-lysine (74). Because of this marked difference in substrate specificity, the work by the two groups has tended to follow different lines and there are not many points at which useful comparisons can be made. A few points of comparison are listed below:

<u>Source of enzyme</u>	<u>pH opt.</u>	<u>preferred substrates</u>	<u>purification obtained</u>
rat kidney	7.2	$\epsilon$ -N-acetyl-L-lysine $\epsilon$ -N-chloracetyl-L-lysine	100x (5)
hog kidney	8.0	$\epsilon$ -N-acetyl-L-lysine $\epsilon$ -N-formyl-L-lysine $\epsilon$ -N-acetyl- $\alpha$ -N-glycyl-L-lysine	162x (9)
chicken kidney	9.0	-----	6x (8)
Pseudomonas KT 83	6.0	$\alpha$ - $\epsilon$ -N,N-debenzoyl-L-lysine	4000x (99)
Aspergillus oryzae No. 10	8.2-8.4	$\epsilon$ -N-benzoyl-L-lysine	10x (7)
Achromobacter pestifer EA	4.8-5.2	$\epsilon$ -N-benzoyl-L-lysine	"pure" (100)

It is uncertain at present whether these enzymes represent a single enzyme with different pH optima and relative substrate specificity or totally different enzymes. A recent publication on the substrate specificity of the bacterial enzyme (74), unfortunately reports few of the substrates which have been tested on preparations of animal origin. Two other factors make it impossible to compare the preparations from microorganisms with those from animal and avian tissues. In the first place, no preparation from

the latter sources has been obtained completely free from acylase I (E.C. No. 3.5.1.b) which acts on many alpha-N-substituted amino acids although only very weakly on  $\alpha$ -N-acyl-L-lysine (101). The fact that enzyme preparations from animal tissues (in contrast to those from bacteria (103)), will act on  $\alpha$ - $\epsilon$ -disubstituted lysine derivatives (5,9), may be due to the combined action of two enzymes. The evidence on this point is conflicting since with  $\epsilon$ -lysine acylase prepared from hog kidney (9) acetyl-L-methionine is attacked - indicating the presence of acylase I, whereas when  $\alpha$ -N- $\epsilon$ -N-diacetyl-L-lysine was the substrate it could be shown that acetate was released without the production of free lysine. From this it was concluded that only one acetyl-amino position was attacked (9).

A second factor which makes it difficult to assess the substrate specificity of animal and bacterial enzyme preparations is the method of determination of activity used with the bacterial preparations. In an early paper (73), the authors used chromatography with pure compounds as markers to identify the products of enzyme reaction. In later work (7,74,103) this was discontinued and an acid ninhydrin method was used (102). This method is claimed to be specific for lysine and negative for  $\epsilon$ -N-acyl-L-lysine (99) but the original paper (102) states that "the reaction seems to require only one amino group  $\alpha$  to the carboxyl group". The original paper also lists several amino acids and derivatives which react in a manner

similar to lysine (102). In the absence of actual data one must question whether the negative results obtained with N,N-diacyl lysine derivatives are valid or whether the reagent used would actually react with an  $\alpha$ -N-acyl-L-lysine if it was produced.

### Conclusion.

From the foregoing discussion it can be seen that acetylation of the epsilon position of lysine may be of biological importance in several systems. In the first place,  $\epsilon$ -N-acetyl-L-lysine is a substrate for several enzymes which do not act, or act only very weakly, on free lysine. The acetylated compound may therefore be an important one in the biosynthesis or degradation of lysine. Secondly, acetylation and deacetylation of the epsilon amino group of lysine may have an important genetic function through its ability to permit or prevent Histone-DNA linkages. Finally, the neutralization of a dibasic amino acid by acetylation of one amino group may be important in controlling the absorption of the amino acid - especially in the kidney.

As has been emphasized by many biochemists, it is almost impossible to clarify the exact steps of any biochemical process unless the enzymes concerned can be isolated and studied under controlled conditions, and the results compared to the overall reaction found in vivo. For this reason the present studies were undertaken to expand the information available on  $\epsilon$ -lysine acylase with the hope that it would help to clarify the role of acetylated lysines in biological systems.

### Experimental Results

A) A survey of the occurrence, subcellular distribution, and pH optima of  $\epsilon$ -Lysine acylase.

#### Introduction.

Earlier studies on  $\epsilon$ -lysine acylase established several interesting differences in this enzyme according to the source from which it was derived. Initially, it was shown that this enzyme occurred in several tissues in mammals, but only in the kidney in birds (8). It was also demonstrated that the enzyme occurred in the soluble cell fraction in the rat and hog (5,9), but in the mitochondrial fraction in chickens (8). Finally, a difference in pH optima had also been reported. The optimum for bacteria being 5.0 (103) or 7.0 (6), for molds 7.5 (7), for rat kidney 7.2 (5), for hog kidney 8.0 (9), and for chicken kidney 9.0 (8).

Such marked differences indicated that it would be profitable to expand the surveys already reported, to determine whether these were species differences. Since the rat and hog enzyme had been investigated in some detail (5,9), it was decided to investigate a variety of birds with a few animals for comparison.

#### Methods

1) Determination of L-lysine with lysine decarboxylase (104).

Duplicate Warburg manometer flasks were prepared containing 2,4,6,8, and 10  $\mu$ moles of L-lysine in 0.06M pH 6.0 phosphate buffer. Lysine decarboxylase (*B. cadaveris* acetone powder) was

tipped in from the side arm after equilibration and the evolution of  $\text{CO}_2$  followed in the usual manner (105). The results are shown in Fig. 7. From this it can be seen that an average of 18.5  $\mu\text{l}$   $\text{CO}_2$  is evolved per umole of lysine. This figure is somewhat lower than that reported by the original workers since no acid was added from the second side arm to release dissolved  $\text{CO}_2$ .

ii) Protein determination by the method of Lowry et al. (106).

Crystalline bovine albumin (Sigma) was dissolved in water to give final concentrations of 0.05, 0.075, 0.10, 0.15, 0.20, and 0.40  $\text{mg/ml}$ . Three stock solutions were prepared:

Solution A - 2%  $\text{Na}_2\text{CO}_3$  in 0.1N  $\text{NaOH}$ ,

Solution B - 2%  $\text{Na}_2\text{K tartrate}$  in  $\text{H}_2\text{O}$ ,

Solution C - 1%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in  $\text{H}_2\text{O}$ .

Immediately before use, solutions A, B, and C were mixed in the proportions of 100:1:1. Five ml of the mixture was added to 1 ml of the protein solution and, after standing ten minutes, 0.5 ml of Folin-Ciocalteu Phenol reagent (obtained as concentrate from Fisher Laboratories) was added with immediate, vigorous shaking. The color was allowed to develop for thirty minutes and then read in a Coleman junior spectrophotometer at 700  $\text{m}\mu$ . The results are shown in Fig. 8, and indicate that a linear relationship can be obtained (between optical density and protein concentration) from 75 to 400  $\delta/\text{ml}$ . Experimental protein solutions were, therefore, diluted to this range before protein determinations were carried out.

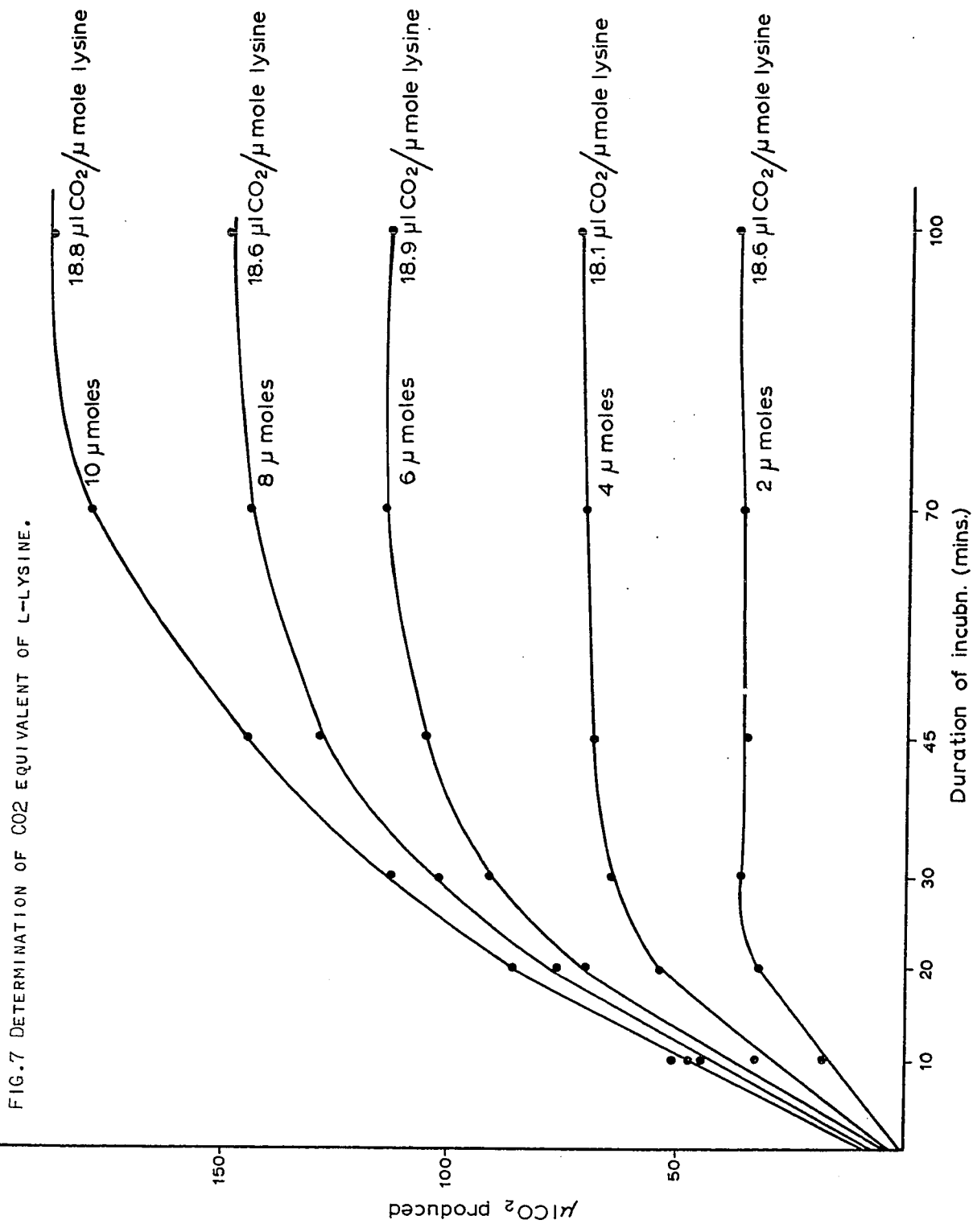
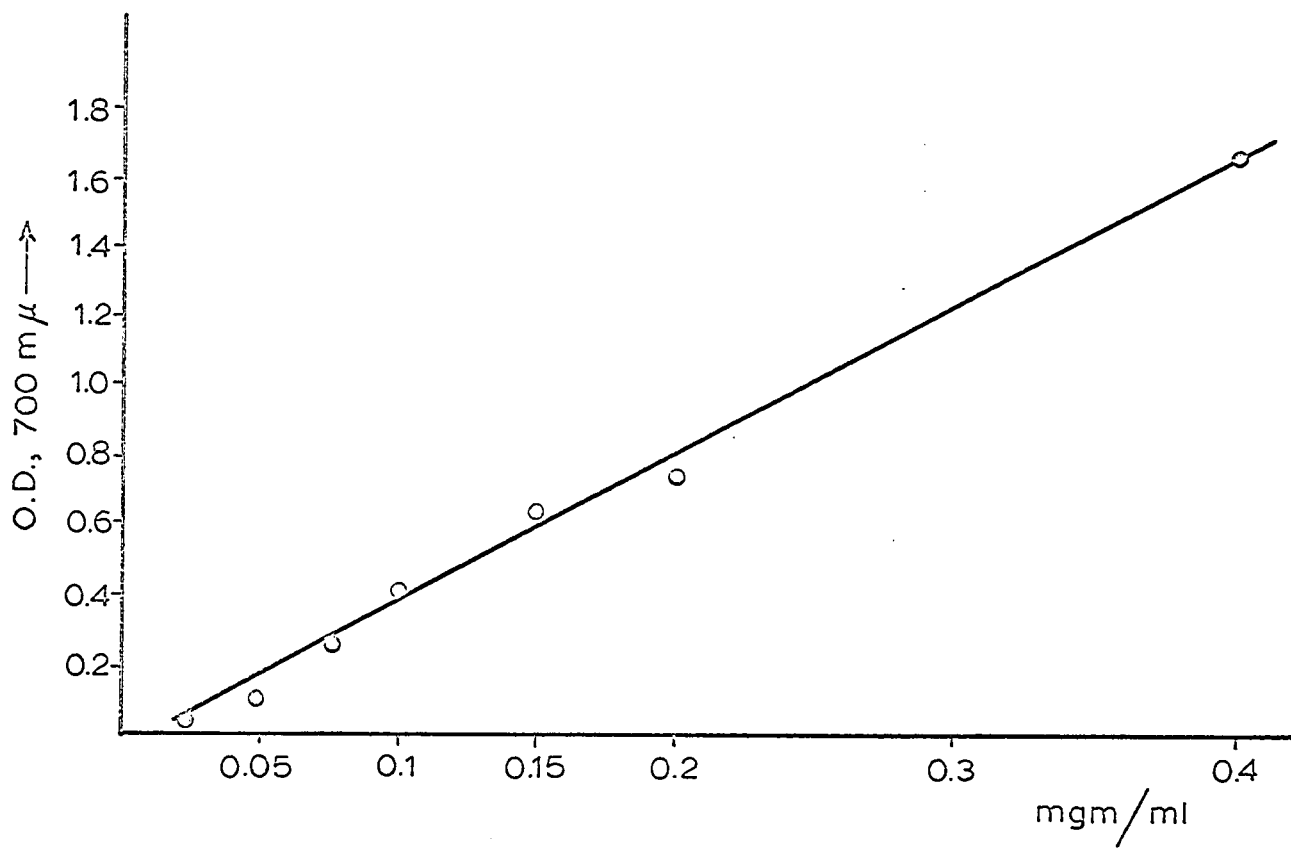


FIG.8 STANDARD CURVE FOR PROTEIN DETERMINATIONS.



111) Preparation of  $\epsilon$ -N-acetyl-L-lysine (107).

$\epsilon$ -N-acetyl-L-lysine was prepared by acetylation of the copper salt of lysine as described by Neuberger and Sanger (72) but using LiOH as base (107,9).

Eighteen grams of L-lysine hydrochloride was placed in a 2L flask containing approximately 750 ml water and brought to a boil. Twenty-eight of  $\text{CuCO}_3$  was added and the total volume brought to 1L. It was refluxed for 1.5 - 2 hours and filtered hot with suction. The filtrate was cooled to  $0^\circ$  to  $-5^\circ$  C in an ice-salt mixture. When cool, 50 ml 2N LiOH was added to the flask. The solution was stirred very vigorously (still in an ice-salt bath) and another 50 ml LiOH and 10.5 ml acetic anhydride were added dropwise, simultaneously, from separate funnels, over a period of one hour. The reaction was kept alkaline at all times by adding the LiOH faster than the acetic anhydride. After all the additions were in the mixture was stirred for a further hour at room temperature.

When precipitation appeared to be complete and the mixture had warmed to room temperature, 4N HCl was added until the precipitate dissolved. Hydrogen sulfide was then bubbled through the mixture for at least 30 mins. with vigorous stirring. A little charcoal mixed with celite was added to settle the sulfide precipitate and air was bubbled through to remove  $\text{H}_2\text{S}$  fumes. The mixture was then filtered through a thin layer of celite, with suction. The pH of the filtrate was adjusted to 5.7 with LiOH. If any blue color appeared the treatment

with  $H_2S$  and filtration were repeated. The colorless filtrate was reduced to a small volume in a flask evaporator at  $50^{\circ} C$ . The residue was an oily liquor.

Crystallization was carried out by the addition of cold ethanol (approximately 3-5 volumes) and standing in the cold overnight. The product was recrystallized from ethanol in the same way. Acetone, in small amounts, aided crystallization, but if used to excess caused free lysine to crystallize out too. Using slow crystallization in ethanol, the product contained free lysine in small amounts. The purity of the product was checked by ascending chromatography in butanol: formic acid: water, 70:15:15, on Whatman No. 1 paper.

#### Assay Procedure

All birds and animals were killed by decapitation. Tissues were excised from healthy, normal birds and animals within 30 mins. of killing (see p for loss of enzyme activity 6 hours after killing) and placed immediately in a dry beaker in an ice bath. When all tissues had been collected, each in turn was trimmed of any fat or extraneous material. Intestine was rinsed three or four times with 0.25M sucrose to reduce the bacterial count; heart was rinsed once to remove blood; other tissues were not rinsed.

Each tissue was then homogenized in a volume of 0.25M sucrose equal to 5x the tissue weight. Homogenization was carried out in a glass homogenizer with a motor-driven teflon head, or in an all-glass homogenizer with a toothed head. The latter

was more efficient for tough tissues such as muscle and intestine. Homogenization was continued until a fairly uniform suspension was obtained but the suspensions still contained some crude material and required large tipped pipettes (obtained from Kimble Glass Co.) to dispense them. The homogenizers were chilled in an ice bath before use and at one minute intervals during use if the homogenization was prolonged. No homogenization was continued more than 4-5 mins. Any unground material remaining at this time was discarded.

Incubation of tissue homogenate with substrate was carried out by placing equal volumes (usually 2.4 ml of each) of homogenate and substrate (dissolved in buffer to give 60 umoles/ml) in a 25 ml Erlenmeyer flask. The flasks were covered with a piece of "Parafilm" (obtained from Marathon Div. of American Can Co.,) and shaken in a Dubonoff shaker at 37° C. The buffer used and the duration of incubation varied with each experiment and are, therefore, recorded under each experiment. However, the incubation time never exceeded 4 hours since the rate/time curve had been shown to be linear for this period (8).

Enzyme reaction was terminated by adding 0.1 ml of 1.0N HCl to each flask. This reduced the pH below 4.0 and stopped enzyme action. The mixture was then placed in a boiling water-bath to destroy the enzyme. Tissue blanks were prepared by incubating the tissue alone, under the same conditions as the experimental flasks, and adding the substrate after the addition of HCl.

After completion of the enzyme action, as described above, the boiled samples were centrifuged at maximum speed in a clinical centrifuge for a few minutes. The supernatant was decanted and the lysine content of 1.0 ml aliquots determined as described, using lysine decarboxylase. To determine the actual amount of lysine produced by the action of  $\epsilon$ -lysine acylase, the  $\text{CO}_2$  evolved by the tissue blanks was subtracted from that of the experimental flasks. This also eliminated the effect of any free lysine in the substrate.

Protein concentration was determined on a small amount of the original homogenate and the enzyme activity expressed as  $\mu\text{moles}$  of lysine produced per hour per  $\text{mgm}$  protein = SPECIFIC ACTIVITY.

Results.

I) Investigation of  $\epsilon$ -lysine acylase activity in various organs from adult chickens.

Tissues were homogenized and incubated as described under "Methods". The buffer used to dissolve the substrate was 0.1M, pH 9.0, glycine/NaOH, and all incubations were carried out for two hours.

The results are shown in Table I. It can be seen that enzyme activity was found only in the kidney as reported previously (8). Also, when investigating the reproductive organs, separate batches of kidney homogenates were found to show no sex differences. During the course of this work, it was also found that, while individual batches of homogenate varied, those prepared at different seasons and those from laying or broody hens, had the same average specific activity as that shown here.

It would appear, therefore, that adult hens and roosters show  $\epsilon$ -lysine acylase activity only in the kidney, and that the level of enzyme is not significantly affected by seasonal or reproductive factors.

II) Investigation of  $\epsilon$ -lysine acylase activity in other birds.

A variety of large and small birds were examined for enzyme activity using the same procedure as described for the chicken.

It was found that in some birds enzyme could be detected in the kidney, but that in others, no enzyme could be detected at all. Possible explanations of these differences are discussed

TABLE I

ε-Lysine Acylase Activity in Adult Chickens

Tissue	Sp. Act.*
Kidney (Male)	0.108
Kidney (Female)	0.129
Liver	0
Spleen	0
Pancreas	0
Intestine	0
Brain	0
Muscle (skel.)	0
Heart	0
Lung	0
Reproductive (Male)	0
Reproductive (Female)	0

\*  $\mu$ moles lysine produced/hr./mgm tissue.

All tissues were tested at least twice.

on p 57 . The specific activities of kidney tissue from birds which showed activity at pH 9.0, under the present assay conditions, are shown in Table II. Tissues in which no enzyme activity was detected are listed in below:

Canary (A mixed breed commercially designated as "Greens with some mixed Cinnamon blood" - ie. the common household cage bird).

Tissues tested and found negative: Kidney, liver, spleen.

African Finch ("Cut-throat" Robin finches from Senegal and N. Nigeria).

Tissues tested and found negative: Kidney and liver.

Budgerigars (common household cage birds of various colours - a mixed breed).

Tissues tested and found negative: Kidney, liver, pancreas, intestine, brain, skeletal muscle, heart, lung, reproductive organs.

Bronze Mannikins (*Spermester Cucullotus*).

Tissues tested and found negative: Kidney, spleen, liver, pancreas, intestine, skeletal muscle, heart, lung, adrenal gland.

Adult chickens (various breeds including Rhode Island Red, White Leghorn, Barred Rock, and Cox Heavy).

Tissues tested and found negative: Liver, spleen, pancreas, intestine, brain, skeletal muscle, heart, lung, female genital organs, male genital organs.

Adult turkey (common barnyard).

Tissues tested and found negative: Liver, spleen, pancreas, intestine, brain, skeletal muscle, heart, lung, adrenal gland.

TABLE II

Activity of Kidney Homogenates from Various Birds

Bird	Sp. Act	Bird	Sp. Act
Chicken	0.124	Turkey	0.113
Duck	0.064	Pigeon	0.178
Wild Duck*	0.041	Pheasant#	0.118

\* Trapped wild but reared in barnyard.

# Farm bred.

Sp. Act. =  $\mu$ moles lysine produced/hr./mgm tissue.

Adult pheasant (barnyard-bred for table use).

Tissues tested and found negative: Liver, spleen, pancreas, intestine, brain, skeletal muscle, heart, lung.

Adult duck (common barnyard).

Tissues tested and found negative: Liver, spleen, pancreas, intestine, brain, skeletal muscle, heart, lung, female reproductive organs, male reproductive organs.

"Wild" duck (a single bird which was trapped wild when young and reared in the barnyard).

Tissues tested and found negative: Liver, spleen, pancreas, intestine, brain, skeletal muscle, heart, lung, adrenal gland.

Pigeon (common blue-grey wild type but reared in captivity).

Tissues tested and found negative: Liver, spleen, pancreas, intestine, brain, skeletal muscle, heart, lung.

Because of the varying size of the birds involved in the above survey, it seemed possible that trace amounts of enzyme activity might not be noticed in small samples - especially in the smaller birds. This was compensated for by taking larger numbers of the small birds. In Table III are listed comparative average weights for birds and their average kidney weights and enzyme activity. From this Table it can be seen that there is no direct relationship between the size of the bird and the degree of enzyme activity, since duck, one of the larger birds tested, has one of the lowest specific activities; and pigeon, the smallest bird to show any activity, has the highest. It

TABLE III

Comparative  $\epsilon$ -Lysine Acylase Activity of  
Kidney Homogenates from Various Birds.

Bird	Av. Wt. Bird (A)	Av. Wt. Kidneys (B)	Minimum Wt. used gms	Ratio B/A	Sp. Act. #	Total Act. ( $\times 10^3$ )
Turkey	3,825	24	7.8	0.0062	0.113	2.66
Duck	1,581	11	4.3	0.0065	0.064	0.72
Chicken	1,350	9	1.6*	0.0065	0.124	1.14
"Wild" duck	1,013	9	5.6	0.0088	0.041	0.156
Pheasant	518	4	3.4	0.0065	0.118	0.45
Pigeon	312	2	3.4	0.0064	0.178	0.34
Budgie	27	0.20	1.4	0.0073	--	--
Finch	18	0.13	0.8	0.0072	--	--
Canary	16	0.20	1.0	0.0125	--	--
Mannikin	9	0.06	0.6	0.0066	--	--

# moles lysine produced/hr./mgm protein

\* see text

can also be seen that the ratio of body-weight to kidney weight does not provide any index of activity (compare "wild" duck and canary). Furthermore the experiment in which the least amount of chicken kidney used was comparable to the amount used for Budgies gave a specific activity of 0.124 (ie. the same as that obtained from the much larger samples usually used).

### III) Investigation of $\epsilon$ -lysine acylase activity in young chickens.

Since the  $\epsilon$ -lysine acylase activity found in the initial experiments did not seem to be a direct function of the size of the bird, it was decided to investigate any possible relationship to developmental age. Accordingly, young chickens were obtained and the enzyme content of their kidneys determined.

The experimental procedure used was the same as that described for adult birds, except that the incubation time was increased to 4 hours for the very young birds, in order to obtain the maximum activity. Eight birds were used to obtain kidney samples from the small chicks, but this was decreased to two as the birds increased in size. The results are shown in Table IV.

In the first experiment with White Leghorn chicks, an interesting gradation of activity with age could be seen. However, subsequent experiments, with Cox Heavy breed and with a second group of White Leghorn chickens, did not show such marked effect. There was a slight increase in activity with age, but even the youngest showed appreciable activity in

TABLE IV

ε-Lysine Acylase Activity in Young Chicks

White Leghorn			Cox Heavy			White Leghorn		
Age days	Body Wt. gms	Sp. Act.	Age days	Body Wt. gms.	Sp. Act.	Age days	Body Wt. gms	Sp. Act.
4	45	0	2	43	0.078	3	42	0.095
			4	58	0.056			
			6	69	0.100	7	59	0.091
			8	84	0.091			
10	73	0.041	10	86	0.079	11	85	0.150
			14	110	0.089			
			18	132	0.130	19	158	0.118
			26	244	0.108			
30	255	0.081						

Sp. Act. =  $\mu$ moles lysine/hr./mgm protein

these experiments. On consulting the breeder (Bray Chicks Ltd., Hamilton, Ontario), it was learned that the laying hens (and the newly hatched chicks before shipment) did not always receive the same diet. The breeders were unable to supply any definite information which would permit further investigation of this point. The difference between these batches of chickens and the small increase in activity with age may, therefore, have been due to dietary factors but the facilities for confirmation of this suggestion were not available.

IV) Investigation of the  $\epsilon$ -lysine acylase activity of chick embryos.

Fertile eggs were incubated at 37° for fifteen and twenty days respectively. At the end of each incubation period, the embryos were removed from the eggs, decapitated, and the kidneys removed and placed in a dry beaker in an ice bath. With fifteen-day embryos it was difficult to separate the kidney entirely from surrounding tissues. With twenty-day embryos accurate dissection was possible. Kidney tissue from sixteen embryos was pooled to make an adequate tissue sample from the fifteen-day old embryos. Seven pairs of kidneys provided sufficient tissue from the twenty-day old embryos.

In each case the tissue was homogenized in 0.25M sucrose as usual. Very little homogenization provided a completely smooth suspension, due to the softness of the tissue. Incubation with 50  $\mu$ moles/ml  $\epsilon$ -N-acetyl-L-lysine in pH 9.0, 0.1M glycine/NaOH buffer was carried out for two hours, and lysine

determined as usual. The results are presented in Table V.

It can be seen that, at both ages tested, chicken embryos showed  $\epsilon$ -lysine acylase activity comparable to that obtained with adult chickens. Although the enzyme activity appears to be lower at the younger age, this is probably not significant. Similar variation is seen between newly hatched chickens and adult hens. Furthermore, the figure obtained for fifteen-day old embryo kidney is probably low due to contamination of the sample with other tissue (as mentioned above).

From these results it would appear that there is no gradation in enzyme activity during the later stages of embryonic development nor coincident with hatching. It must, however, be emphasized again that it was not possible to control the diets of these birds, and this may have an important role in the appearance of enzyme activity. For a fuller discussion of this point see p 57 .

#### V) Investigation of the $\epsilon$ -lysine acylase activity of animals.

In a previous investigation into the occurrence of this enzyme (5,8,9), several animals had been shown to possess  $\epsilon$ -lysine acylase activity in a variety of organs. A few others were therefore studied to expand the original survey. Since the pH optima for rat and hog kidney were different (5,9), a preliminary experiment was run in which the enzyme activity of each animal kidney was determined at different pH values. The activity of other tissues was then determined at the pH found optimum for kidney. For some buffers it was

TABLE V

ε-Lysine Acylase Activity of Chick Embryo Kidney Homogenates

Age of Embryos days	Wt. of Kidney used, gms	Protein mgm/ml.	Sp. Act.
15	1.6	36.9	0.086*
20	1.5	34.5	0.108

\* Sp. Act./mgm protein (This figure would probably be higher if pure kidney tissue used).

Note: av. sp. act. for newly hatched chicks (2-4 days old)  
= 0.091, av. sp. act. for adult hens = 0.118.

found necessary to add 0.2 ml 1N HCl to reduce the pH below 4.0 when stopping the reaction. These buffers are marked "0.2" in the list below. All incubations were carried out for 2 hours.

Buffers used in animal experiments:

Mouse:           pH 7.2 - 0.05M Veronal  
                      - 0.06M Phosphate  
                      pH 8.0 - 1.0M Tris/HCl (0.2)  
                      - 0.2M Tris/Maleate (0.2)  
                      - 0.1M Phosphate  
                      pH 9.0 - 0.1M Glycine/NaOH

Rabbit:           pH 7.0 - 0.2M Tris/Maleate (0.2)  
                      To  
                      pH 8.6 - 0.2M Tris/Maleate (0.2)

Dog and Human: pH 6.0 - 0.06M Phosphate  
                      pH 6.5 - 0.06M Phosphate  
                      pH 7.1 - 0.06M Phosphate  
                      pH 8.0 - 1.0M Tris/HCl (0.2)  
                      pH 9.0 - 0.2M Glycine/NaOH

Since all these buffers were used to dissolve the substrate, whereas the homogenate was prepared in sucrose, the final concentration of buffer in the incubation mixture was half that given in the list above.

Results obtained with mouse tissues.

308<sub>1</sub>J mice approximately eight months old were used. In the initial experiment, with kidney homogenates, pH 8.0 was selected as the optimum pH for this animal. Accordingly, all

other tissues were tested at pH 8.0. Liver was also tested at other pH values to confirm that the lack of activity was not due to a difference in optimum pH between tissues.

As can be seen from Table VI, no activity was found in liver at any pH tested. The maximum activity found in kidney was approximately one sixth that found in chicken kidney. Only traces of activity were found in other organs.

Results obtained with other animal tissues.

When rabbit kidney and liver, and dog kidney, were tested for their  $\epsilon$ -lysine acylase activity, it was found that rabbit kidney showed some enzyme activity over a wide range of pH values. Rabbit liver was inactive both at pH 7.2 and at pH 8.0. Dog kidney was entirely inactive. These results are summarized in Table VII.

Attempts to demonstrate  $\epsilon$ -lysine acylase activity in human kidney.

A normal human kidney was obtained from autopsy within 6 hours of death. The subject was an adult male. Using the same buffers as had been employed for experiments on dog kidney, attempts were made to demonstrate enzyme activity in homogenates of kidney cortex. The results were completely negative at all pH's tested.

The effect of autolysis on enzyme activity.

Since it was not possible to obtain human kidney without several hours delay from the time of death, the question arose

TABLE VI

ε-Lysine Acylase Activity in Mouse Tissues.

Organ	pH	Sp. Act.
Liver	7.2	0
	8.0	0
	9.0	0
Kidney	7.2	0.012
	8.0	0.019
	9.0	0
Spleen	8.0	0.004
Intestine	8.0	0.008
Brain	8.0	0
Skel. mus.	8.0	0
Heart	8.0	0
Lung	8.0	0

TABLE VII

ε-Lysine Acylase Activity of Animal Tissues.

TISSUE	pH							
	6.0	6.5	7.0	7.2	7.6	8.0	8.5	9.0
Rabbit kidney	-	-	0.013	0.020	0.021	0.016	0.010	-
Rabbit liver	-	-	-	0	-	0	-	-
Dog kidney	0	0	-	0	-	0	-	0

Figures are sp. act. =  $\mu$ moles lysine/hr./mgm protein.

whether such delay might have caused destruction of the enzyme. It was decided to test the effect of autolysis on the  $\epsilon$ -lysine acylase activity of chicken kidney to determine whether autolysis might be responsible for the negative results obtained with human kidney.

Two chickens were killed, opened, and allowed to stand at room temperature for six hours before the kidneys were removed. In order to see the maximum possible difference between enzyme activity in these kidneys, and that in kidneys removed from the bird as rapidly as possible, determination of enzyme activity was not carried out on the crude homogenates but on mitochondria isolated from them (see p 51 for isolation procedures). The determinations were carried out in the usual way using pH 9.0, 0.1M glycine/NaOH buffer and a two-hour incubation.

The specific activity of the preparation held at room temperature for six hours was 70% of that prepared in the normal way. If one can assume that the autolytic processes are the same in the human and the chicken kidney, this result would imply that autolysis was not responsible for the absence of activity in the human kidney sample - unless the original activity was exceedingly low.

The results obtained with a single, human autopsy sample, and the effect of autolysis on chicken kidney, cannot be considered conclusive. They do, however, suggest that the human kidney, like that of the dog, has little or no  $\epsilon$ -lysine acylase activity. Some possible implications of this suggestion have been discussed on p 143.

VI) Attempts to induce  $\epsilon$ -lysine acylase activity in canaries.

Twelve canaries were maintained on a diet which included added  $\epsilon$ -N-acetyl-L-lysine for two weeks. It was learned from the supplier that these birds prefer to obtain their water from moist food rather than from a water trough. For this reason, a solution of  $\epsilon$ -N-acetyl-L-lysine was poured into the food daily. Not all would be used due to scattering of food by the birds, but what they ate would contain a small amount of the added amino acid.

Five ml of a 40  $\mu$ mole/ml solution of  $\epsilon$ -N-acetyl-L-lysine in water was added to the food each day for two days. Since no ill-effects were seen, the volume was increased to ten ml per day for four days. At this time one bird died and several looked "bloated" therefore the dose was reduced to five ml per day for the remaining time. No more birds died and the survivors all looked healthy at the end of the two weeks. No gross abnormalities were seen at autopsy.

The livers and kidneys were removed from the surviving eleven birds at the end of two weeks. Each tissue was homogenized and tested in the usual way. The results are presented in Table VIII.

It can be seen that the amount of activity obtained is very small. In fact, in view of the small amount of carbon dioxide evolved, it is questionable whether it is significant. However, the fact that liver was even more negative than kidney, and that two runs with kidney samples yielded very similar

TABLE VIII

ε-Lysine Acylase Activity in Canaries After  
Addition of ε-N-Acetyl-L-Lysine to Diet.

Sample	<u>HOMOGENATE</u>		
	Protein (mg/ml)	μCO <sub>2</sub> /ml	Specific Activity
Kidney * untreated	43.2	0	0
Kidney treated	1) 65.5	14.8	0.012
	2) 40.5	5.3	0.014
Liver treated	57.7	2.5	0.002

\* "Untreated kidney" was removed from birds which had received the same food as the treated ones but without added ε-N-acetyl-L-lysine.

specific activities, suggests that there may have been an induction of enzyme activity.

VII) Effect of storage of kidneys on  $\epsilon$ -lysine acylase activity.

Since the removal and preparation of chicken kidneys required considerable time, several experiments were run to determine whether the kidneys could be stored frozen and used as required.

Kidneys were removed from several chickens at once, trimmed as usual and divided into several containers. One batch was homogenized immediately and the enzyme activity determined the same day. The others were stored at  $-4^{\circ}$  C. Individual samples were thawed and tested after various time intervals. The experiments were also carried out with one batch of pigeon kidney for confirmation that the stability of the enzyme was a general phenomena. The results are presented below:

<u>Tissue:</u>	<u>Specific Activity:</u>						
	Fresh	1wk.	2wks.	1mth.	2mths.	3mths.	4mths.
Whole Chick. Kidney	0.12	-	-	-	-	0.12	0.13
	0.13	-	-	-	-	0.11	-
	0.19	0.19	-	-	-	-	-
	0.25	-	0.20	-	-	-	-
Whole Pigeon Kidney	0.17	-	-	-	0.19	-	-

All figures on the same horizontal line constitute one experiment.

From these results it is obvious that the enzyme is completely stable when whole kidneys are stored at  $-4^{\circ}$  C for months. The

procedure adopted was, therefore, to kill large groups of birds at one time and store the kidneys until required.

VIII) The subcellular distribution of  $\epsilon$ -lysine acylase activity in birds and animals.

It had been shown in earlier work (5,8,9), that  $\epsilon$ -lysine acylase was to be found in the soluble fraction of rat and hog kidney cells, and in the mitochondrial fraction of chicken kidney cells. It was decided to prepare cell fractions from some of the more active tissues and determine whether this was a consistent difference between birds and animals.

Crude cell fractions were prepared as described below. After it had been ascertained which cell fraction contained the major part of the enzyme activity, active fractions were washed with 0.25M sucrose, recentrifuged, and their activity redetermined. In this way it could be demonstrated whether the enzyme activity remained distributed throughout several fractions, or tended to become concentrated in one fraction as its purity increased.

Preparation of cell fractions.

Homogenates of tissue in five volumes of ice-cold 0.25 M sucrose were prepared by grinding in a chilled Waring blender for 30 seconds. The crude homogenate was filtered through cheesecloth, and ground in a glass grinder, with a teflon grinding head, until uniform. The homogenizer was chilled in an ice bath before use and after every two progressions of the head up and down. The homogenate itself was kept in ice at all times.

After grinding was completed the homogenate was centrifuged at 3,000 rpm in a No. SS 34 head in a refrigerated Servall centrifuge ( $1,085 \times g$ ), for ten minutes. The precipitate was taken as the crude nuclear fraction. The supernatant was decanted and centrifuged at 9,000 rpm ( $9,750 \times g$ ), under the same conditions. The precipitate was taken as the crude mitochondrial fraction. The supernatant was decanted and centrifuged in a refrigerated Spinco model No L, head No. 40, at 40,000 rpm ( $105,000 \times g$ ), for one hour. The precipitate was taken as the crude microsomal fraction and the supernatant was taken as the soluble fraction. Since no washings were employed in the initial stages, the soluble fraction did not become diluted and was suitable for use in testing for enzyme activity.

Determination of enzyme activity was carried out as described for the homogenates except that the following buffers were used:

For all avian kidney cell fractions the substrate was dissolved in 0.2M, pH 9.0 glycine/NaOH buffer.

For experiments with rabbit kidney cell fractions the substrate was dissolved in 0.05M, pH 7.2, phosphate buffer.

For experiments with dog kidney cell fractions, each crude cell fraction was tested at all the different pH values used for the whole homogenate, employing the same buffers as used for the homogenate (see p 43).

The results of cell fractionation studies are shown in Table IX. From this table it can be seen that  $\epsilon$ -lysine acylase

TABLE IX

ε-Lysine Acylase Activity of Crude Kidney  
Fractions from Various Birds and Animals.

<u>Cell Fraction</u>	<u>Sp. Act.</u>	<u>Effect of Washing</u>
<u>a) Pigeon</u>		
Nuclear	0.06	Washing nuclear fraction once, removed half its enzyme activity to the mitochondrial fraction.
Mitochondrial	0.27	
Microsomal	0.04	
Soluble	0.006	
<hr/>		
<u>b) Duck</u>		
Nuclear	0.24	Washing nuclear fraction once caused two thirds of its activity to move to the mitochondrial fraction.
Mitochondrial	0.30	
Microsomal	0.031	
Soluble	0	
<hr/>		
<u>c) Turkey</u>		
Nuclear	0.08	After 4 sequential washings of the nuclear fraction three quarters of its activity moved to the mitochondrial fraction.
Mitochondrial	0.07	
Microsomal	0.01	
Soluble	0	
<hr/>		
<u>d) Rabbit</u>		
Nuclear	0.009	—
Mitochondrial	0	
Microsomal	0	
Soluble	0.11	

activity is located primarily in the mitochondria of all three birds tested, and entirely in the soluble fraction of rabbit kidney cells. All fractions of dog kidney cells were negative at all pH's tested.

In the case of avian samples, the fact that the second highest enzyme activity was found in the nuclear fraction in each case is not surprising since this fraction would also contain any unbroken cells. The fact that the major portion of this activity became transferred to the mitochondrial fraction by washing and recentrifuging would indicate that enzyme activity is actually located only in the mitochondria and that the lesser amounts of activity found in other fractions were due to contamination.

IX) Confirmation of negative results using stronger buffer.

As this programme of work developed, it was discovered that the buffer used during this survey was not strong enough to maintain the pH during the incubation period. Since the substrate was dissolved in 0.1M glycine/NaOH the final concentration during incubation was 0.05M. Other buffers used for animal tissues were much stronger therefore there was no question about them but with avian tissues, and this buffer, the question arose whether negative values were perhaps due to inadequate buffer strength, and consequently, low pH during incubation.

One other error was also discovered. During the survey just reported, buffer was prepared to the desired pH and

substrate dissolved in it. The low concentration of glycine/NaOH buffer was not sufficiently strong to be used this way. It was found that this procedure yielded a substrate solution of approximately pH 8.6-8.7 rather than 9.0 which it was assumed to be. (See pp 95-100 for a full discussion of these problems).

Accordingly, three experiments were repeated with stronger buffer, adjusted to the exact pH after the addition of substrate, to check whether the apparent negative results were valid. Homogenates were prepared as described on p 29. Fresh chicken kidney homogenate was run as a positive control. The results are presented in Table X.

It can be seen that correction of the pH of the incubation mixture did not alter the results obtained. The negative results obtained in this survey were, therefore, valid and not due to faulty pH. The use of chicken brain in this experiment made this conclusion particularly strong because, in one of the four original experiments a trace of activity had been noted - so low that it was assumed to be an artifact. If faulty pH values had affected the results this, of all tissues, was the most likely to show positive values when the pH was corrected.

#### Discussion and Summary.

The survey (8) of the occurrence of  $\epsilon$ -lysine acylase has been expanded to include duck, pheasant, wild duck, budgie, finch, mannikin, mouse, dog, rabbit, and human tissues. The results obtained have confirmed previous indications that this enzyme can be found in many mammalian tissues in small

TABLE X

Confirmation of Results Using Stronger Buffer During Incubation

Sample	Protein mgm/ml.	$\mu\text{CO}_2$ /ml	Sp. Act.	pH.
Canary kidney	37.9	0	0	9.0
Chicken kidney	71.2	77.6	0.11	9.0
Chicken liver	55.6	4.6*	0.004*	9.0
Chicken brain	35.9	0	0	9.0

Sp. Act.  $\mu\text{moles lysine/hr./mgm protein}$

\* Probably an artifact. Cannot be considered significant  
because of low  $\text{CO}_2$  value.

amounts but only in the kidney in birds. Although the results are not conclusive, there are indications that the enzyme may be inducible in birds. This suggestion arises from two points. First, it is known that  $\epsilon$ -N-acetyl-L-lysine is a good dietary supplement for barnyard fowl and is used in some commercial diets (I08, I09). The fact that one group of young chicks showed no enzyme activity and developed it after several days in the laboratory could not be correlated with diet because of lack of specific information on their dietary history. However, in view of the fact that other batches of young chickens, and of chicken embryos, showed marked enzyme activity, the clear-cut pattern of developing enzyme activity in this one group can be more easily visualized as a result of diet than as anything else.

Second is the fact that small cage birds - which receive a totally different commercial diet - showed no activity. No correlation to body weight or kidney weight could be shown. There has not been any intensive investigation of the biochemical processes of such birds therefore differences in overall metabolic processes can neither be postulated nor dismissed. However, of the known factors, diet is again the most likely difference between these birds and those which are deliberately fed for their meat quality or egg-laying.

This speculation is also supported by the one sample of wild duck that was obtained. This bird was trapped wild and reared in the barnyard. The specific activity of  $\epsilon$ -lysine

acylase in its kidneys was considerably less than that of ducks which were hatched and reared in the barnyard. It is possible that the enzyme level was increasing, in response to dietary factors, at the time the bird was killed. In addition, there is the indication of a possible trace of enzyme activity in canary kidney after their diet had been supplemented with  $\epsilon$ -N-acetyl-L-lysine. Furthermore,  $\epsilon$ -lysine acylase in *Aspergillus* is an inducible enzyme (7).

The extended survey reported in the present work has also indicated that the subcellular distribution of the enzyme is distinctive. All animals tested to date show enzyme activity in the soluble fraction if at all. All the birds tested show enzyme activity in the mitochondria if at all. This difference in subcellular distribution has been shown with many other enzymes (II9). For example catechol oxidase is found in the chloroplasts of tea leaves but in the cytoplasm of spinach leaves (III). In many other examples enzymes are found in two fractions of the same cell, and show different pH optima and other physical differences, eg. L-glutamate-oxaloacetate transaminase from rat liver (II2), malic dehydrogenase from rat liver (II3). The examples available are sufficient to warrant the major part of one chapter in a recent text book (II4). At the present stage of knowledge of  $\epsilon$ -lysine acylase no functional purpose can be related to this difference in distribution.

## Experimental Results

### B) Attempts to purify $\epsilon$ -lysine Acylase from Chicken Mitochondria.

#### Introduction:

Previous attempts to isolate this enzyme from mitochondria had resulted in some purification, but low yield (6). A variety of methods were therefore tested in an attempt to obtain purification of the enzyme with significant yield.

Throughout this part of the work, the results obtained with any procedure were not fully reproducible. Some reasons for the variations were found, and are discussed at the end of this section. However, not all causes of variation were discovered, and eventually the attempts to purify the enzyme were abandoned. For this reason, the results reported in this section must be regarded as tentative. Those which are presented here are those which appeared to offer reasonably clear facts when all experiments were averaged, and might, therefore, be of future use after the variable factors have been eliminated.

#### Methods

The methods of preparation and storage of chicken kidneys has been described on p 29 and 50. The methods of preparation of kidney homogenate and the isolation of mitochondria are described on p 51. The assay procedure used is described on pp 29-31. In the present section various modifications of these procedures are introduced in individual experiments. In such cases, the changes are described under the appropriate experiments.

## Results

### I) Attempt to solubilize $\epsilon$ -lysine acylase by preparation of acetone powder.

Chicken mitochondria were prepared as usual. The packed crude mitochondrial pellet was suspended in an equal volume of ice-cold distilled water. To this suspension was added 10 volumes of cold acetone (chilled overnight to  $-10^{\circ}\text{C}$ ). The slurry was filtered in a Buchner with suction. The precipitate was resuspended in a further 10 volumes of cold acetone and filtered until dry but not allowed to warm to room temperature. It was then allowed to air-dry in the cold ( $+4^{\circ}\text{C}$ ) for 4 hours. The dried powder was stored in the freezer in a dessicator.

Two such preparations were made. In both cases the untreated mitochondria and the freshly prepared acetone powder were tested. The original mitochondrial preparations had a specific activity of 0.108 and 0.097. The specific activity of the acetone powders was 0.002 and 0.000 respectively. It was concluded that the preparation of acetone powder was not advisable for this enzyme.

### II) Attempt to Solubilize $\epsilon$ -Lysine Acylase with Triton X-100\*.

Mitochondria were prepared and suspended in water to give a volume 2x the original kidney weight. The suspension was

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\* Obtained from Hartman-Leddon Co., Philadelphia, Pa.

divided into five parts and treated as follows:

- 1) No treatment (hold in ice bath).
- 2) Incubate at 37° C for ten minutes.
- 3) " " " " " thirty minutes.
- 4) As 2) but with Triton X-100 added to give a final concentration of 0.1%.
- 5) As 3) but with Triton X-100 at 0.1%.

The ten minute samples were held in ice until the others were finished, then all samples were centrifuged in a refrigerated Spinco at 105,000 x g for one hour. After centrifugation the supernatants were decanted and used as such. The precipitates were each resuspended in 5 ml distilled water with gentle hand grinding. The results are presented in Table XI.

As shown in this table, Triton X-100 did not cause any increased solubilization of the enzyme when compared to simple dilution with water.

III) Attempts to Solubilize  $\epsilon$ -Lysine Acylase by freezing and thawing mitochondria and grinding with sand.

Mitochondria were prepared as described on p 51 and resuspended in distilled water. Different dilutions were used ranging from 5x to 20x the volume of mitochondria. These preparations were frozen and thawed repeatedly up to ten times, either by a "slow" or "rapid" process. The "slow" process involved freezing at -4° C (which required up to 17 hours) with thawing at room temperature (which required

TABLE XI

Effect of Triton-X on Chicken Kidney Mitochondria

Sample	Protein mgm/ml.	$\mu\text{CO}_2$ determd.	Sp. Act.	Total Act.	o/o Act. in SN.
Control SN	4.4	24.3	0.302	479	58
ppt	8.4	39.4	0.250		
10 mins 37°					
SN	4.2	12.8	0.16	349	43
ppt	8.5	39.4	0.25		
10 mins 37°					
+ Triton-SN	4.7	12.3	0.14	340	43
ppt	7.8	37.9	0.27		
30 mins 37°					
SN	4.2	8.5	0.108	217	44
ppt	9.7	23.5	0.13		
30 mins 37°					
+ Triton-SN	4.7	15.3	0.16	351	58
ppt	8.2	40.6	0.27		

1-2 hours). The "rapid" process consisted of shell-freezing in dry ice and acetone and thawing at 37° C in a waterbath with gentle swirling (both of which required only a few minutes). At no time was the mitochondrial suspension allowed to become warm. Frequent gentle mixing was employed during the thawing periods to prevent any part of the suspension becoming warm. As soon as the suspension was thawed it was placed in an ice bath.

The treated preparations were then centrifuged at 105,000 x g in a refrigerated Spinco centrifuge.  $\epsilon$ -lysine acylase activity was determined as described on pp21-31 using 0.1M pH 9.0 glycine/NaOH to dissolve the substrate and a 2 hour incubation period. The results of a typical experiment are given below.

In this particular experiment the mitochondrial suspension was divided into three lots which were diluted 5, 10, and 20x respectively, with distilled water. All three were frozen and thawed four times by the slow procedure:

Sample	$\mu$ lCC <sub>2</sub> /ml	Protein (mgm/ml)	Volume	Spec. Act.	Total Act.	Rela- tive Act.
Mitoch.	60	59.0	6.0	0.03	10.6	2.2 <sub>p</sub>
5x ppt.	79.0	17.9	5.0	0.19	17.0 )	3.7 <sub>p</sub>
Sn.	6.6	1.0	17.0	0.23	3.9 )	
10x ppt.	61.0	16.3	5.0	0.12	10.9 )	2.2 <sub>p</sub>
Sn.	0.0	1.7	23.5	0.0	0.0 )	
20x ppt.	99.5	39.0	8.0	0.09	28.1 )	3.9 <sub>p</sub>
Sn.	10.4	2.7	48.1	0.146	18.9 )	

These activities are expressed in  $\mu\text{moles lysine/mgm protein/hour}$ . The "relative activity" is a calculated value showing the amount of activity per ml that would have been obtained if the whole sample of mitochondria had been treated in any one of the ways used in this experiment.

This table shows several features typical of these experiments:

- a) a batch of mitochondria which had a low activity - for which no explanation was discovered.
- b) the enzyme activity released into the supernatant was not sufficient to yield significant  $\text{CO}_2$  evolution for the determination of meaningful specific activity values.
- c) although some shift of activity to the supernatant can be seen in one sample its meaning is questionable in view of a) and b) and it could not be duplicated in further experiments.
- d) the "relative activity" figures show that no consistent loss of activity occurred due to either the dilution or the freezing and thawing.

Similar experiments were carried out in which the mitochondrial suspension was ground in a mortar with acid-washed sand. The dilutions of mitochondria employed, the grinding time, and the number of elutions of the sand residue, were all varied. As with freezing and thawing, the results indicated that there was no consistent loss of enzyme activity but neither was there any reproducible, significant release of activity into the supernatant.

IV) Storage of mitochondria.

Obviously, in order to have convenient amounts of mitochondria on hand for various experiments, it was preferable to prepare them ahead of time. The storage of frozen mitochondria was therefore investigated. Short term storage of mitochondria at other temperatures was also examined.

Mitochondria were prepared as usual. In order to have comparable samples, the mitochondrial pellet was first resuspended in an equal volume of distilled water and then divided into tubes for storage. One tube was thawed and tested at each time interval. Thawing was by gentle swirling in a waterbath at 37° C. The mitochondrial suspension was not allowed to become warm.

The results presented below represent several experiments. All figures on the same horizontal line are part of the same experiment. The figures shown are specific activity ( $\mu$ moles lysine produced/hour/mgm protein).

Storage at 1/2 dilution at -4° C.

<u>Fresh</u>	<u>4 days</u>	<u>15 days</u>	<u>23 days</u>	<u>30 days</u>	<u>47 days</u>	<u>61 days</u>
0.140	-	0.169	0.17	-	0.108	0.14
0.188	0.188	-	-	-	-	-
0.124	-	-	-	0.150	-	-

From these figures it is apparent that mitochondria can be stored up to two months without damage. Normally, however, they were not stored more than one week.

Storage under other conditions.

<u>Dilution</u>	<u>Temp.</u>	<u>Fresh</u>	<u>20mins.</u>	<u>40mins.</u>	<u>3hrs.</u>	<u>24hrs.</u>
2x orig. wt. of kidney	30°C	0.227	0.270	0.230	-	-
	0° C*	0.180	-	-	0.180	0.201

\* in an ice bath.

It can be seen that short periods of time at room temperature do not destroy the activity of this enzyme in mitochondria. Neither do longer periods near freezing point. A few attempts to store mitochondria frozen when diluted to 2x the original kidney weight yielded very poor results. No controlled experiment was made under these conditions but it was concluded that frozen storage should only be used for concentrated mitochondrial suspensions. For this reason, the routine procedure adopted was to prepare mitochondria (as described on p 51), decant the supernatant, and freeze the concentrated pellet directly in the centrifuge tube. When required for use the pellet was thawed for a few minutes in a 37° waterbath and resuspended in distilled water.

V) Attempts to Solubilize  $\epsilon$ -lysine Acylase by Rupturing the Mitochondria with Ultrasonic Vibrations.

The machine employed was the Biosonic, 20 Kilocycle model, produced by Bronwill Scientific Co. It consisted of a metal chamber in which material could be placed for treatment. Through the center of the chamber ran a cooling rod containing circulating fluid.

Preliminary experiments, using water in the chamber, indicated certain features of the machine itself:

- a) tuning the machine by ear (which can be done with some instruments of this type) was not satisfactory. The sharpest tone did not always produce the maximum cavitation.
- b) the best results were obtained if the instrument was adjusted to maximum visible cavitation (a faint mist seen above water) with a known volume of water. If the tuning was left at this setting and samples were prepared to this volume, fairly consistent results could be obtained with mitochondrial preparations.
- c) the chamber required thorough chilling for at least one hour before use. If this was not carried out the temperature within the chamber rose to approximately 25° C regardless of the temperature of the circulating fluid.

Initially, water, chilled in a bath of dry ice and acetone, was employed as the circulating cooling fluid. This was very unsatisfactory because it required constant supervision. Furthermore, the temperature fluctuated and at temperatures near zero the water was very liable to freeze and block the system. Eventually, ethylene glycol was employed as the circulating fluid and cooled by an electric refrigerator motor.

Using the conditions described above, the Biosonic was then tested for the rupture of mitochondria. Mitochondria were prepared as before and diluted with distilled water to a suitable volume. A small sample was held in ice as a control

and the remainder was placed in the Biosonic chamber. Ultrasonic treatment was carried out for various periods of time.

The samples were then centrifuged and the enzyme activity determined in the whole mitochondria, the supernatant and the precipitate. Initially, centrifugation was carried out in a Spinco at 105,000 x g for one hour, but little enzyme activity was found in the supernatant unless ultrasonic treatment was very prolonged (30-40 mins). Under these conditions there was a great loss of activity. For this reason, the routine centrifugation employed was 20 mins at 15,000 rpm (27,000 x g) in a No. 55 34 head, in a refrigerated RC 2 Servall centrifuge.

Several problems were encountered in the design of these experiments. If the total volume was placed in the Biosonic chamber initially, and samples removed at intervals, the volume in the chamber and, consequently, the efficiency of cavitation, varied. If the volume removed was replaced by water or other solutions, the concentration of protein varied and this was also found to affect the efficiency of the machine. If, on the other hand, the suspension of mitochondria was divided into separate lots and sonicated individually, it was necessary to allow the machine to cool for prolonged periods between runs. The last few samples of mitochondria were therefore held in the cold for prolonged periods. Since they were in dilute sucrose, it was uncertain whether enzyme had been released into the supernatant by ultrasonic treatment or simply by standing in the cold in a hypotonic solution.

After the introduction of the better cooling system, separate lots of mitochondria could be run without too great delay. At this time the experiments on ultrasonic treatment were repeated and confirmed those obtained initially. However, the bulk of the experiments on ultrasonic treatment were run with the poor cooling system by the procedure described below.

After examining the various possible ways of carrying out experiments on the effect of ultrasonic treatment, it was found that the best results were obtained if a large volume of mitochondria were used and the machine tuned for slightly less than this volume. Small samples were removed at the required time intervals and the major portion of the sample remained until the maximum time. For example, if 50 ml of mitochondrial suspension was used, the machine was tuned for 40 ml. Two 10 ml samples could be removed at time intervals leaving 30 ml for the final sample.

In Table XII the combined results of seven experiments are presented. Although the five-minute samples shown here had considerable activity, in many runs they were totally inactive - very little mitochondrial rupture having occurred. Five minutes was, therefore, not considered suitable. Similarly, 20 minutes was not considered suitable because the machine frequently overheated during this prolonged period and destroyed enzyme activity. One such sample, with a specific activity of 0.030 and a recovery of only 19%, is shown here.

The degree of variation encountered can be seen from this

TABLE XII

Effect of Ultrasonic Treatment on  $\epsilon$ -lysine  
Acylase of Chicken Kidney Mitochondria.

Time in Biosonic	Sp. Act. SN	Total Act. as % of Mitoch.
5 mins	0.335	67
	0.129	55
10 mins	0.097	54
	0.129	145
	0.113	84
	0.132	55
	0.138	69
	0.156	91
15 mins	0.151	74
	0.097	40
	0.140	56
	0.291	81
	0.216	71
20 mins	0.210	72
	0.200	70
	0.140	78
	0.167	89
	0.032	19
	0.259	105
	0.163	8
	0.129	50

table. From this data fifteen minutes sonication was selected for routine use since it appeared to offer the best compromise between specific activity and yield.

The effect of temperature during sonication

The experiments to determine the optimum time for sonication of mitochondria were all run with the circulating fluid at approximately zero degrees. In the next series of experiments sonication was carried out for various periods of time with the cooling fluid set at different temperatures. The results are presented in Table XIII. From Table XIII it can be seen that low temperatures were unsatisfactory. Since, at these temperatures, protein material was frequently found frozen to the cooling coil which runs through the chamber, the poor results may have been due to loss of enzyme protein rather than actual destruction. In future experiments the temperature of the circulating cooling fluid was maintained at approximately +8° C.

This experiment also illustrates certain other aspects of the use of the Biosonic. As can be seen from the large total activity in each case, compared to the previous table, these samples were run individually after the introduction of the better cooling system. They show that maximum release of enzyme activity into the supernatant with minimum destruction occurs with a sonication time between 10 and 20 minutes (see SM, +10° and +50°).

Summary of conditions used for the preparation of sonicated mitochondria in further experiments.

The tuning of the Biosonic was adjusted for a fixed volume of water. The Biosonic was precooled for one-two hours until the chamber was thoroughly chilled. The ultrasonic generator was also run (while the chamber contained water) for about fifteen minutes before the first use each day.

Mitochondria were suspended in distilled water to give the volume required for the ultrasonic chamber tuning. The amount of mitochondria used was selected so that the final volume of suspension was approximately 2x the original kidney weight. After the installation of the better cooling system large volumes of mitochondria were obtained by dividing a large volume of mitochondrial suspension into suitable volumes, running each volume separately in the Biosonic, and pooling the supernatants obtained.

Mitochondria were ice cold when placed in the chamber and the circulating fluid was maintained at approximately +8° C during sonication. Sonication was carried out for fifteen minutes.

The treated mitochondria were removed from the chamber and centrifuged at 27,000 x g in a No. 55 34 head in a refrigerated Servall for 20 mins. The supernatant was decanted and used for experiments. This preparation will be referred to throughout the thesis as "USSN".

TABLE XIII

Effect of Temperature During Ultrasonic Treatment

Sample	T <sup>o</sup> fluid	Time of Sonicn.	Sp. Act.SN	Total Act.SN	Act. SN as % of Mitoch.
Mit	-	-	0.227	37.9	-
SN	+ 10 <sup>o</sup>	10 min	0.270	29.6	78
		20 min	0.230	24.1	63
		40 min	0.102	11.3	29
SN	+ 5 <sup>o</sup>	10 min	0.097	8.2	21
		20 min	0.170	17.9	47
		40 min	0.097	10.3	21
SN	0 to -3 <sup>o</sup>	10 min	0.108	10.5	27
		20 min	0.072	6.7	17
		40 min	0.086	10.9	28

VI) Attempts at further Purification of  $\epsilon$ -Lysine Acylase  
from USSN.

Having obtained a crude enzyme preparation free from whole mitochondria several procedures were used in an attempt to fractionate the active protein selectively.

i) Ammonium sulfate treatment,

A sample of frozen mitochondria was thawed and diluted to 2x the original kidney weight with distilled water. Five ml was used as a control to determine the specific activity of the untreated mitochondria. The remainder was sonicated for fifteen minutes and centrifuged at 27,000 x g for twenty minutes. Five ml of the supernatant was used as a control sample of USSN. To the remainder, solid ammonium sulfate was added slowly, with constant stirring, to give the required concentration. (70.5 gms% was taken as saturation). The mixture was kept in ice throughout the procedure. Addition of ammonium sulfate took approximately twenty minutes for 50% saturation and approximately ten minutes for 10% saturation. The mixtures were allowed to stand in ice for ten minutes after all the ammonium sulfate was added. The preparation was then centrifuged for 20 mins at 27,000 x g in a refrigerated Servall.

Two layers formed - a clear pink supernatant and a pinkish-white precipitate - with 50% saturation. With smaller concentrations the increasing amount of protein remaining in the supernatant was clearly visible. The precipitate was resuspended in distilled water. The volume required to

resuspended all the precipitates varied. Gentle hand grinding was also necessary at this step.

The two fractions were dialyzed against running tap water until the effluent was negative for sulfate. This required from 2.5 to 5 hours depending on the flow rate and was carried out in the cold. The dialysis sacs were then transferred to a dehydrating solution also in the cold. Two methods were used to reduce the volume - either standing overnight in 0.88M sucrose or standing for two hours in moist "carbomax" Peg 20M, (obtained from Union Carbide, Canada, Ltd. (II5)). Enzyme activity and protein concentration were then determined on the original mitochondria, the untreated US<sub>SN</sub>, and the two ammonium sulfate fractions.

A total of twelve experiments, each employing several concentrations of ammonium sulfate, were run in this manner. They showed great variation in the percentage of US<sub>SN</sub> activity recovered after ammonium sulfate treatment. However, in three experiments all the US<sub>SN</sub> activity was recovered therefore it would appear that ammonium sulfate treatment per se was not harmful. The variable causing poor recovery in the remaining experiments was not discovered. Presumably it was not pH since the same variation occurred whether ammonium sulfate was added to US<sub>SN</sub> at its original pH of 5.7 or to US<sub>SN</sub> which had been adjusted to pH 9.0.

One consistent fact did emerge from these experiments and that was that the bulk of the enzyme activity and the

bulk of the protein were always found in the same fraction. This is illustrated in Table XIV where one experiment is given in detail together with the average results obtained. From these results it appeared that no useful purification would be obtained by ammonium sulfate precipitation and this approach was abandoned.

ii) Precipitation at acid pH.

A large batch of US<sub>5N</sub> was divided into separate beakers - all kept in an ice bath. The US<sub>5N</sub> in each beaker was adjusted to a different pH by the addition of 0.1N HCl or 0.1N NaOH. The acid or alkali were added slowly dropwise from a long-tipped 1 ml pipette with continuous stirring. Each sample was then centrifuged fifteen minutes at maximum speed (approximately 2,500 rpm) in a clinical centrifuge in the cold room. A sample of the US<sub>5N</sub> was held in ice without treatment. Its pH was 6.7. Only those samples adjusted to less than pH 6.0 formed precipitates.

Where a precipitate formed the supernatant was decanted and the precipitate resuspended in distilled water to give a total volume of five ml. Small amounts of the samples at low pH were mixed with an equal volume of pH 9.0 0.1M glycine/NaOH buffer and the necessary pH adjustment to bring the incubation mixture to pH 9.0 calculated.  $\epsilon$ -lysine acylase activity was then determined on each sample in the usual manner but including the pH adjustment as required. The results are shown in Table XV.

TABLE XIV

Effect of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> Addition to USSN

°/o Saturn		Vcl	ulCO <sub>2</sub>	Protein	Sp.Act	Total/	°/o Protein	°/o Act.
		ml		(mgm/ml)		Act.	in SN	in SN
10	SN	10.5	21.1	11.0	0.190	11.55	80	100
	ppt	4.0	0.7*	2.9	0	0		
20	SN	10.0	10.8	6.2	0.054	3.97	50	50
	ppt	14.5	7.7	6.1	0.046	4.07		
30	SN	12.1	3.6*	16.1	0.007*	1.36	37	60*
	ppt	1.6	37.5	34.3	0.036	1.97		
50	SN	54.6	0	0.88	0	0	4	0
	ppt	11.7	39.2	21.0	0.070	17.2		

Average of duplicate experiments

°/o Saturation	°/o Protein in SN	°/o Activity in SN
10	85	96
20	45	50
30	37	40
50	5	0

\* Not significant

# Total activity = umoles lysine produced if entire sample used.

TABLE XV

Effect of pH on US<sub>SN</sub>

pH		Sp. Act	Protein (mgm/ml)	Volume ml	Total Act.	Recovery *	
						Activ./ml.	%
4.5	SN	0.05	4.0	7.2	1.4	0.66	21
	ppt	0.173	7.8	5.0	6.7		
5.0	SN	0	3.0	7.1	0	0.73	24
	ppt	0.130	13.7	5.0	8.9		
5.6	SN	0	4.0	9.4	0	1.04	34
	ppt	0.400	7.5	5.0	15.0		
6.0	SN	0.070	11.9	8.0	6.5	0.81	26
	ppt	-	-	-	-		
6.7**	SN	0.24	12.9	5.0	15.4	3.08	100
	ppt	-	-	-	-		
8.2	SN	0.119	11.2	7.0	9.33	1.33	43
	ppt	-	-	-	-		
9.0	SN	0.160	10.4	8.0	13.31	1.66	54
	ppt	-	-	-	-		

\*  $\frac{\text{Total Activity SN} + \text{Total Activity ppt}}{\text{Total Volume (SN + ppt)}} = \text{Activity/ml.}$

\*\* Untreated US<sub>SN</sub>

From this Table it can be seen that there was a selective precipitation of active protein at pH 5.6, as shown by the increase in specific activity. There was also considerable loss of total enzyme activity in all samples especially at low pH. Despite this fact the use of acid precipitation was investigated further because these results were entirely reproducible. During the course of further experiments it was found that the range of pH 5.5-5.7 gave reproducible results, and that 0.1N HCl and 0.1N HAc were equally satisfactory. It was also demonstrated that the precipitate obtained with either acid could be stored frozen for a few days, as shown below.

Storage of pH 5.5 precipitate.

A batch of  $U_{5N}$  was divided into two and each half adjusted to pH 5.5 in the cold. For one half 0.1N HCl was used and for the other 0.1N HAc. The precipitates were collected and resuspended with just enough distilled water to make a slurry. Each was divided into three tubes. The enzyme activity of one tube of HCl precipitate and one tube of HAc precipitate was determined immediately. The remaining tubes were stored at  $-4^{\circ}$  C. One tube of each was thawed and tested at three and five days respectively. The results are presented below:

<u>No. days storage</u>	<u>Acid used</u>	<u>Sp. Act.</u>	<u>Prot./ml</u>
Fresh	HCl	0.33	2.7
	HAc	0.18	3.8
3 days	HCl	0.33	2.4
	HAc	0.157	3.3
5 days	HCl	0.23	2.1
	HAc	0.23	2.9

From these figures it can be seen that although acetic acid gave a poorer preparation than HCl, both could be stored frozen for a few days.

iii) Attempts to extract  $\epsilon$ -lysine acylase from pH 5.5 precipitate.

The first attempt to extract enzyme activity from the pH 5.5 precipitate was carried out as follows:

The precipitate was suspended in 4 volumes of 0.06M phosphate buffer at pH 6.0 and stirred gently for twenty minutes in an ice bath. It was then centrifuged at maximum speed in a clinical centrifuge in the cold for ten minutes. The supernatant was decanted and the precipitate resuspended in 4 volumes of 0.06M phosphate buffer pH 8.0 and treated in the same way. After centrifugation, the final precipitate was dissolved in pH 9.0 glycine/NaOH buffer (0.1M). Each fraction was tested for enzyme activity in the usual manner. Since it was found that there was considerable activity extracted at pH 8.0, but even more at pH 9.0, the pH 8.0 extract was reprecipitated at pH 5.5 and redissolved at pH 9.0 in the same manner as before.

From the result of this experiment (see Table XVI), it appeared that a pH between 6.0 and 8.0 might extract inactive material leaving the enzyme behind. Accordingly this procedure was tried next. A fresh sample of pH 5.5 precipitate was extracted with 0.06M phosphate buffer at pH 7.2, allowed to stand and centrifuged in a clinical centrifuge as before. The resulting precipitate was then dissolved in pH 9.0 glycine/NaOH buffer. In different runs of this experiment,

TABLE XVI

Extraction of  $\epsilon$ -lysine Acylase from pH 5.5 precipitate

Experiment I)

pH used to extract	Sp. Act.	Protein (mgm/ml)	Volume ml	Total Activ.
6.0	0	4.8	23.5	0
A) 8.0	0.163	5.9	21.5	23.2
9.0 (residue)	0.44	1.2	4.0	2.1

<u>Reprecipitation of A) at pH 5.5 + extraction at pH 9.0</u>				
pH	Sp. Act.	Protein (mgm/ml)	Volume ml	Total Activ.
5.5	0	2.9	5.0	0
9.0	0	0.26	9.1	0

The original pH 5.5 ppt in this experiment had a specific activity of 0.162, Total Activity 20.4.

% Recovery in this experiment = 124 %

TABLE XVI (continued)

Experiment 2a)

pH used to extract	Sp. Act.	Protein (mgm/ml)	Volume ml	Total Activ.
7.2	0.027	4.0	11.5	1.20
9.0 sol	0.34	9.6	4.9	16.0
residue	0.40	6.1	4.5	10.9

Original pH 5.5 precipitate Sp. Act. = 0.318 Total Activity = 34.6  
 o/o Recovery = 61 %

Experiment 2b)

pH used to extract	Extraction Time	Sp. Act.	Protein (mgm/ml)	Volume ml	Total Activ.
7.2	20 mins	0	1.4	39.2	0
9.0 sol	20 mins	0.238	2.3	6.6	3.5
residue		0.130	8.3	6.6	7.3
9.0 sol	45 mins	0.146	3.4	6.6	3.3
residue		0.113	7.9	6.6	5.9
9.0 sol	120 mins	0.40	3.5	6.6	9.2
residue		0.124	7.8	6.6	6.7

Original pH 5.5 ppt Sp. Act. = 0.118 Total Activ. = 25.4  
 o/o Recovery = 141 %

TABLE XVI (continued)

Experiment 3)

pH used to extract		Centrifug'n	Sp. Act.	Protein (mgm/ml)	Volume ml	Total Activity
9.0	sol	10 mins	0.167	3.9	44.0	28.7
	res.	Clinical	0.102	2.3	4.4	1.0
9.0	sol	27,000 x g	0.054	4.8	40.0	10.3
	res.	/ 20 mins	0.054	3.6	5.5	1.1

Original pH 5.5 ppt Sp. Act. = 0.194 Total Activ. = 43.9

% Recovery = 96 %

the speed of centrifugation of the pH 9.0 extract and the length of time it was allowed to stand in ice (with occasional stirring) before centrifugation was varied. The results are shown as experiments 2a and 2b in Table XVI.

A third type of experiment was also employed. In this the pH 5.5 precipitate was extracted directly with pH 9.0 buffer for approximately 20 minutes. The extract was then divided and each half centrifuged at different speeds; one in a clinical centrifuge at maximum speed, the other in a refrigerated Servall at 27,000 x g for twenty minutes. These results are also shown in Table XVI.

Certain conclusions can be drawn from a comparison of these results:

- a) Extraction at pH 7.2 removes appreciable amounts of protein leaving the active enzyme in the precipitate. At pH 8.0 some of the enzyme also dissolves.
- b) Subsequent treatment of the pH 7.2 insoluble material at pH 9.0 dissolves some of the enzyme, but appreciable amounts of enzyme remain in the precipitate unless it is either given a preliminary treatment at pH 8.0 or extracted for long periods of time.
- c) As can be seen from the various experiments in Table XVI, unless the original pH 5.5 precipitate had a high specific activity initially the activity recovered by any treatment was quite low. The theoretical recovery of 141% in Experiment 2b) is probably due partly to the low apparent activity of the original precipitate. The further dilution caused by repeated extraction would only exaggerate this problem, and, as can be

seen from Experiment I), attempts to concentrate the material by reprecipitation at pH 5.5 destroyed all activity.

Since precipitation at pH 5.5 entailed some loss of enzyme activity in itself, these attempts to extract the active enzyme did not yield a sufficient increase in specific activity to warrant further investigation at present.

iv) Attempts to purify  $\epsilon$ -lysine acylase by selective adsorption on Calcium Phosphate gel.

In earlier attempts to purify this enzyme, calcium phosphate gel had been used to remove some of the inactive protein (8).

In the first experiment, a sample of frozen stored pH 5.5 precipitate was thawed and resuspended in an equal volume of distilled water with gentle hand-grinding. To 4.1 ml of this suspension was added 1.0 ml of calcium phosphate gel (17.5 mg/ml). The pH of the mixture was 5.9 and was adjusted to pH 7.0 with 0.1N NaOH. The mixture was allowed to stand in an ice bath for 30 mins with occasional gentle mixing. It was then centrifuged very lightly (5 mins at half-maximum speed in a clinical centrifuge) in the cold. The supernatant was pipetted off and the precipitate resuspended in five volumes of pH 9.0, 0.05M glycine/NaOH buffer (final pH 8.5-8.7), and treated in the same way as the gel mixture. This extraction procedure was repeated twice more. The second extraction was carried out for 15 mins, and the third for 2 hours. Enzyme activity was determined in the original precipitate and in each supernatant.

The amount of CO<sub>2</sub> generated during the determination of enzyme activity was too low to provide reliable values, but there were indications that enzyme was selectively extracted in the second and third extractions with buffer. There was, however, great loss in total enzyme activity.

The experiment was, therefore, repeated using ten minutes in an ice bath both to add the gel to the resuspended precipitate (at pH 7.3), and for each extraction with buffer. The results of two such experiments are presented in Table XVII. In this table the results from two experiments are tabulated in order. It can be seen that only in the case of the second extraction, in the first experiment, was sufficient CO<sub>2</sub> evolved during enzyme determination to have any meaning. Furthermore, the duplicate experiment did not yield any activity in this fraction.

From these results it was concluded that, as with extraction of the pH 5.5 precipitate at different pH values, the procedure was useless when the original material had a low activity per se.

v) Attempt to combine pH 5.5 precipitation with ammonium sulfate precipitation.

This experiment was run in duplicate on two separate batches of pH 5.5 precipitates. In each case the precipitate was resuspended in three volumes of pH 9.0, 0.05M glycine/NaOH buffer. This gave a volume roughly equal to the original volume of the US<sub>5N</sub> and provided a suspension which did not settle out on standing in an ice bath. Solid ammonium sulfate was added slowly, in the cold, with continuous stirring, to

TABLE XVII

Treatment of pH 5.5 Precipitate with Calcium Phosphate Gel

Sample	$\mu\text{lCO}_2/\text{ml}$	Protein/ml	Volume (ml)	Sp.Act.	Total Activity as % of original ppt.	
5.5 ppt.	a) 9.2	3.3	16.8	0.159	8.6 = 100%	
	b) 12.5	2.9	5.6	0.230	3.7 = 100%	
gel SN.	a) 0	0.4	4.0	0	0	0
	b) -	-	-	-	-	-
1st extr.	a) 0	1.7	6.1	0	0	0
	b) 4.3	1.1	11.0	0.216	2.6	73
2nd extr.	a) 16.4	1.4	6.8	0.65	6.2	70
	b) 0	1.4	8.6	0	0	0
3rd extr.	a) 4.4	1.9	7.1	0.130	1.7	20
	b) 2.2	0.7	6.3	0	0	0
4th extr.	a) 2.3	0.8	6.0	0.130	0.6	7
	b) 5.8	0.6	12.3	0.53	3.9	104

a), b) = duplicate experiments.

give 20% saturation. The addition took 15 mins. The mixture was then centrifuged for ten minutes at  $9,750 \times g$  in a refrigerated Servall. The supernatant was decanted and the precipitate resuspended in a small volume of distilled water. Both were dialyzed against running tap water until the effluent tested negative for sulfate. This required about two hours. The volume of the dialysate in the first experiment was then reduced by suspending the dialyzing sac in 0.68M sucrose for two hours at  $+4^{\circ} C$ . In the second experiment reduction of volume was carried out by packing moistened "Carbowax" (obtained from Union Carbide, Canada, Ltd., (IIS)) around the sacs and leaving them in the cold for twenty minutes. The enzyme activity of all fractions was then determined. Although there was sufficient activity in the supernatant after ammonium sulfate fractionation to produce significant  $CO_2$  evolution, there was not sufficient increase in specific activity to justify the loss in total enzyme incurred in all the steps from US<sub>5K</sub>.

The effect of anaerobic conditions on  $\epsilon$ -lysine acylase activity.

Two single experiments were run to determine the effect of aeration on  $\epsilon$ -lysine acylase activity. The preparation of mitochondria and US<sub>5N</sub> were carried out as usual. Two ml samples of tissue were incubated with an equal volume of substrate in 0.1M glycine/NaOH buffer, pH 9.0. Before incubation three flasks containing mitochondria and four flasks containing US<sub>5N</sub> (together with their respective controls) were gassed

with nitrogen for two minutes and then sealed with gas-tight white rubber stoppers (IIS). Incubation was stopped after various time intervals (as shown in Table XVIII) and enzyme activity determined in the usual manner.

It can be seen from Table XVIII that anaerobic conditions had no major effect on the activity of this enzyme. It is possible that nitrogen was to be preferred for the longer incubation times but for the two hour period usually employed the difference was not sufficient to warrant further investigation.

#### Effect of lyophilization.

US<sub>5N</sub> was prepared as usual. Part was tested immediately, part frozen overnight, and part lyophilized. The lyophilized sample was reconstituted next day, using distilled water, to give the same protein concentration as the frozen sample. Considerable hand grinding was necessary to dissolve the lyophilized sample and it was found that the protein which actually dissolved (and, was therefore, used in the determination of the specific activity of the sample) was only half as much as that in the frozen sample. One part of the redissolved lyophilized sample was centrifuged in a Spinco at 105,000 x g and the supernatant collected.

The enzyme activity of all these samples was determined in the usual way, using a 2 hour incubation period. (It should be noted that these experiments were carried out after the discovery of the problems of buffer strength and pH discussed

TABLE XVIII

Effect of Aeration on  $\epsilon$ -Lysine Acylase Activity

Sample	Gas.	Incubation Time	$\mu\text{CO}_2$	Sp. Act.
A) Mitochon.	$\text{N}_2$	1 hour	13.0	0.253
		2 hours	26.6	0.308
		3 hours	45.8	0.313
	Air	1 hour	13.6	0.238
		2 hours	21.7	0.201
B) US <sub>SN</sub>	$\text{N}_2$	1 hour	10.2	0.081
		2 hours	26.5	0.146
		3 hours	30.1	0.124
		4 hours	54.5	0.175
	Air	1 hour	23.9	0.188
		2 hours	20.8	0.108
		3 hours	28.4	0.115
		4 hours	26.4	0.081

Sp. Act. =  $\mu\text{moles lysine produced/hr./mgm protein.}$

A) B) Two individual experiments  $\therefore$  protein and volume same for all A) samples, and for all B) samples. Figures can therefore be compared directly on the basis of  $\mu\text{CO}_2 + \text{sp. act.}$  within each experiment.

on p95. They were, therefore, carried out using substrate dissolved in 0.2M glycine/NaOH buffer at exactly pH 9.0).

The results are presented below:

<u>Sample</u>	<u>Protein (mg/ml)</u>	<u>µl CO<sub>2</sub> /ml</u>	<u>Volume</u>	<u>Sp. Act.</u>	<u>Total Act.</u>
US <sub>5</sub> N fresh	17.7	56.7	33 ml	0.182	106.3
frozen	26.6	109.2	33 ml	0.230	201.9
lyoph.	13.8	47.6	33 ml	0.190	86.5
Spinco SN	2.3	0	20.8 ml	0	0

It can be seen that lyophilization does not destroy any major portion of the activity present in the fresh sample. The higher total activity in the frozen sample is, obviously, due to the higher protein concentration of this sample, since the total activity is almost double the fresh sample but the activity/mg protein (Sp. Act.) is only 24% greater.

A further sample of lyophilized chicken US<sub>5</sub>N was reconstituted in 0.1M glycine/NaOH buffer, pH 9.0, and the substrate also dissolved in this buffer, to give the same final concentration of buffer as the first experiment, and centrifuged in the Spinco as above. The activity of the Spinco supernatant was again zero showing that lyophilization had not solubilized the enzyme. However, it is also apparent that lyophilization does not destroy this enzyme.

Other methods of extracting  $\epsilon$ -lysine acylase from the pH 5.5 precipitate with appreciable activity were also unsuccessful. It was therefore decided that a totally different

approach to the problem would be necessary. However, during the course of these unsuccessful experiments it was observed that the activity of the pH 5.5 fraction, and even of the  $US_{SN}$  could not be reproduced. Throughout this part of the work there had been considerable variation. This can be seen from the specific activities recorded for identical fractions in different experiments. When a greater variety of reagents were used (including deoxycholate, butanol, chymotrypsin, ethanol, and Cetab) the variation made the experiments totally useless. Enzyme activity varied from zero to excellent under what were apparently identical conditions. It was obvious that some part of the assay procedure was not standardized.

#### VII) Investigation of the source of variation.

##### i) Product inhibition.

In the experiments carried out so far, the  $\epsilon$ -N-acetyl-L-lysine substrate had contained a small, variable amount of free lysine. Since this was a variable factor, the first investigation undertaken was a study of the effect of lysine contamination of the substrate.

##### Preparation of pure $\epsilon$ -N-acetyl-L-lysine.

$\epsilon$ -N-acetyl-L-lysine was prepared as before. The remaining lysine was removed by the procedure of Klonisalo et al. (II7), as modified by S. Kim (personal communication).

The procedure used was as follows: Dowex-50  $H^+$ form ( $X_4$  200-400 mesh) was washed with distilled water two or three times

and the fine particles discarded.  $\epsilon$ -N-acetyl-L-lysine, containing small amounts of lysine, was prepared as described on p 28, and was dissolved in the minimum volume of distilled water. Resin was then added to the solution, in a beaker, with constant stirring, until the pH dropped to 1.5-2.0. It was allowed to stand five minutes, then filtered on a Buchner. The filtrate was discarded and the resin resuspended in a little distilled water containing dilute phosphate buffer (about 0.005M) and adjusted to pH 6.0-6.3 with 2N KOH. Care was taken not to overshoot the limit of pH 6.3 at any time. The resin was then poured into a 15 x 1 cm column and eluted with 0.005M phosphate buffer at pH 6.3 until the eluate tested ninhydrin negative. This procedure eluted  $\epsilon$ -N-acetyl-L-lysine while leaving free lysine on the column. At higher pH lysine was also eluted and at lower pH some of the acetyl lysine remained on the column.

The eluate was then condensed to a small volume in a flash evaporator at 37° C. Two or three volumes of ethanol were added and the product allowed to crystallize overnight at +4° C. Provided sufficient care was taken in adjusting the pH, the product contained virtually no free lysine when chromatographed.

Using this product (to which known amounts of free L-lysine were added as required) for substrate, the determination of enzyme activity was carried out as usual. The results obtained with whole mitochondria as the enzyme preparation

are shown below. It is apparent that with an active enzyme preparation there is no inhibition by substrate even in great amounts.

<u>Sample</u>	<u>Amt. lys. added</u>	<u><math>\mu\text{CO}_2</math></u>	<u>Amt. lys.* found</u>	<u>Amt. lys. due to enzy. actn.</u>
Control <sup>†</sup>	none	124.4	27 $\mu\text{m}$	27 $\mu\text{m}$
+8.3% lys.	12 $\mu\text{m}$	161.0	35 $\mu\text{m}$	23 $\mu\text{m}$
+16.7% lys.	24 $\mu\text{m}$	244.6	54 $\mu\text{m}$	30 $\mu\text{m}$
+33.3% lys.	48 $\mu\text{m}$	320.3	70 $\mu\text{m}$	22 $\mu\text{m}$
+50% lys.	72 $\mu\text{m}$	482.0	104 $\mu\text{m}$	32 $\mu\text{m}$
			Average	26.8 $\mu\text{m}$

\* obtained by multiplying  $\mu\text{CO}_2/\text{ml}$  by the total volume of 4.1 ml and dividing by the number of  $\mu\text{CO}_2/\mu\text{m}$  lysine derived earlier (18.5).

<sup>†</sup> 50 umoles  $\epsilon$ -N-acetyl-L-lysine/ml.

The high  $\text{CO}_2/\text{ml}$  values were obtained by using 0.5, 0.3, and 0.1 ml of reaction mixture in the Warburg flask, instead of 1.0 ml, and calculating back. Since the amount of lysine generated by the enzyme acting in the absence of added lysine is 27  $\mu\text{m}$ , and the average amount due to enzyme action, in the presence of up to 50% contamination with lysine, is 26.8  $\mu\text{m}$ , there can be no suggestion that the low, variable contamination of substrate with free lysine was responsible for the variable results.

Although these results did not show the cause of variation they did, indirectly, reveal one variant. When large amounts

of lysine were present in the substrate, it was noted that the pH of the reaction mixture was not what it was supposed to be. This led to a general investigation of buffer strength, as a result of which some of the variation was eliminated.

ii) Investigation of pH and buffer strength.

In earlier work on this enzyme it had been shown that 0.06M glycine/NaOH buffer was superior to 0.15M for the chicken kidney enzyme. Accordingly, the substrate used for the survey of enzyme occurrence had been dissolved in 0.1M buffer to give a final concentration of 0.05M when diluted with an equal volume of unbuffered tissue. Similarly, during attempts to purify this enzyme which involved buffered solutions, the enzyme had been dissolved in 0.05M buffer and the substrate in 0.05M buffer also giving a final concentration of 0.05M. Significantly, these latter experiments were the only attempts at purification which showed any degree of reproducibility. It seemed, therefore, that an investigation of buffer strength was warranted. Furthermore, an error was discovered in some of the more recent (and unsuccessful) experiments. Substrate in 0.05M buffer had continued to be used even when the tissue was not itself extracted with buffer. The final concentration of the buffer, during incubation of the reaction mixture for determination of enzyme activity, had therefore, been only 0.025M.

Investigation of all these buffer strengths revealed that when  $\epsilon$ -N-acetyl-L-lysine was dissolved in 0.025M or

0.05M buffer (previously adjusted to pH 9.0) the pH of the solution fell and continued to fall during the incubation period. In fact, the incubations had been carried out at pH values well below the optimum.

A complete survey of the effect of pH and buffer strength was carried out as follows: The appropriate tissue sample (mitochondria, USSN) was prepared in the usual way. Glycine/NaOH buffer was prepared at 0.05M, 0.1M, 0.2M.  $\epsilon$ -N-acetyl-L-lysine was dissolved in each buffer to give a final concentration of 50  $\mu$ moles/ml as usual. The solution of substrate was adjusted to the required pH with NaOH. (In some experiments the substrate was added to the tissue preparation and kept in an ice bath while the pH of this mixture was checked. However, it was found that the tissue did not noticeably affect the pH of the mixture, therefore this was discontinued). Incubation was carried out for two hours at 37° C. At the end of the incubation period the mixtures were poured into chilled beakers in an ice bath (to slow enzyme reaction) and the pH determined again as rapidly as possible. After this step HCl was added as usual to stop enzyme reaction, and the samples placed in a boiling water bath for three minutes. The specific activity was then determined as usual. Since the use of stronger buffers might affect the pH in the Warburg flasks, these were also checked individually and adjusted if necessary (see also p 98 ).

In the table below is shown a typical experiment in which three strengths of buffer were used, each at two pH values.

The enzyme preparation consisted of whole mitochondria diluted with distilled water to give a volume 2 x the original kidney weight. The protein concentration was 30 mgm/ml. Incubation was carried out for 2 hours.

<u>Buffer Strength</u> <u>of incubn. mixt.</u>	<u>pH at start</u> <u>of incubn.</u>	<u>pH at end</u> <u>of incubn.</u>	<u>Average</u> <u>pH</u>	<u>mlCO<sub>2</sub>/ml</u> <u>evolved</u>	<u>Sp. Act.</u>
0.025M	8.0	7.6	7.8	27.1	0.047
	9.2	8.8	9.0	48.9	0.091
0.05M	8.8	8.5	8.65	37.6	0.064
	9.3	9.0	9.15	55.7	0.100
0.1M	9.0	8.7	8.85	56.7	0.100
	9.4	9.0	9.2	49.7	0.091

The first pH given for each buffer was the unadjusted pH when  $\epsilon$ -N-acetyl-L-lysine was dissolved in it. The second pH given for each buffer was arrived at by addition of 0.05 ml 1.0N NaOH. It can be seen that the pH dropped approximately the same amount during incubation, regardless of the buffer strength. However, with the strongest buffer this was still within the optimum pH range for this enzyme. When the average pH during incubation, and the apparent specific activity, are listed in order, it is apparent that a pH range of 8.85 to 9.2 has little effect on enzyme activity:

Av. pH	7.8	8.65	8.85	9.0	9.15	9.2
Sp. Act.	0.047	0.064	0.100	0.091	0.100	0.091

The effect of buffer strength and pH on the specific activity of  $\epsilon$ -lysine acylase from chicken mitochondria and

US<sub>5N</sub> are shown in Figs. 9 and 10 respectively. Each line is the average of at least three experiments. The pH values shown on the figures are themselves averages of the pH encountered during incubation (ie. averages of the pH before and after the two hour incubation period).

In Fig. 9 it can be seen that 0.1M buffer is better than 0.05M but the optimum pH is sharper. It can also be seen that the very dilute buffer (used in error in the unreported experiments mentioned above) probably accounted for many of the failures. It is totally inadequate.

In Fig. 10, with US<sub>5N</sub>, the beneficial effect of the stronger buffer is more marked. With the ruptured mitochondria, unlike the previous figure, the stronger buffer does not show a narrow optimum range.

After examining these results it was decided to employ 0.1M glycine/NaOH buffer in all future experiments. Although the pH was checked routinely after substrate solutions were prepared, it was found that with this stronger buffer no adjustment was necessary.

The change in buffer during the incubation required some slight changes in the subsequent determination of lysine with lysine decarboxylase. When HCl was added to the better buffered reaction mixtures to stop  $\epsilon$ -lysine acylase action, 0.1 ml of 0.1N HCl was still sufficient to stop enzyme action, but, when 1.0 ml of the resulting boiled preparation was added to 1.7 ml of 0.06M phosphate buffer the pH now remained at 5.0.

FIG.9 EFFECT OF PH AND BUFFER STRENGTH ON  $\epsilon$ -LYSINE ACYLASE ACTIVITY  
IN WHOLE MITOCHONDRIA.

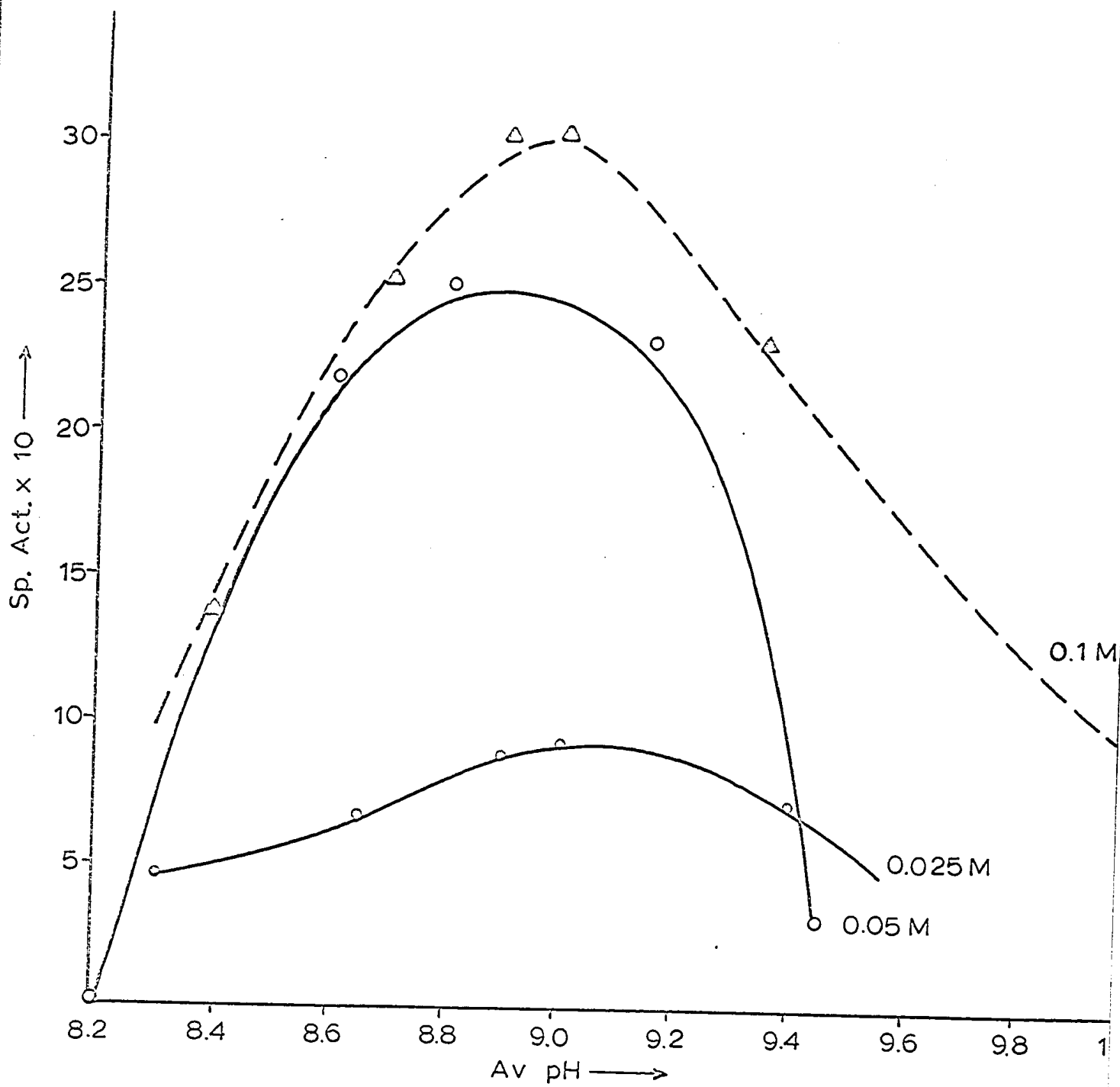
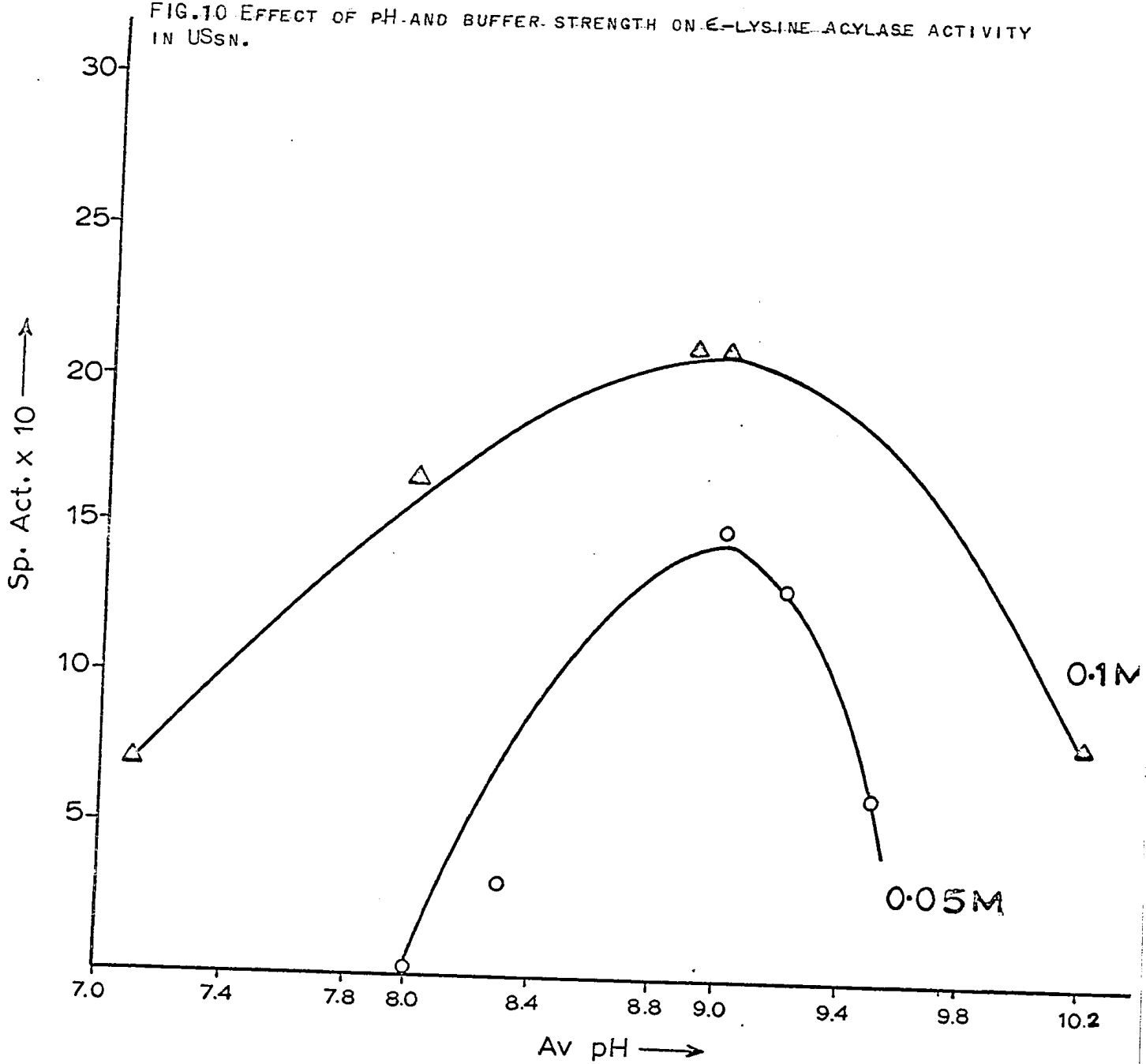


FIG.10 EFFECT OF PH-AND BUFFER-STRENGTH ON  $\epsilon$ -LYSINE-ACYLASE ACTIVITY IN USSN.



Previously, when determining the  $\text{CO}_2$  equivalent of lysine (see p 25), it had been found that the actual pH in the Warburg flasks was 5.5 to 5.6. This was also confirmed when using different buffers in the survey (see p 40). This pH was desirable since only small amounts of  $\text{CO}_2$  would be in solution (105). Under the present conditions, with the Warburg reaction being carried out at pH 6.0, not only would much more  $\text{CO}_2$  remain in solution, but small changes in pH would alter this amount greatly.

It was found that if 0.06M phosphate buffer was prepared at pH 5.8, the resulting pH on adding the experimental samples always fell between pH 5.2 and 5.5. Within this pH range the amount of  $\text{CO}_2$  dissolved does not vary appreciably with pH. Accordingly, the buffer used in the Warburg was prepared at pH 5.8 from this time onward.

#### VIII) Repetition of isolation procedures under new conditions.

Having found a major source of variation in the methods used so far, some of the most potentially useful isolation attempts were repeated. The procedures were exactly as described before except for the change in buffers described above (0.2M glycine/NaOH used to dissolve the substrate and give a final concentration of 0.1M, and pH 5.8 buffer used in the Warburg). Incubation time was 2 hours. The results are presented in Table XIX.

TABLE XIX

Effect of Isolation Steps (carried out under  
Controlled Conditions) on  $\epsilon$ -Lysine Acylase Activity.

Preparation	Sp. Act.	Average
Mitochondria	0.356	0.250 $\pm$ 0.021
	0.254	
	0.259	
	0.270	
	0.156	
	0.259	
USSN	0.443	0.292 $\pm$ 0.030
	0.354	
	0.216	
	0.324	
	0.146	
	0.281	
5.5 ppt.	0.432	0.270 $\pm$ 0.032
	0.281	
	0.324	
	0.151	
	0.432	
pH 9.0 extract of	0.356	---
pH 5.5 ppt.		

It can be seen from this table that no significant purification had occurred. The apparent effect of extraction at pH 9.0 in previous experiments undoubtedly resulted from the better pH actually obtained during incubation of these preparations with substrate.

Discussion and summary.

The attempts to purify  $\epsilon$ -lysine acylase, reported in this section, were unsuccessful. Variation in pH during the determination of enzyme activity nullified many attempts. Despite this fact some experiments were repeated so often that a general trend became apparent, over and above the individual variations. The general indications of these experiments can thus be considered reliable, even if the purification obtained was useless.

Three such experiments, when viewed together, lead to one interesting conclusion. When mitochondria were ruptured by treatment with ultrasonic vibrations, the bulk of the active protein did not sediment in the precipitate when centrifuged at 27,000 x g. Neither did it remain in solution when centrifuged at 105,000 x g. This would indicate that the enzyme might be attached to other bulky protein - smaller than whole mitochondria which sediment at 9,750 x g, and larger than the common soluble enzymes - which remain in suspension at 105,000 x g. This suggests that  $\epsilon$ -lysine acylase might be attached to the broken mitochondrial membranes. Experiments in which protein was precipitated by ammonium sulfate or acid support this suggestion since it can be seen from these experiments

that the enzyme activity was found in the same fraction as the bulk of the protein (see Table XIV for example). Certainly, the fact that this enzyme was not separated selectively from other major protein constituents in the USSN, by any means tried nor was it adsorbed on calcium phosphate gel (8), lends strength to the supposition that it may be attached to mitochondrial membranes.

Secondly, the fact that all enzyme activity was destroyed by acetone treatment suggests that lipid may be involved. The lipid may be part of the active molecule, or might be part of a protective feature whose removal rendered the enzyme liable to destruction by manipulations to which it was previously resistant.

In all the experiments reported in this section, some properties of  $\epsilon$ -lysine acylase are revealed. It is very resistant to freezing and thawing. It can be stored frozen and stored without loss of activity in the form of whole mitochondria, USSN, and as a concentrate precipitated at pH 5.5. It can also be held at +4° or even at 30° C for considerable periods of time without significant loss in activity. It can also withstand dialysis.

All these properties, of course refer to a very crude preparation. It is possible that a purified preparation might not be so stable - particularly if the suggestions above concerning lipid should prove to be correct.

C) Comparison of some of the Properties of  $\epsilon$ -Lysine Acylase prepared from various sources.

Introduction.

Having failed to find any method of purifying  $\epsilon$ -lysine acylase from chicken mitochondria without great loss of activity, it was decided that a better understanding of the basic properties of the crude enzyme was necessary before purification would be possible. The present section of the work, therefore, covers an investigation of the kinetics of the crude enzyme, the effect of inorganic ions on whole and disrupted mitochondrial enzyme, and the substrate specificity of this enzyme compared with that from other sources.

Methods.

The methods of preparation of mitochondria are described on p 51, the preparation of disrupted mitochondria (US<sub>SN</sub>) on pp 66-72. The assay procedure is described on pp 29-31 with modifications on p 99. Any other methods used are described under the appropriate experiment.

I) Comparison of the effect of pH on the activity of chicken, duck and pigeon mitochondria.

Mitochondria were prepared from all three sources as before. The buffers used were:

pH 7.2	0.065M phosphate
pH 8.2	0.1M Tris (required 0.2 ml HCl to stop reaction)
pH 8.5	0.2M glycine/NaOH

pH 9.0      0.2M glycine/NaOH

pH 9.5      0.2M glycine/NaOH

Substrate was dissolved in each buffer to give 60  $\mu\text{m}/\text{ml}$  and the pH adjusted if necessary. Incubation was carried out for two hours and the amount of lysine produced determined in the Warburg as usual.

The results are presented in Fig. II. It can be seen that pH 9.0 is the most suitable pH for comparison of these preparations differ in the sharpness of their pH optima.

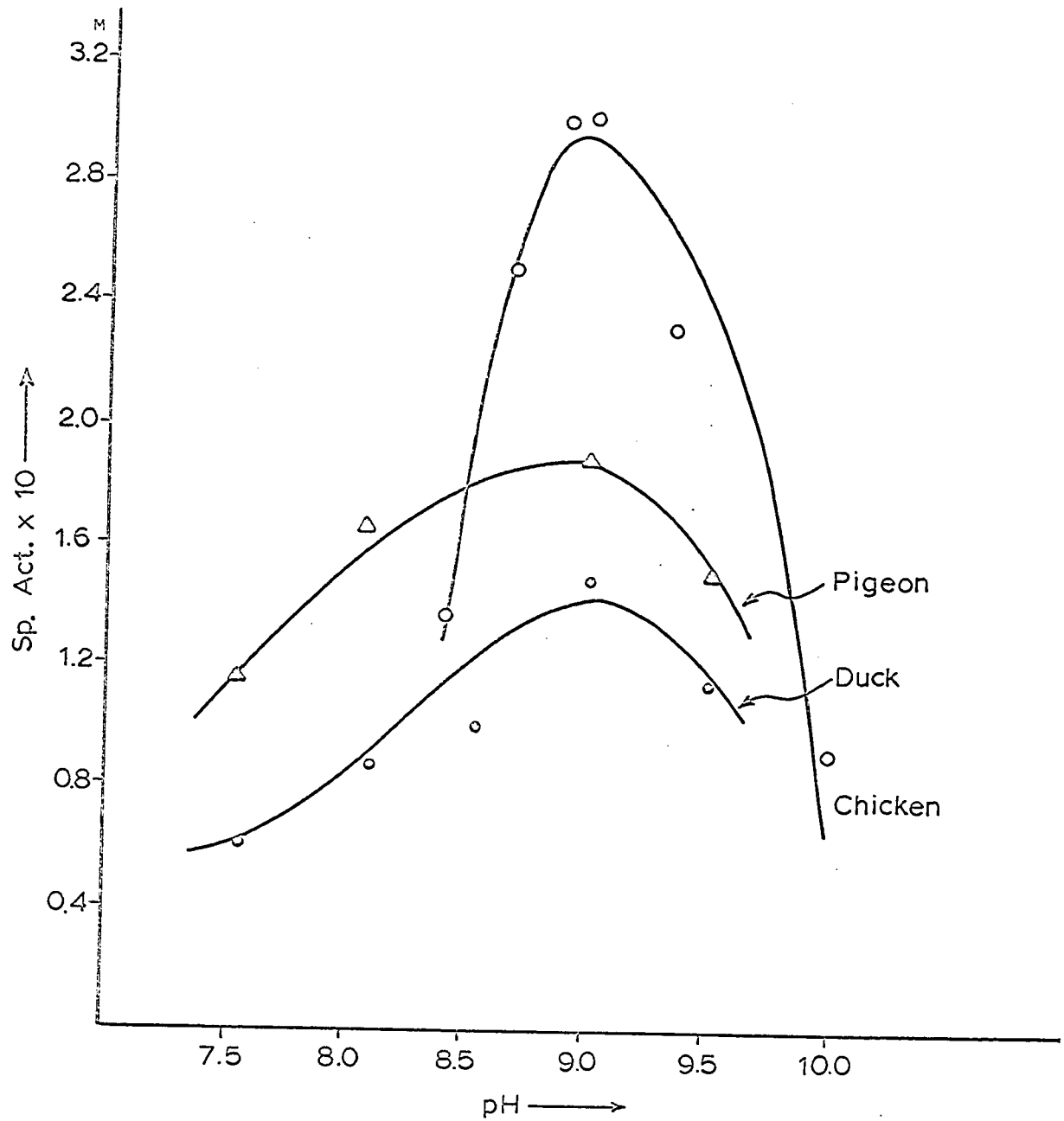
## II) Kinetics.

The following experiments were carried out on whole mitochondria and on US<sub>SN</sub>. However, US<sub>SN</sub> prepared in the usual way was not sufficiently concentrated and did not produce enough free lysine to give a significant evolution of CO<sub>2</sub> when determined in the Warburg. For this reason, the US<sub>SN</sub> was concentrated by precipitation at pH 5.7 (see pp 76-80). The precipitate was resuspended in distilled water and adjusted to pH 9.0 with the careful addition of 0.1N NaOH before use.

a) The effect of incubation time on product formation.

After the tissue sample was prepared it was divided into six 25 ml Erlenmeyer flasks (2 ml/flask). To each flask except the last one was added 2 ml of substrate (60  $\mu\text{moles}/\text{ml}$  pure  $\epsilon$ -N-acetyl-L-lysine, dissolved in 0.2M glycine/NaOH buffer, at exactly pH 9.0). The flasks were incubated at 37° C with gentle shaking in a Dubonoff shaker. At the end of 1, 2, 3,

FIG. II COMPARISON OF THE EFFECT OF PH ON  $\epsilon$ -LYSINE ACYLASE  
ACTIVITY OF CHICKEN, PIGEON AND DUCK KIDNEY MITOCHONDRIA.



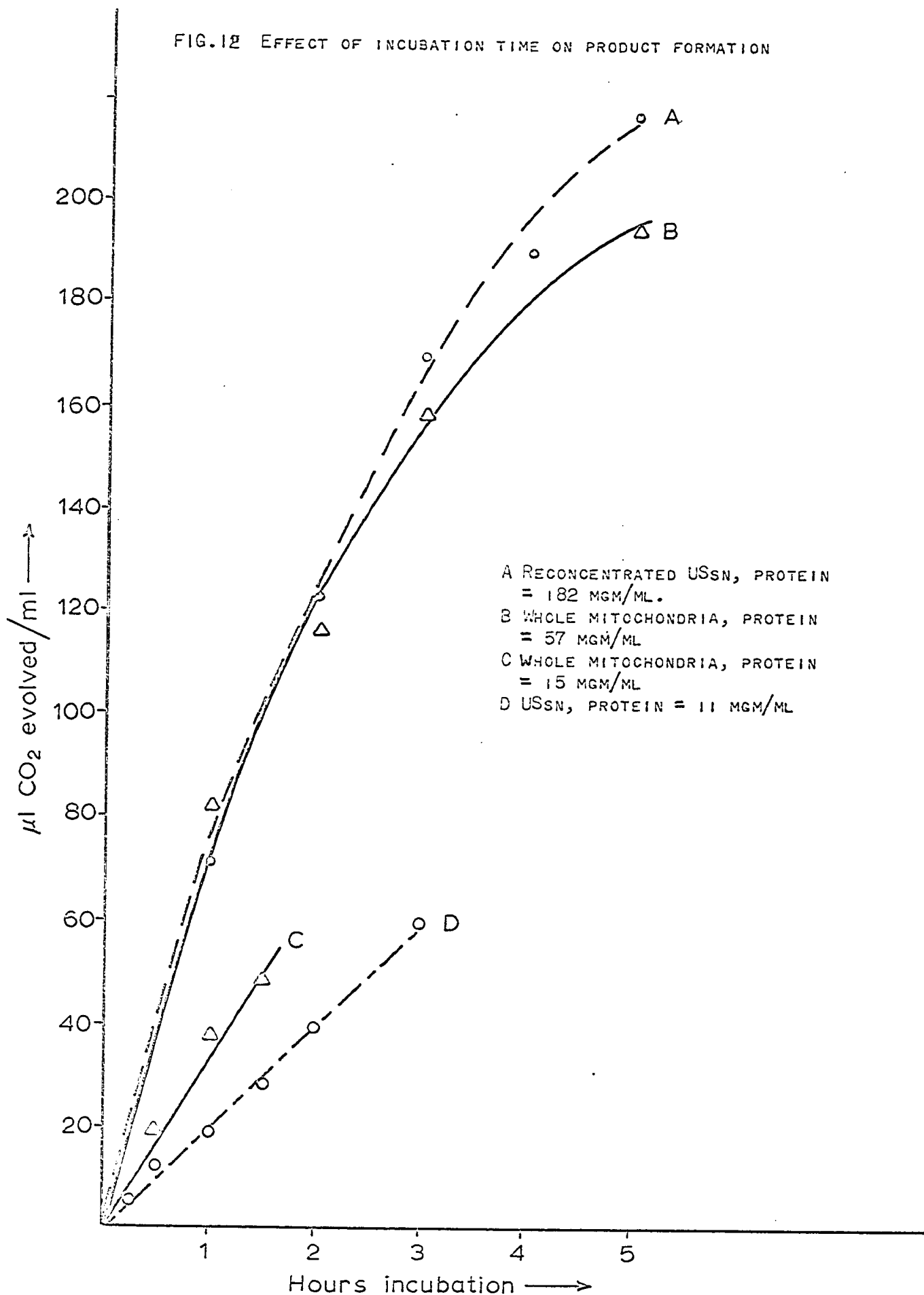
and 4 hours a flask was removed, 0.1 ml 1.0N HCl added to stop enzyme reaction, and the enzyme destroyed by placing it in a boiling waterbath for three minutes. At the end of 5 hours, both the remaining flasks were removed from the shaker. The one containing substrate was treated as the earlier ones. The one without substrate received 0.1 ml 1.0N HCl first and then 2 ml substrate, before being placed in the boiling waterbath. All samples were then centrifuged at maximum speed in a clinical centrifuge for a few minutes. Duplicate 1.0 ml samples of the supernatant were then analyzed for their lysine content in a Warburg manometer using the method of Gale and Epps (104). The sixth flask served as a control for the lysine content of the tissue.

The results are presented in Fig. 12. As shown on p26, 16.5  $\mu$ l  $\text{CO}_2$  are equivalent to 1  $\mu$ m lysine. These lines demonstrate that both whole and sonically disrupted mitochondria decarboxylate the substrate at a linear rate for up to 2 hours. The lines C and D show that lysine production is linear even during short incubation times when the enzyme concentration is low. Lines A and B represent the limiting conditions when massive amounts of enzyme are present. From this figure it is obvious that 2 hours is a satisfactory incubation time for a wide range of enzyme concentrations.

b) Relationship between enzyme concentration and product formation.

Since the experiment above indicated that a 2 hour incubation period was suitable for a range of enzyme concentrations, this

FIG.12 EFFECT OF INCUBATION TIME ON PRODUCT FORMATION



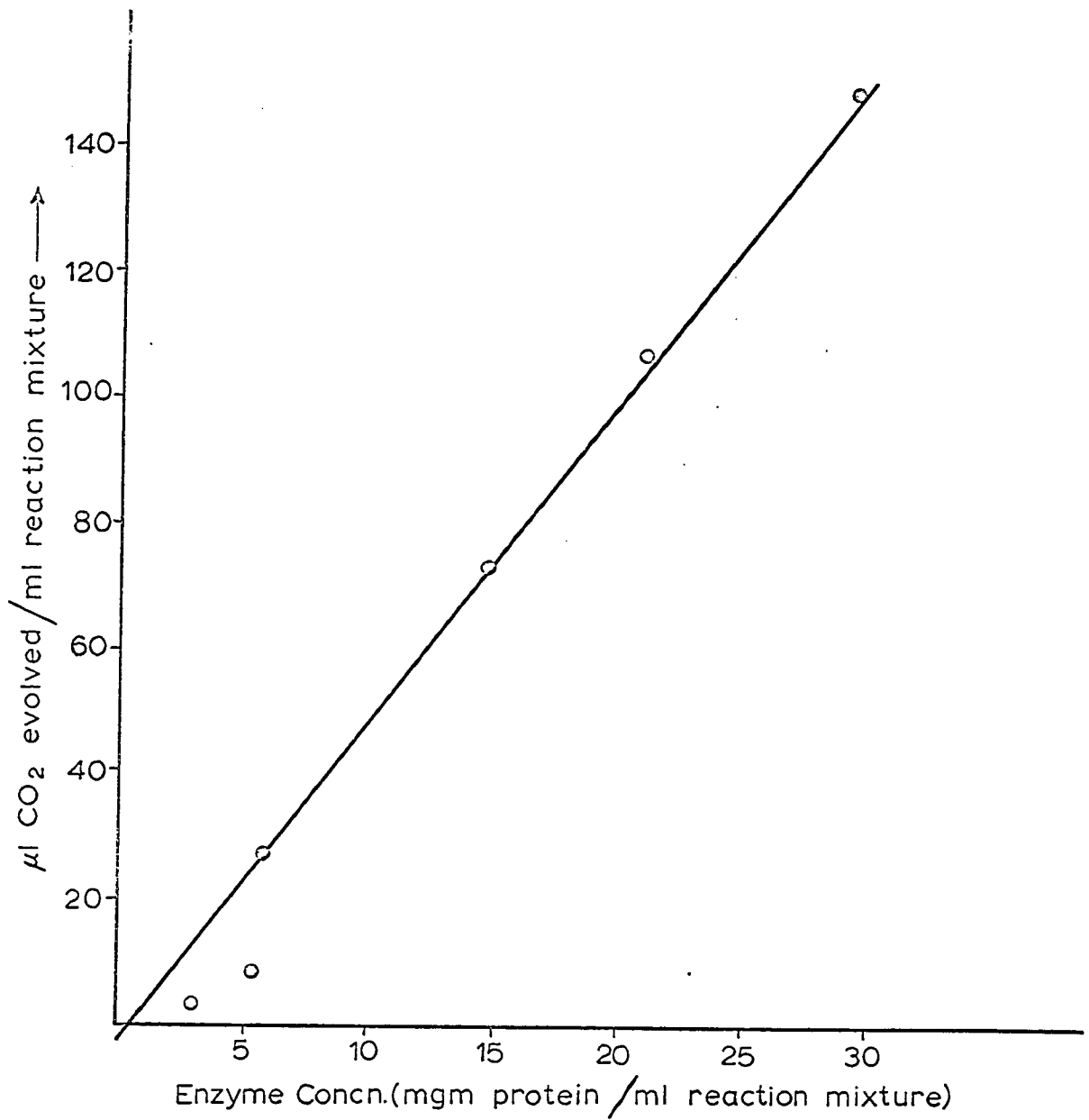
was employed in the following experiment. The experiment was carried out as above except that the amount of enzyme in each flask was varied. The volumes of enzyme used were 2.0, 1.5, 1.0, and 0.5 ml. Two different preparations of different protein concentration were used to arrive at a range of final protein concentrations in the incubation mixture. The volume was brought up to the standard volume of 4 ml with distilled water. This experiment was carried out on the resuspended pH 5.7 precipitate only.

From the results shown in Fig. 13 it can be seen that the relationship is linear but that below 6.5 mgm/ml protein the values obtained are not on the straight - line portion of the curve. They do not therefore reflect a direct proportionality. From this curve it was concluded that experiments in which the evolution of  $\text{CO}_2$ /ml of reaction mixture was less than 20  $\mu\text{l}$  could only be considered significant if frequent repetitions revealed a consistent average value.

c) The effect of substrate concentration and derivation of  $K_m$

Experiments on resuspended pH 5.7 precipitate were carried out as before, using a two hour incubation period. A substrate solution of 50  $\mu\text{moles/ml}$   $\epsilon$ -N-acetyl-L-lysine in 0.2M glycine/NaOH buffer was diluted with the same buffer to give the required concentrations. All solutions were exactly pH 9.0. This experiment was not run on whole mitochondria since they offered an even more crude enzyme preparation than the ruptured preparation.

FIG 13 RELATIONSHIP BETWEEN ENZYME CONCENTRATION AND PRODUCT FORMATION.



The actual values obtained in one experiment are shown in Table XX, and the Lineweaver and Burk plot from which the  $K_m$  value was obtained is shown in Fig. 14.

Calculation of  $K_m$ .

By the Lineweaver and Burk plot, the y-intercept is equal to  $1/V_{max}$ , and the slope of the line is equal to  $K_m/V_{max}$ . Hence, from this figure, it can be calculated that  $K_m = 7.2 \times 10^{-2}$ , and  $V_{max} = 0.99 \mu\text{m/hr./mgm protein}$ . In repeated experiments the following values were found:

$K_m$	Av.	$V_{max}$	Av.
$7.2 \times 10^{-2}$		0.99	
$8.05 \times 10^{-2}$		0.80	
$3.9 \times 10^{-2}$	$6.2 \times 10^{-2}$	0.75	$0.85 \mu\text{m/hr./mgm protein}$ .

For comparison, the  $K_m$  and  $V_{max}$  values obtained with partly purified hog  $\epsilon$ -lysine acylase are presented for three substrates. These determinations were carried out by Dr. W. K. Paik.

Attempts were made to determine the  $K_m$  value for the chicken mitochondrial enzyme with  $\epsilon$ -chloracetyl-L-lysine but the activity of this preparation on this substrate was not sufficient to yield meaningful values.

FIG. 14 DETERMINATION OF  $K_M$  WITH  $\epsilon$ -N-ACETYL-L-LYSINE AT PH 9.0

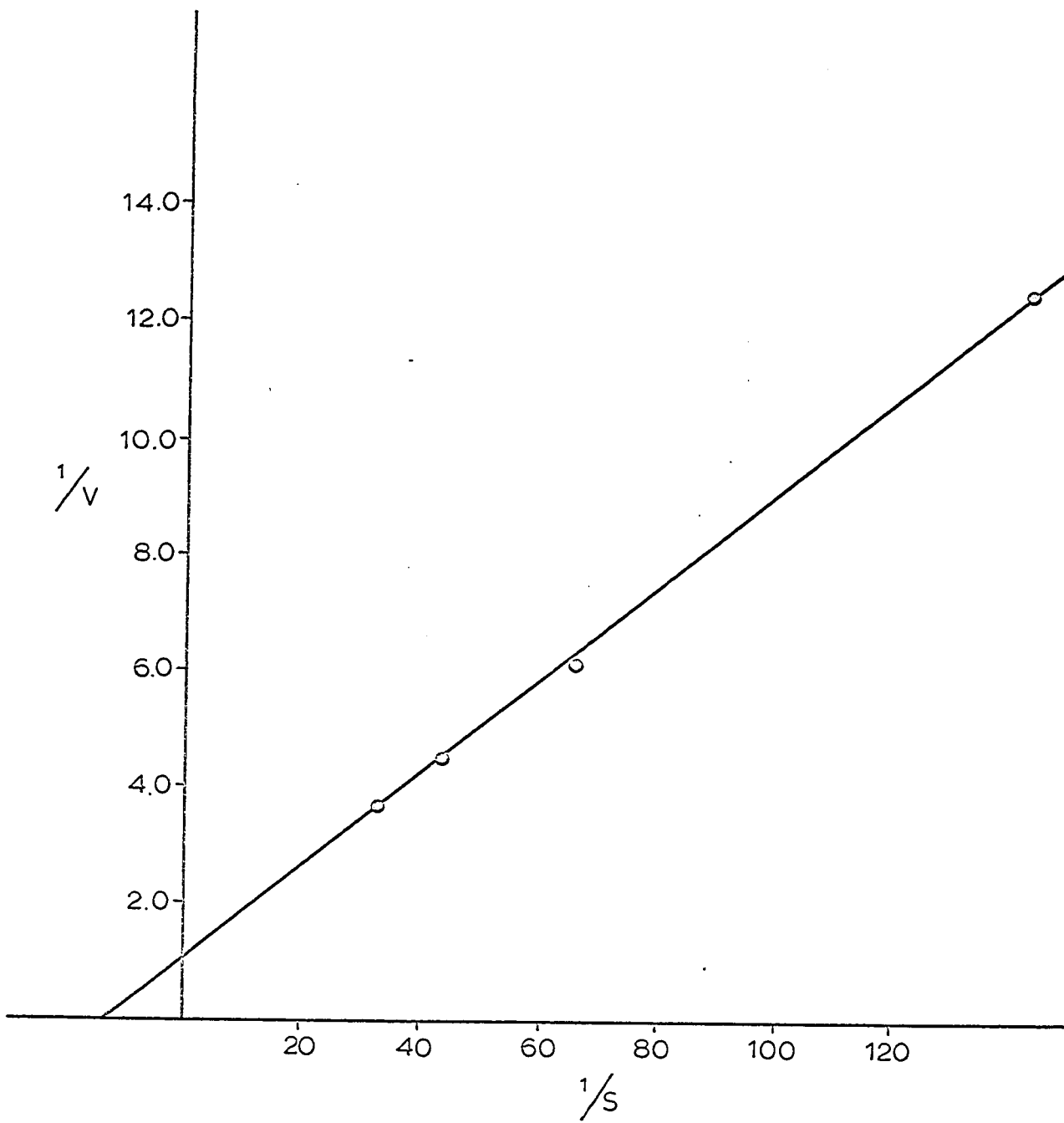


TABLE XX

Determination of Apparent  $K_m$

Substr. concn.	$\mu\text{lCO}_2$ /ml	$\mu\text{m lysine}$ /ml	Velocity*	$1/v$	$1/s^{\#}$
7.5 $\mu\text{m/ml}$	46.5	2.3	0.080	12.5	133
15.0	92.8	4.57	0.153	6.13	66
22.5	125.1	6.26	0.219	4.56	44
30.0	159.9	8.03	0.281	3.59	33

\* Velocity =  $\mu\text{m lysine produced/hr./mgm protein}$

$\#$  Substrate concn. in moles/L.

Source of enzyme	pH	Substrate	K <sub>m</sub>	V max.*
Chicken US <sub>SN</sub> (pH 5.7 ppt)	9.0	ε-N-acetyl-L-lysine	6.2 x 10 <sup>-2</sup>	0.85
Partly purified hog enzyme	8.0	"	4.58 x 10 <sup>-3</sup>	7.74
"	8.0	α-keto-ε-acetamido-caproic acid	7.55 x 10 <sup>-3</sup>	6.47
"	8.0	ε-N-formyl-L-lysine	1.02 x 10 <sup>-2</sup>	7.56

\* μmoles product/hr./mgm protein.

d) The energy of activation.

Mitochondria and US<sub>SN</sub> were prepared as before. The US<sub>SN</sub> was concentrated by precipitation at pH 5.7 and resuspended in 0.1M pH 9.0, glycine/NaOH buffer. Substrate (ε-N-acetyl-L-lysine at 60 μmoles/ml) was dissolved in a 0.1M solution of the same buffer for use with reconcentrated US<sub>SN</sub>, and in a 0.2M solution for use with whole mitochondria. The final concentration of buffer during incubation was therefore 0.1M in each case.

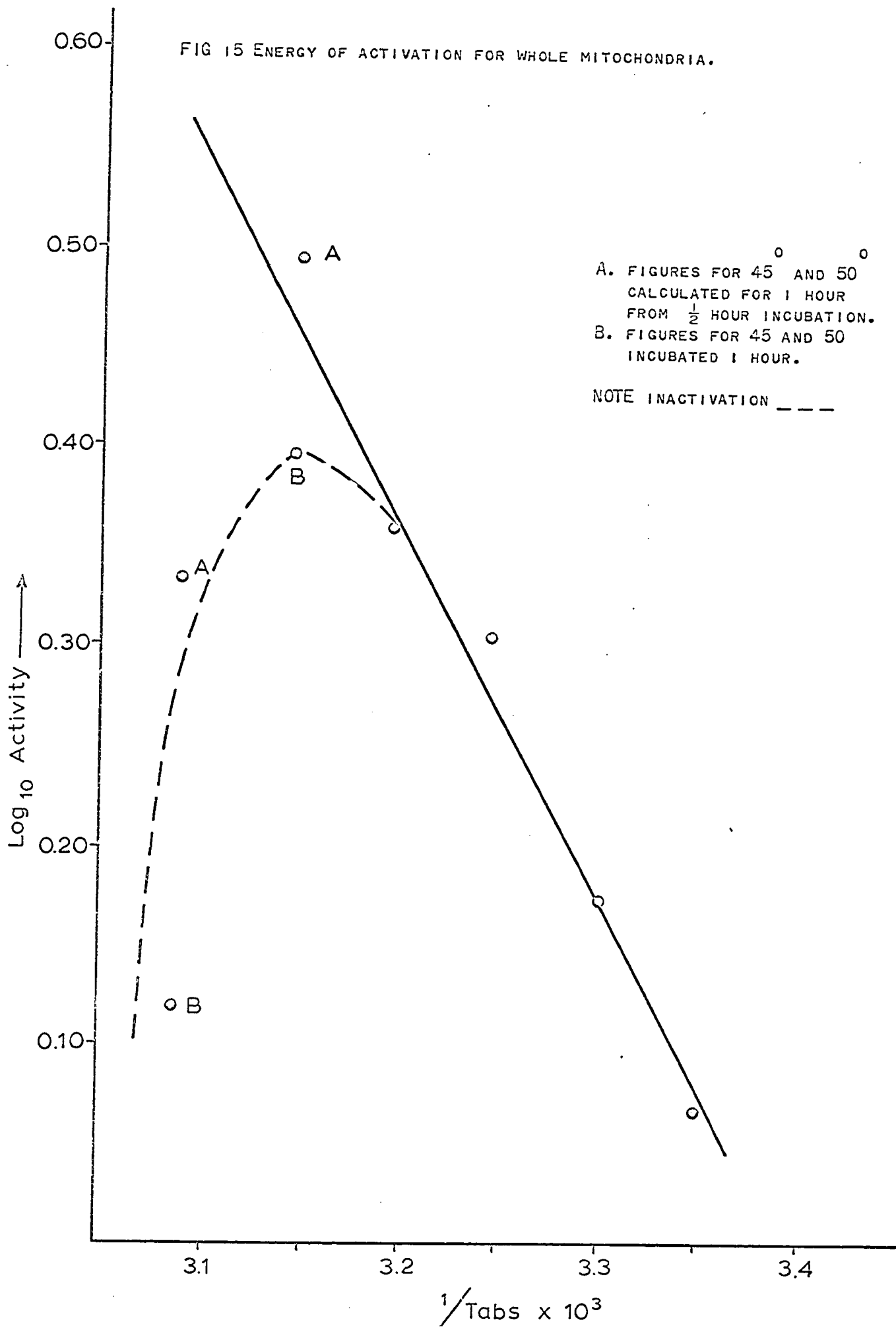
Incubations were carried out using 2 ml tissue sample and 2 ml substrate in each case; duplicate samples were incubated for 1 and 1.5 hours at low temperatures and for 0.5 and 1 hour for high temperatures. The experiment was terminated and the amount of lysine/ml determined in the usual way.

Calculation of activation energy.

The activation energy (A) was calculated from a plot of log<sub>10</sub> Activity/ I/Temp.Abs., derived from the standard equation:

$$\log_{10} (\text{act.}) = \log_{10} \text{Constant} - \frac{A}{2.303RT} \quad (\text{II8}).$$

FIG 15 ENERGY OF ACTIVATION FOR WHOLE MITOCHONDRIA.



In these experiments the activity is expressed as  $\mu$ moles lysine produced/hr./mgm protein. The hourly rate is derived from the average figures except at 45° + 50° C where it is apparent that inactivation occurred. The values obtained for one hour and half an hour at 45° and 50° are therefore shown separately.

The experimental data and calculations are shown in Table XXI, and the resulting plots in Figs. 15 and 16.

From these figures the activation energy can be calculated as follows:

1) Mitochondria

$$y\text{-intercept} = \log_{10} C = 0.580$$

$$\text{slope} = - \frac{A}{4.6} \quad \text{therefore } A = 8.46 \text{ k cal/mole}$$

$$\text{Average of two experiments: } A = 8.50 \text{ k cal./mole.}$$

2) Reconcentrated US<sub>5</sub>N

$$\text{Slope} = 0.80, \quad \log_{10} C = 0.635$$

$$A = 3.7, \text{ k cal./mole}$$

$$\text{Average of two experiments: } A = 4.3 \text{ k cal./mole}$$

Dixon and Webb (119) have criticized the determination of activation energy using a single substrate concentration at all temperatures. They point out that the concentration of substrate necessary to saturate the enzyme may vary with temperature. In the present case, the use of a single concentration of substrate would appear to be valid for two reasons. First, it can be seen from the experiment on

FIG. 16 ENERGY OF ACTIVATION FOR RECONCENTRATED USSN.

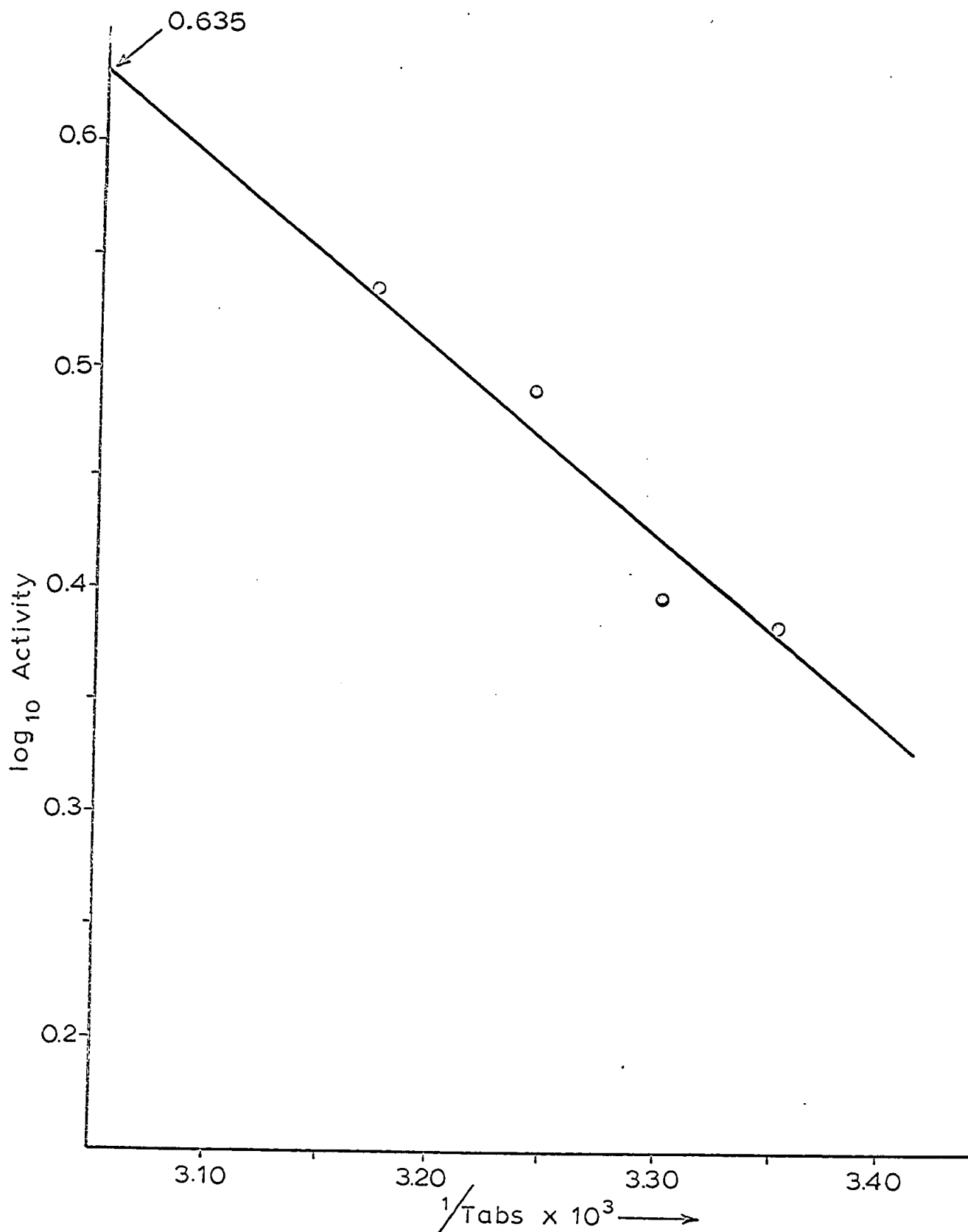


TABLE XXI

Determination of Energy of Activation

Mitochondria

$T^{\circ}$ abs.	$\mu\text{CO}_2/\text{hr}$	$k^*$	$\text{Log}_{10} k$	$I/T_{\text{abs.}}^{\circ} \times 10^3$
298	45	1.13	0.0531	3.35
303	59	1.46	0.1702	3.30
308	60.5	2.02	0.3053	3.24
313	93	2.33	0.3673	3.19
318a	124	2.57	0.4099	3.14
b	96	2.46	0.3909	
323a	104	2.04	0.3344	3.09
b	63	1.31	0.1173	

US<sub>SN</sub>

$T^{\circ}$ abs.	$\mu\text{CO}_2/\text{hr}$	$k^*$	$\text{Log}_{10} k$	$I/T_{\text{abs.}}^{\circ} \times 10^3$
298	30	2.42	0.384	3.35
303	31.0	2.49	0.396	3.30
310	39.3	3.15	0.498	3.24
315	43.0	3.44	0.536	3.17

\*  $k = (\mu\text{m lysine}/\text{hr.}\mu\text{gm}) \times 100$        $18.5 \mu\text{CO}_2 = 1 \mu\text{m lysine}$

a = hourly rate calculated from  $\frac{1}{2}$  hr. incubation

b = incubation carried out for 1 hr - note inactive.

possible product inhibition (p 92 ), that, even with far more enzyme present than in the present experiment, only half the substrate was consumed at 37<sup>0</sup> C. Therefore, in the present experiment, with less enzyme present, the substrate was in great excess. Secondly, by direct calculation it was found that at no time did the actual amount of lysine produced exceed 7  $\mu$ moles/ml.\* Since substrate was present at 30  $\mu$ moles/ml of reaction mixture, this would appear to be ample excess to keep the enzyme saturated at all times.

These activation energies are surprisingly low in view of the fact that several proteolytic enzymes fall in the range of 13 to 19 kcal./mole (120), and invertase, acting on sucrose, shows an energy of activation of approximately 9 k cal./mole.

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\* Foot-note: At 45<sup>0</sup>, with 30 mins incubation, the amount of CO<sub>2</sub> evolved per ml was 62  $\mu$ l. By calculation this was equivalent to 6.7  $\mu$ moles of substrate broken down per hour. This was the highest rate obtained in any sample.

III) The effect of inorganic ions on  $\epsilon$ -lysine acylase in whole and ruptured chicken mitochondria.

An investigation of the effect of some inorganic ions was carried out with whole mitochondria. The salts which gave the most interesting results were then tested on ruptured mitochondria, to determine whether the effect was on the enzyme per se, or on the mitochondrial structure and permeability.

All experiments were carried out by preparing the required concentration of salt, dissolved in the usual 50  $\mu$ moles/ml substrate solution, and adjusted to pH 9.0 with the appropriate acid or alkali if necessary. For example, sodium salts were adjusted with NaOH, phosphates with phosphoric acid etc. The amount of anion or cation added this way was allowed for in calculating the final concentration if it was significant. Incubation and determination of lysine were carried out as usual using a two hour incubation period.

With any salts which showed marked effect, separate determinations of their effect on lysine decarboxylase were carried out. Only in the case of the iron salts was this of any significance - as will be discussed under the appropriate experiment.

Initially, a variety of salts were tested at high concentrations. The most interesting ones were repeated over a broader concentration range and others added for

comparison. Thus, the results shown for mitochondria consist of seven overlapping experiments, and those for  $US_{SN}$  of three overlapping experiments.

The results obtained with whole mitochondria are presented in the next five tables (Tables XXII, XXIII, XXIV, XXV, XXVI). In the first of these tables the inhibitory effects of chloride ion are summarized. It can be seen that there is a moderate inhibition at concentrations ranging from molar to tenth molar with monovalent cations. The inhibition is much more severe, but decreases more sharply, when magnesium is the cation.

These results raised some question about the use of HCl to make a pH 5.5 precipitate from  $US_{SN}$ . However, on examining the earlier data, it was found that the volume of 0.1N HCl used to adjust the pH to 5.5 to 5.7 was always approximately 1/10 of the volume of the supernatant. The final concentration of  $Cl^-$  would therefore be approximately  $1 \times 10^{-2}$  (ie. below the inhibitory level).

The second table (Table XXIII) shows the comparative effect of various cations when tested as sulfates. Sulfate was selected as the anion because it did not seem to be inhibitory per se in the preliminary experiments. This conclusion was based on the fact that the behaviour of various salts could be grouped according to their cations regardless of whether the anion was sulfate, phosphate or acetate. Sulfate was selected in preference to phosphate or acetate because a wide variety of cations could be

TABLE XXII

Salts Tested as Chlorides on Whole Mitochondria

Cation	Molar concns.				
	1.0	$6 \times 10^{-1}$	$3 \times 10^{-1}$	$1 \times 10^{-1}$	$6 \times 10^{-2}$
Na +	-	42	23	36	-
K +	60	37	-	-	-
NH <sub>4</sub> +	-	67	40	26	-
Mg ++	-	-	100	32	0

Figures are % inhibition calculated from the amount of CO<sub>2</sub> evolved compared to control sample with no added salt.

100% Activity (0 inhibition) = 80.9 - 83.2  $\mu$ lCO<sub>2</sub>/ml.

Values vary  $\pm$  15% in duplicate runs  $\therefore$  all values < 15%

considered zero.

TABLE XXIII

Salts Tested as Sulfates on Whole Mitochondria

Cation	Molar concns.						
	$3 \times 10^{-1}$	$1.5 \times 10^{-1}$	$7 \times 10^{-2}$	$5.5 \times 10^{-2}$	$2.5 \times 10^{-2}$	$6 \times 10^{-3}$	$1 \times 10^{-3}$
Na <sup>+</sup>	16	0	0	-	-	-	-
NH <sub>4</sub> <sup>+</sup>	0	0	0	-	-	-	-
Li <sup>+</sup>	-	-	-	0	#20	#23	0
Ca <sup>++</sup>	-	-	-	#69*	#107*	#23*	0
Mn <sup>++</sup>	-	-	-	0*	#40	#28	0
Co <sup>++</sup>	-	-	-	85	17	#27	0
Mg <sup>++</sup>	-	48	-	41	26	-	-
Ni <sup>++</sup>	-	-	-	68	0	0	0
Cu <sup>+</sup>	-	100	-	100	-	-	-

\* Not soluble at this concentration at pH 9.0, used as suspensions.

# = % Stimulation

All figures are % Inhibition or Stimulation calculated from  $\mu\text{CO}_2$  evolved/ml in the absence of added salt.

0 Inhibition = 60.9 - 117  $\mu\text{CO}_2$ /ml in different expts.

obtained as sulfates. Some solubility problems were encountered as indicated in the table.

With monovalent cations, little inhibition or stimulation is seen in Table XXIII. The divalent cations however, can be separated into three groups. In the first group are found calcium, manganese and cobalt - all of which show stimulation at one or more level. Calcium stimulates the reaction up to 100% even above its solubility point. Manganese is slightly inhibitory when insoluble and the stimulation is less than with calcium but occurs at the same concentrations. Cobalt is extremely inhibitory at the highest concentration but does show a small stimulation at lower levels.

The second group consists of magnesium and nickel. Both of these cations produce definite inhibitions - nickel more so than magnesium. Finally, copper is completely inhibitory even at a concentration where other divalent cations show only moderate inhibition or actual stimulation.

In Table XXIV are shown three cations tested as acetates. It can be seen that potassium is stimulatory, sodium has little effect, and copper, this time in the cupric form, is again extremely toxic.

Nitrate, nitrite, monophosphate and triphosphate were also tested. The results are shown in Table XXV. It can be seen that sodium nitrate is more inhibitory than the potassium salt. Strangely, it is also more inhibitory than sodium nitrite. Neither form of phosphate would appear to have much

TABLE XXIV

Salts Tested as Acetates on Whole Mitochondria

Cation	Molar concns.					
	$6-7 \times 10^{-1}$	$3 \times 10^{-1}$	$1 \times 10^{-1}$	$5 \times 10^{-2}$	$2-3 \times 10^{-2}$	$7 \times 10^{-3}$
$K^+$	#27	#78	-	-	-	-
$Na^+$	33	-	0	-	0	0
$Cu^{++}$	-	-	100	100	80	-

# = Stimulation

All figures are % Inhibition or Stimulation compared to control values without added salt. 0 Inhibition =  $34.0 - 110 \mu\text{LCO}_2/\text{ml}$  depending on protein concentration of sample.

Values vary  $\pm 15\%$  in duplicates  $\therefore$  figures of  $< 15\%$  considered zero.

TABLE XXV

Other Salts Tested on Whole Mitochondria

Cation	Molar Concns.							
	$8 \times 10^{-1}$	$6 \times 10^{-1}$	$5.5 \times 10^{-1}$	$4 \times 10^{-1}$	$2 \times 10^{-1}$	$6 \times 10^{-2}$	$2.5 \times 10^{-2}$	$6 \times 10^{-3}$
a) Nitrates:								
Na <sup>+</sup>	=	100	-	68	-	-	-	-
K <sup>+</sup>	77	-	-	0	-	-	-	-
b) Phosphates:								
NaH <sub>2</sub>	-	-	0	-	17	-	-	-
Na <sub>3</sub>	-	-	-	-	16	-	-	-
K <sub>3</sub>	-	-	-	-	-	0	0	0
CaH	-	-	-	47	21	-	-	-
c) Other salts:								
NaNO <sub>2</sub>	-	-	42	-	31	-	-	-

F = % Stimulation

Figures are % Stimulation or Inhibition compared to  $\mu\text{CO}_2$  evolved

by control with no added salts. 0 Inhibition =  $80.9 - 110 \mu\text{CO}_2/\text{ml}$ .

Values < 15% considered zero.

effect except as the calcium salt. This stimulation is undoubtedly due to the calcium ion per se.

In order to clarify the differences between various cations, these results have been summarized in Table XXVI. The stimulatory effect of potassium ions can be seen not only in actual stimulation obtained, but also in the fact that, with an inhibitory anion such as nitrate, the potassium salt was less inhibitory than the sodium salt. The most interesting point which emerges from this summary is the association of potassium and calcium as stimulatory cations while sodium and magnesium show inhibition or no effect. This association suggested that the physical condition of the mitochondria, or transport mechanisms, might be involved - rather than the enzyme itself. Accordingly, a few selected cations were tested on ruptured mitochondria using non-inhibitory anions.

Effect of cations on  $\epsilon$ -lysine acylase in ruptured mitochondria.

US<sub>5</sub>N was prepared as usual and used without reconcentrating. The salts to be tested were prepared as described on p 113; incubation was carried out for 2 hours.

The results are presented in Table XXVII and XXVIII, together with a comparison with whole mitochondria. Certain major differences between whole and ruptured mitochondria are to be seen:

a) The effect of potassium ions has changed from a stimulatory to an inhibitory one, while the effect of sodium ions is virtually the same as before. The effect of lithium ions is now a marked inhibition at all concentrations tested, while

TABLE XXVI

Summary of Effects of Cations on Whole Mitochondria

Monovalent:

Cation	Anions tested	Result
Na <sup>+</sup>	SO <sub>4</sub> , Cl, NO <sub>3</sub> , NO <sub>2</sub> CH <sub>3</sub> COO, PO <sub>4</sub> , H <sub>2</sub> PO <sub>4</sub>	Marked inhibition as NO <sub>3</sub> . Mod. inhib. or inhib. by high levels of:- CH <sub>3</sub> COO, Cl, NO <sub>2</sub> . Little or no effect as SO <sub>4</sub> , PO <sub>4</sub> , H <sub>2</sub> PO <sub>4</sub> .
K <sup>+</sup>	Cl, CH <sub>3</sub> COO, PO <sub>4</sub> , NO <sub>3</sub>	Acetate gave marked stimulation Nitrate gave less inhibition than Na salt. Cl, PO <sub>4</sub> as Na salts.
NH <sub>4</sub> <sup>+</sup>	SO <sub>4</sub> , Cl	As respective Na salts.
Li <sup>+</sup>	SO <sub>4</sub>	Sl. stim. at low level.

Divalent:

Cation	Anions Tested	Result
Ca <sup>++</sup>	HPO <sub>4</sub> , SO <sub>4</sub>	Both stimulatory (esp. SO <sub>4</sub> )
Mn <sup>++</sup>	SO <sub>4</sub>	Mod. stimulation
Co <sup>++</sup>	SO <sub>4</sub>	Inhibitory at 5x10 <sup>-2</sup> . Sl. stim. at low level.
Ni <sup>++</sup> Mg <sup>++</sup> Cu <sup>++</sup>	) ) ) SO <sub>4</sub> , Cl, CH <sub>3</sub> COO	Inhibition only.

TABLE XXVII

Effect of Monovalent Cations on  $U_{SN}$  and  
Comparison with Whole Mitochondria.

Salt	Concn. Tested	% Inhibition	Effect on whole Mitochondria
KAc *	$6 \times 10^{-1}$	88	#27%
	$3.3 \times 10^{-1}$	0	#78%
	$6 \times 10^{-2}$	24	#15%
Na Ac	$6 \times 10^{-1}$	53	-33%
	$6 \times 10^{-2}$	0	0
$(NH_4)_2SO_4$	$6 \times 10^{-1}$	0	0 This level is saturated.
	$6 \times 10^{-2}$	0	0
$Li_2SO_4$	$6 \times 10^{-2}$	67	0
	$2 \times 10^{-2}$	74	#20
	$6 \times 10^{-3}$	75	#23
	$1 \times 10^{-3}$	79	0

Ac = acetate.

# = Stimulation

TABLE XXVII

Effect of Divalent cations on  $US_{SN}$  and  
Comparison with whole Mitochondria.

Salt	Concn. Tested	% Inhibition	Effect on whole Mitochondria
MgSO <sub>4</sub>	2 x 10 <sup>-1</sup>	60	-48%
	5 x 10 <sup>-2</sup>	48	-41%
	2 x 10 <sup>-2</sup>	0	-26%
CaHPO <sub>4</sub>	3.6 x 10 <sup>-2</sup>	32	#47%
CaSO <sub>4</sub>	6 x 10 <sup>-2</sup>	49	#69%
	3 x 10 <sup>-2</sup>	23	#107%
	6 x 10 <sup>-3</sup>	0	#23%
MnSO <sub>4</sub>	6 x 10 <sup>-2</sup>	55	-15%
	3 x 10 <sup>-2</sup>	32	#40%
	6 x 10 <sup>-3</sup>	#21	#28%
	1 x 10 <sup>-3</sup>	28	0
CoSO <sub>4</sub>	5 x 10 <sup>-2</sup>	100	-85%
	3 x 10 <sup>-2</sup>	84	-17%
	6 x 10 <sup>-3</sup>	43	#27%
	1 x 10 <sup>-3</sup>	28	0
NiSO <sub>4</sub>	6 x 10 <sup>-2</sup>	74	-68%
	3 x 10 <sup>-2</sup>	42	0
	1 x 10 <sup>-3</sup>	56	0

# = Stimulation

Figures calculated as Tables XXII - XXV

Control values 87 - 110  $\mu$ lCO<sub>2</sub>/ml.

ammonium ions still have no effect even at saturation concentration.

- b) there is no correlation between the valency of cations and the concentration at which inhibition becomes negligible.
- c) all divalent cations now produce more inhibition, or inhibit at lower concentrations than before except for manganese which, at one concentration shows the same small stimulation as it did with whole mitochondria. The change of the calcium ion effect from one of marked stimulation to one of moderate inhibition is particularly noticeable.

No experiment was run to determine the effect of anions on this preparation but, from this same table, it can be seen that the anions employed are relatively inert. For example with acetate the potassium salt shows a marked inhibition whereas the sodium salt shows an effect very similar to that obtained before. There is therefore no consistent effect for the two acetates. Sulfate is obviously innocuous since even a saturating concentration of ammonium sulfate is without effect. Similarly, calcium is now inhibitory regardless of whether the salt is sulfate or phosphate.

From this series of experiments on ruptured mitochondria some generalizations can be drawn:

- a) most of the stimulatory effects seen earlier were effects on the mitochondrion rather than on the enzyme itself. The only exception to this is the small stimulation caused by  $6 \times 10^{-3}M$   $MnSO_4$ . It is questionable what significance this

stimulation has since the values obtained varied  $\pm$  15%, and concentrations on either side of this value show inhibitions. However, it would be interesting to check this salt on a purified preparation when one is available.

b) Ammonium sulfate is obviously safe to use at concentrations up to saturation in any future purification attempts.

c) Since almost all cations cause some inhibition it would be wise to use deionized water in future work.

#### Effect of ferrous and ferric sulfates

Iron salts have not been discussed in either of the foregoing sections because they proved to be a special case. The same procedure was followed for the preparation of mitochondria, ruptured mitochondria, and substrate solutions as with other salts. The results obtained with ferrous sulfates are listed below:

<u>Salt</u>	<u>Molar concentration</u>	<u>% Inhibition</u>	
		<u>whole mitoch.</u>	<u>ruptured mitoch.</u>
Fe SO <sub>4</sub>	3 x 10 <sup>-2</sup>	100	100
	1.8 x 10 <sup>-2</sup>	49	-
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	1.9 x 10 <sup>-2</sup>	0	0
	9.8 x 10 <sup>-2</sup>	0	-

These results indicate an apparent inhibition by ferrous sulfate. However, when these salts were tested in the Warburg manometers alone, it was found that ferrous sulfate caused an uptake of gas even when phosphate buffer was the only other

solution present. Ferric sulfate on the other hand, had virtually no effect whether the manometer contained buffer alone or a known solution of lysine plus lysine decarboxylase. The apparent inhibition shown above was, therefore, the net result of two separate reactions - CO<sub>2</sub> evolution due to decarboxylation, and O<sub>2</sub> uptake due to the ferrous salt.

The real effect of ferrous sulfate on  $\epsilon$ -lysine acylase was therefore determined by measuring the acetate released rather than the lysine formed. The procedure for determination of acetate will be described in detail in the next series of experiments. The effect of ferrous sulfate was tested only on ruptured mitochondria and the results are presented below:

FeSO <sub>4</sub>	3 x 10 <sup>-2</sup> M	% Inhibition = 22%
	1.8 x 10 <sup>-2</sup> M	" = 30%

Since variation of  $\pm$  15% was obtained throughout these experiments the inverse relationship between concentration and % inhibition cannot be considered significant.

It was concluded therefore that ferrous sulfate had a slight inhibitory effect on  $\epsilon$ -lysine acylase, and ferric sulfate no effect.

#### IV) Substrate specificity of $\epsilon$ -lysine acylase from chicken mitochondria and other sources.

A variety of possible substrates for this enzyme were tested on whole mitochondria, and on US<sub>SN</sub> prepared from chicken kidney. Some of these were also tested on preparations from duck and pigeon kidneys, and on purified  $\epsilon$ -lysine

acylase prepared from rat kidney. Substrates which, on deacylation at the position, would yield free lysine, were tested by determining the amount of lysine formed - using lysine decarboxylase as before. Other substrates were tested by determining the amount of acetate released.

Method of determining acetate (M. Soodak (I2I)).

The following assay mixture was prepared and stored frozen:

<u>Reagent</u>	<u>Proportions mixed to make assay mixture</u>
0.02M Sulfanilamide	2.0 ml
0.1N Crystalline Na ATP	3.0 ml
0.1M MgCl <sub>2</sub>	0.5 ml
1.0M K citrate	2.0 ml
CoA 500 µ/ml	2.0 ml.

The coenzyme A used varied between 71 and 75% pure (Sigma).

The required amount was calculated for each sample used (pure CoA = 413 µ/mgm - Merck Index, 1950) because excess CoA can inhibit the reaction (I22). Tubes of this mixture were thawed individually as required, and any excess discarded.

Other stock solutions prepared and stored in the cold:

1.0M Tris pH 8.0.

0.5M cysteine HCl (stored frozen)

5% Trichloroacetic acid

4N HCl

0.5% NH<sub>4</sub> sulfamate

0.1% N-(1 naphthyl)ethylene diamine dihydrochloride ( in dark bottle - renew when solution becomes highly colored)

0.005M KAc. solution

0.1% NaNO<sub>2</sub> was also prepared but only immediately before use.

Enzyme:

A partially purified extract of pigeon liver acetone powder.

This was prepared as follows: (123,124).

Pigeons were killed by decapitation and the livers removed and placed in a dry beaker in an ice bath. The liver was trimmed and then chopped with scissors and homogenized in a chilled Waring blender with acetone cooled (overnight) to -10° C. Different references (124,125) recommend various volumes of acetone. The best results were obtained here with 10 volumes and 1 minute homogenization. The homogenate was filtered on a Buchner with suction but not allowed to pack too tightly. The filter-cake was pulverized with a glass rod, resuspended in another 10 volumes cold acetone and refiltered. The filter-cake was placed on a large sheet of filter paper and air dried. Although this enzyme is stable at room temperature for many hours, the best preparation was obtained if the cake was broken up with a glass rod, fibrous material removed, and the powder stirred frequently. By this means it could be dried in about 1.5 hours at room temperature, and a much pinker preparation was obtained. (it has been claimed (124) that denaturation of hemoglobin and loss of enzyme activity appear to run parallel and that a pink preparation

is a more active one). The acetone powder was stored in a tightly sealed container in the freezer.

When required, 5 gm acetone powder was allowed to soak, in an ice bath, in 100 ml 0.02M  $\text{KHCO}_3$  for about 30 mins. It was then homogenized very gently by hand in a loose fitting glass homogenizer and centrifuged 20 mins at 2,000 rpm in a refrigerated Servall. The supernatant was decanted and frozen overnight (this precipitates ATPase(I24)). The next morning the preparation was thawed and allowed to stand at room temperature for 4 hours (this destroys CoA (I22)). The preparation was then centrifuged for 30 mins at 30,000 rpm in a Spinco. The volume of supernatant was measured and it was stored frozen (overnight freezing was convenient but not essential at this stage). 50-85 ml of the ice cold, aged extract was made 70% saturated with solid  $\text{NH}_4\text{SO}_4$  (take 65 gm/100ml as 100% saturated). It was allowed to stand at room temperature for 30 mins, then centrifuged at 3-4,000 rpm for 30 mins in a refrigerated Servall. The residue was dissolved in 0.02M  $\text{KHCO}_3$  and brought to the volume of the original aged extract. This was brought to 40% saturation with solid ammonium sulfate and treated as before. After this centrifugation the supernatant was saved and brought up from 40% to 70% saturation with solid  $\text{NH}_4\text{SO}_4$ , allowed to stand and centrifuged as before. The resulting precipitate was dissolved in the minimum quantity of ice cold 0.02M  $\text{KHCO}_3$  and dialyzed overnight against 10 L of distilled water containing 50 gm KCl, 20 gm  $\text{KHCO}_3$  and

2 gm cysteine hydrochloride. The dialyzed enzyme was stored frozen in small amounts.

Assay procedure:

Incubation of substrate with  $\epsilon$ -lysine acylase was carried out as before except that HCl was not added before boiling. Graded levels of the supernatant obtained from these incubations or of standard acetate solution were used in each tube as "acetate". Dilutions were made with distilled water where required. Each tube contained:

Tris 0.05 ml, Assay mixture 0.05 ml, Cysteine HCl 0.01 ml, "acetate" 0.24 ml, enzyme 0.15 ml. A blank tube was prepared containing all the ingredients but with water instead of acetate. All tubes were gassed 1 minute with  $N_2$  and sealed with gas-tight white rubber stoppers (I16). With most enzyme preparations it was necessary to incubate 2 hours at 37° C to obtain sufficient activity.

Enzyme action was stopped by adding 2 ml TCA to each tube. After ten minutes standing, the tubes were centrifuged in a clinical centrifuge and the supernatant used for acetate determination by the Bratton-Marshall method (I26).

Determination of acetate by the Bratton-Marshall method:

This method consists of the determination of non-acetylated sulfanilamide from which the amount of acetate present in the original mixture can be calculated.

The following solutions were placed in large tubes, in order:

1.0 ml supernatant prepared as described above.

9.0 ml water,

0.5 ml 4N HCl,

1.0 ml freshly prepared 0.1%  $\text{NaNO}_2$ .

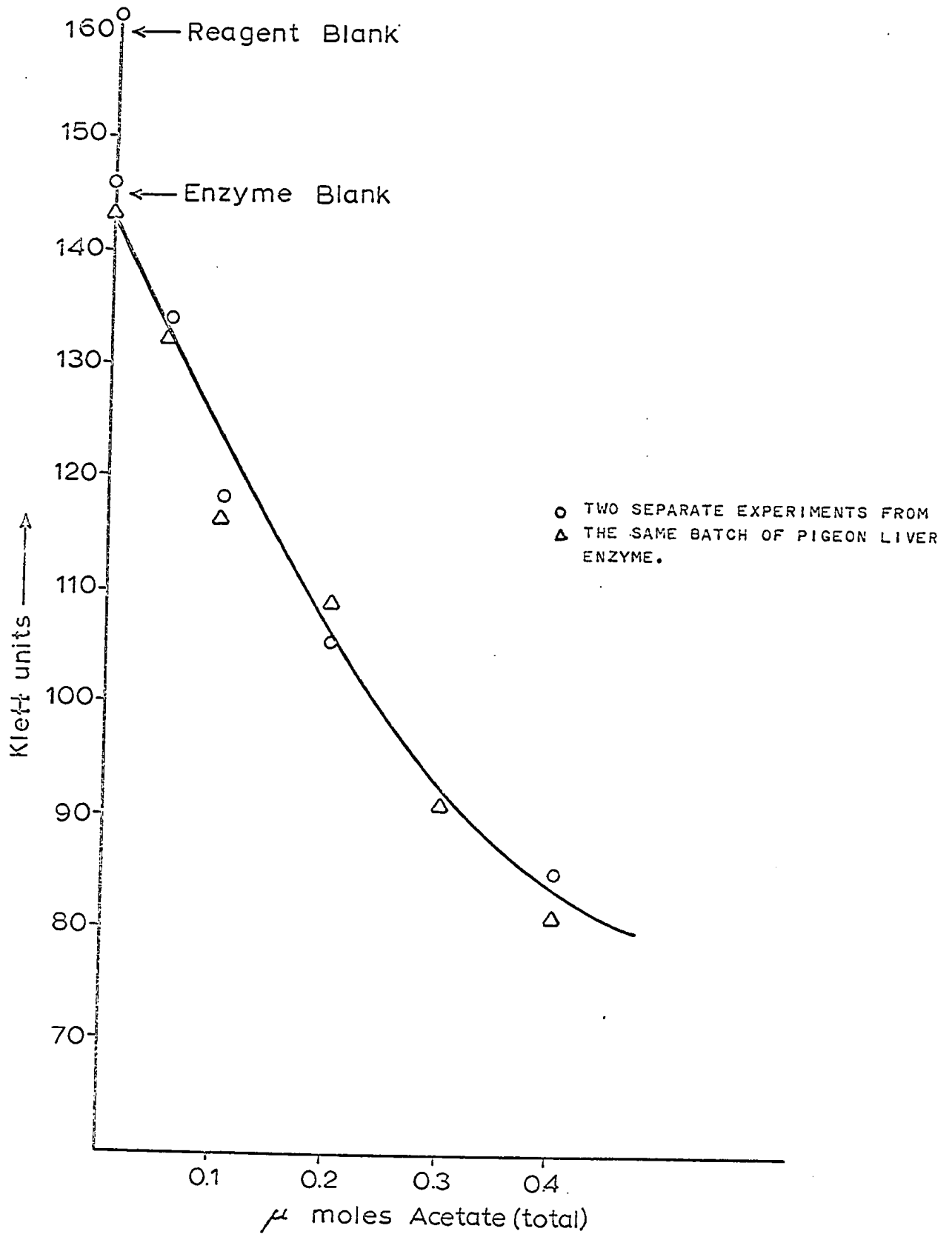
After mixing and standing 3 minutes, 1.0 ml 0.5%  $\text{NH}_4$  Sulfamate was added. Thorough mixing was required here and the best procedure was found to be to invert the tubes using a piece of "parafilm" over the end. After 2 mins standing the Bratton-Marshall reagent was added, and the whole mixed and read in a Klett colorimeter with a No. 54 (green) filter.

During the course of these experiments it was necessary to prepare several batches of pigeon liver transacetylase (EC No. 2.3.1.5.). A separate standard curve was prepared for each batch of enzyme using a solution of K $\bar{a}$ c. The figures shown in the following experiments were therefore derived from different standard curves. All curves showed the same type of result but the absolute value of any given Klett reading varied slightly due to variation in the amount of free acetate in the final enzyme preparation (121). One of the standard curves is shown in Fig. 17.

Preparation of tissue samples ( $\epsilon$ -lysine acylase):

In order to compare the substrate specificity of the chicken mitochondrial enzyme with a variety of other sources of enzyme, mitochondria and  $\text{US}_{\text{SN}}$  were also prepared from pigeon and duck kidneys. The procedures used for these latter tissues was exactly as described for chicken mito-

FIG.17 STANDARD CURVE FOR THE DETERMINATION OF ACETATE.



chondria and US<sub>SN</sub> (see p 51, p 54, pp 66-72, and final modifications p 72). The incubation period was 2 hours.

Partly purified enzyme was prepared from rat kidney by the method of Paik et al. (5). The purification was followed exactly as described, and is not therefore, repeated in detail here. In three separate runs the final purification obtained varied from 23x to 33x. When rat enzyme was to be used a few mgms. of the lyophilized product was dissolved in 0.066M phosphate buffer pH 7.2, with gentle mixing with a glass rod, to give a solution containing approximately 8 mgm/ml protein. This concentration was found to give a significant liberation of product during a 2 hour incubation period. Incubation was carried out using 60  $\mu$ moles/ml of each substrate dissolved in 0.066M phosphate buffer at pH 7.2. Reaction was terminated by placing the reaction mixture in a boiling waterbath for three minutes. The mixture was centrifuged to remove protein and the supernatant used for the determination of lysine or acetate.

NOTE: When acetate was to be determined rather than lysine, no HCl was added to stop the reaction. The incubated mixtures of substrate and enzyme were placed directly in a boiling waterbath.

The results obtained in present studies are shown in Table XXIX. Also listed for comparison purposes, are results obtained by other workers. These latter are indicated in the table by reference numbers to the publications quoted. Results

TABLE XXIX

Substrate	Source of Enzyme						
	<u>Chicken</u>		<u>Duck</u>		<u>Pigeon</u>	<u>Rat</u>	<u>Hog</u>
<u>ε-lysine</u>							
derivs. (A)	Mit	SN	Mit	SN	SN	-	-
Formyl	0	0	Tr	0	0	57	114(9)
Chloroacetyl	13	19	Tr	0	-	(161(5) 0	52(9)
Benzoyl	Tr	11	0	Tr	0	0	0(9)
Tosyl	0	0	-	-	-	0	0(9)
Carbobenzoxy	0	0	-	-	-	0	0(9)
<u>α-lysine derivatives (A)</u>							
Acetyl	Tr	38	-	-	0	-	4.3(9)
Benzoyl	Tr	0	-	-	15	-	37.7(9)
<u>Others (B)</u>							
ε-N-acetyl-L-ornithine	82	53	-	-	-	0	0(9)
α-N-ε-N-diacetyl-L-lysine	91.5*	103*	-	-	-	127*	79.5*(9)
α-N-glycyl-ε-N-acetyl-L-lysyl-glycine	75.5	68	-	-	-	168	70.3(9)
Acetyl-L-methionine	197	29	-	-	-	187	105.7(9)

(A) Lysine determined (B) acetate determined

All figures are  $\mu\text{m}$  product/hr./mgm protein, expressed as relative to  $\epsilon\text{-N-acetyl-L-lysine} = 100$ .

# See discussion in text.

\* The apparent activity of acylase I (as indicated by activity on acetyl methionine) must be taken into account in these figures.

Source of compounds:-

ε-N-Formyl-L-lysine obtained from Cyclo Chemical Corpn., Los Angeles, Calif

Acetyl-L-methionine obtained from Nutrit. Biochem. Corpn.

All other compounds tested were prepared and donated by the courtesy of Dr. N. L. Benoiton and Mr. J. Leclerc (9,127,128 and unpublished data).

obtained with bacteria and molds (6,7,73,74,103) have been omitted from this table because there are not sufficient points of comparison. The letters "A" and "B" beside groups of substrates indicate the determination of lysine or acetate respectively.

The case of  $\epsilon$ -chloroacetyl-L-lysine as a substrate for the rat enzyme is interesting and, at the moment, unexplained. Previous studies (5) had indicated that this substrate was a very good one. When compared to  $\epsilon$ -N-acetyl-L-lysine, its activity was 151% (5).

In the present studies completely negative results were obtained on repeated occasions. The only obvious difference between the two series of experiments was the method of preparation of the substrate. In earlier work, chloroacetyl lysine was prepared by digesting  $\alpha, \epsilon$ -dichloroacetyl-DL-lysine with renal acylase I (5). The preparation used in the present studies was synthesized by the method of Benoiton and Leclerc (127) using p-nitrophenyl chloroacetate as the acetylating agent. This preparation had a slightly yellow color in solution. It seemed possible therefore, that traces of p-nitrophenol contaminating the preparation might be acting as inhibitors. An experiment was, therefore, carried out to determine the effect of p-nitrophenol on  $\epsilon$ -lysine acylase from rat kidney.

Lyophilized  $\epsilon$ -lysine acylase purified from rat kidney (5) was dissolved in 0.066M pH 7.2 phosphate buffer to give 6 mgm protein/ml. The enzyme was incubated with an equal

volume of substrate (50  $\mu\text{m}/\text{ml}$   $\epsilon$ -acetyl lysine in the same buffer) to which had been added two different concentrations of p-nitrophenol. The top level of p-nitrophenol was 10 x the maximum impurity which could have been present in the  $\epsilon$ -chloroacetyl lysine sample\*. A parallel experiment was made in which p-nitrophenol was added to a solution of 10  $\mu\text{m}/\text{ml}$  L-lysine dissolved in 0.06M pH 5.8 phosphate buffer.

The rat enzyme mixture was incubated, boiled, and lysine determined in the Warburg in the usual way. The L-lysine solutions were determined directly in the Warburg as a check on any possible effect which p-nitrophenol might have on lysine decarboxylase. The results are shown in Table XXX.

The maximum p-nitrophenol contamination possible (none was ever proved but this figure represents the limit of sensitivity of the method) was less than 0.2 moles/100 moles chloroacetyl lysine\*. This would be equivalent to 0.12  $\mu\text{m}/\text{ml}$  under the present conditions. As can be seen from Table XXX up to ten times this amount had no effect on the rat enzyme and only a slight effect on lysine decarboxylase. It was concluded that the failure to obtain activity with  $\epsilon$ -chloroacetyl lysine in the present experiments was not due to p-nitrophenol contamination. Since the compound was recrystallized repeatedly, and yielded very similar results

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\* I am indebted to Mr. J. Leclerc for this determination.

TABLE XXX

Effect of p-nitrophenol on determination  
of  $\epsilon$ -lysine acylase activity.

Sample	$\mu\text{CO}_2/\text{ml}$	$\text{CO}_2$ evolved as % of Control
A) $\epsilon$ -N-Acetyl- L-lysine	26	(= 100%)
+0.48 $\mu\text{m}/\text{ml}$ pNP	37	141%
+1.2 $\mu\text{m}/\text{ml}$ pNP	31	119%
B) L-lysine	209	(107% Recovery)
+0.12 $\mu\text{m}/\text{ml}$ pNP	182	87%
+0.48 $\mu\text{m}/\text{ml}$ pNP	197	94%
+1.2 $\mu\text{m}/\text{ml}$ pNP	177	85%

A) Incubated with rat kidney  $\epsilon$ -lysine acylase, followed by determination of lysine with lysine decarboxylase.

B) Determination of effect on lysine decarboxylase only.

after different steps in the recrystallization, it seems highly unlikely that any contaminant could be responsible for the negative results. Unfortunately, none of the original preparation remained, therefore there was no way to check whether it had been contaminated with any other product.

When  $\epsilon$ -N-acetyl-L-lysine was prepared by the p-nitrophenol acetate method it gave exactly the same results as that prepared by the method described in this thesis (p 28 ). This would also indicate that the new method of preparation of chloroacetyl lysine was unlikely to have contributed any inhibitory contaminant.

One other feature of Table XXX which is of particular interest is the fact that the chicken enzyme deacetylates  $\delta$ -N-acetyl-L-ornithine. This is the first time that an enzyme of this type (ie. one active on  $\epsilon$ -N-acetyl-L-lysine) has been shown to act on a compound with other than 6 carbons. The positive result obtained in this case was confirmed by chromatography. When the reaction mixture (after incubation of chicken mitochondria with  $\delta$ -N-acetyl-L-ornithine) was chromatographed by ascending chromatography in formic acid: t-butanol: water, 15:70:15, for three hours, clear separation of acetyl ornithine and free ornithine was obtained. These compounds were identified by comparison with the pure samples. No free ornithine was seen in either the tissue preparation or the acetyl ornithine substrate under the same conditions.

Discussion and summary.

Some of the characteristics of  $\epsilon$ -lysine acylase from chicken kidney mitochondria have been determined. The following values were obtained:

	<u>K<sub>m</sub></u>	<u>V max.</u>	<u>Activn. E.</u>
Whole mitoch.	-	-	8.6 kcals/mole
Rupt. mitoch.	$6.2 \times 10^{-2}$	0.85 $\mu$ m/ hr./mgm	4.3 kcals/mole

The high  $K_m$  value, even with ruptured mitochondria, and the decrease in activation energy on rupturing the mitochondria, probably reflect the crudeness of the enzyme preparation. It has already been pointed out that the best enzyme preparations obtained were not solublized, and that the enzyme behaved as if it was masked in some manner (either physically - by membrane fragments to which it was attached, or by some chemical or conformational barrier). For this reason the values obtained in this study can only be regarded as preliminary.

The fact that the activation energy for ruptured mitochondria is half that for whole mitochondria may reflect the removal of part of this theoretical "masking". Certainly, rupture of the mitochondria would decrease any permeability barrier. However, it is also known that mitochondrial glutamic-oxaloacetic transaminase is activated by brief treatment with ultrasonic (112). Other "latent" mitochondrial enzymes are known to be unmasked by ultrasonic treatment (129). The present data is insufficient to permit any conclusions but, in view of the above considerations, it is possible

that the activity of the  $US_{SN}$  represents a balance between the unmasking of a "latent" enzyme and destruction of some of that enzyme. If this were true then the fact that the  $US_{SN}$  has approximately the same specific activity as the mitochondrial preparation ( $p^{100}$ ) would be purely fortuitous. In this connection it would be interesting to determine the effect of brief ultrasonic treatment on mitochondria which had previously been ruptured by some other means.

In studying the effects of inorganic ions on this enzyme it was found that most of the effects were on the mitochondria, rather than on the enzyme. However, these results are not entirely consistent with simple swelling of the mitochondria. For example, it has long been known that magnesium and manganese stabilize mitochondria against swelling, and calcium causes swelling (I30). Yet, as can be seen from Table XXVI, calcium and magnesium are stimulatory, while manganese is inhibitory.

The change of the effect of lithium ions - from a stimulatory one on whole mitochondria to an inhibitory one on ruptured mitochondria - is also interesting. It is known that sodium ions are required for optimum amino acid transport in the kidney cortex (I31), but that active transport of lysine and histidine continues in the absence of sodium, whereas active transport of neutral amino acids does not (I31). In this system (of kidney slices), lithium would not replace sodium, but in isolated nuclei lithium would replace sodium to some extent (I32). It is obviously not wise to

compare tissue slices with isolated subcellular particles. Unfortunately, published work on the effects of inorganic ions on isolated mitochondria seems to have been concerned primarily with oxidative phosphorylation and high energy compounds (133,134). One other report which is of interest in the present context (despite the fact that the test system was rat diaphragm slices) is that of Levinsky et al. (135). These authors report that when rat diaphragm was incubated in a medium rich in lysine, up to one third of the tissue  $K^+$  was lost into the medium. This might suggest that lysine was acting as a simple cation. On the other hand, it has been shown that lysine cannot substitute for  $Na^+$  in the transport of neutral amino acids in the kidney (131). No correlation can be made between these findings and the present results until the effects of inorganic ions can be tested on a purified enzyme preparation. It is interesting, however, to note that lysine again shows properties which set it apart from most other amino acids.

A third point of interest is the complete lack of inhibition by ammonium sulfate under any conditions - even up to saturation. The results obtained with ferrous and ferric salts confirm those obtained when this enzyme was incubated in an atmosphere of nitrogen i.e. the oxidative systems have little effect on this enzyme.

The results obtained with a variety of substrates tested with enzyme preparations from different tissues have

been discussed in the text and will be referred to again in the general discussion on isoenzymes (p 149). Only one further comment is required here. It can be seen from Table XXIX that the activity of the chicken kidney preparation towards acetyl-L-methionine drops sharply when whole mitochondria are converted to  $U_{SN}$ . This provides a useful index for the removal of much of the acylase I of the original preparation by this procedure.

### GENERAL DISCUSSION

For many years review articles on lysine metabolism have emphasized the need for detailed studies with isolated enzyme to clarify the many uncertainties (136,137). One of the uncertainties in the catabolic pathway of lysine in animals, is the role of  $\epsilon$ -N-acetyl-L-lysine. The original suggestion by Neuberger and Sanger (4), that the formation of this compound was the first step in lysine degradation is still not proved. Moreover, it has not yet been found as a free amino acid in vivo (35) although it has been shown that it can be formed enzymatically by a beef liver preparation (36). One approach to these problems lies in investigations of  $\epsilon$ -lysine acylase activity. Since this enzyme is widely distributed (5,6,8,9), and is able to deacylate  $\epsilon$ -N-acetyl-L-lysine, investigations of this enzyme must, eventually, provide an indication of the relationship of its substrate to other metabolites. For example, it has been shown that this enzyme is also able to deacylate  $\alpha$ -keto- $\epsilon$ -acetamidocaproic acid (35), which is also implicated in the degradative pathway of lysine. For this reason, the present studies have been undertaken to continue the basic investigations into what this enzyme does, in various tissues, under various conditions.

One question considered at the beginning of this thesis was that of the distribution of  $\epsilon$ -lysine acylase, and whether this might reveal any implications as to its

metabolic function. Extension of the survey of enzyme occurrence, begun by Paik et al. (5,8,9), has revealed that the localization of any significant, measurable amounts of enzyme in the kidney, in birds, is highly specific. If the enzyme can be detected at all in birds, it is in the kidney, and only in the kidney. Furthermore, the experiments with duck, pigeon, turkey, and rabbit kidney preparations have confirmed that this enzyme is characteristically a mitochondrial enzyme, with a pH optimum of 9.0 in birds, whereas it is characteristically a soluble enzyme with a pH optimum of 7.2 - 8.0 in mammals. The significance of the subcellular distribution is not apparent at present. It is one of the characteristics by which  $\epsilon$ -lysine acylase in fowl kidney shows a greater resemblance to the chicken kidney mitochondrial enzyme which deacylates aromatic compounds (138) than it does to  $\epsilon$ -lysine acylase isolated from mammals. (This resemblance will be discussed in more detail later in this discussion).

With regard to the presence or absence of  $\epsilon$ -lysine acylase in various birds, nothing conclusive can be stated, but two speculations are attractive on the basis of the present results. In the first place, all birds and animals which showed marked enzyme activity were either barnyard birds, or laboratory animals on commercial diets. Those which gave negative results were either on a more varied diet (such as mongrel dogs and the one human sample) or on a totally different commercial diet (such as the small cage

birds). The indication that this distribution pattern may be due to enzyme induction as a result of diet have already been discussed (pp 55-58).

The second speculation arises from the negative results obtained with dog and human kidney. Rosenberg et al. (95), studying amino acid transport in the rat kidney, comment on some interesting differences between the cat and the rat on one hand, and the dog and the human on the other. In human cystinuria, the renal tubular reabsorption of all four dibasic amino acids - arginine, ornithine, lysine, and cystine - is defective (I39) and competitive (I40). The methods used in these studies could not distinguish between non-competitive inhibition due to toxic or osmotic effects, and true competitive inhibition due to common transport systems (95). Rosenberg and co-workers, therefore used an in vitro system to investigate this question. However, whereas other workers had used human or canine subjects, Rosenberg et al. used rat tissue. They found that in their system cystine neither inhibited the transport of the other three nor was inhibited by them. It has also been shown that the Kenya genet (a cat with genetic cystinuria) excretes only cystine (I41). This is in contrast to human cystinurics who excrete all four amino acids with cystine and lysine showing an increase in the urine before arginine and ornithine. These authors therefore postulate that dog and human have a common renal transport system for all four amino acids, whereas the rat (and possibly the cat) have a different system in which cystine

does not compete with the other three amino acids.

It is interesting that the two species which appear to have one type of renal transport system for basic amino acids show  $\epsilon$ -lysine acylase activity in the kidney, and two species which have a different renal transport system do not. Such nebulous ideas cannot even be called a speculation, but they to emphasize that it would be interesting to watch for any possible correlation between this enzyme and amino acid reabsorption in the kidney - as suggested at the start of this work (see p 21 for other references on this topic).

As was mentioned in the text (p 59), attempts to purify  $\epsilon$ -lysine acylase from chicken mitochondria were unsuccessful, and very few conclusions can be drawn because the experimental conditions were unsatisfactory. Amongst these attempts were some which seemed hopeful and which were therefore, repeated as many as twelve times in a search for variable factors. The general indications of these experiments can thus be considered reliable even if the purification obtained was useless. Three of these experiments are the use of ultrasonic vibrations to rupture mitochondria, the attempts to precipitate active enzyme with ammonium sulfate, and the precipitation of enzyme at acid pH followed by attempts to dissolve it. Both types of precipitation indicate that the enzyme precipitated or dissolved with the bulk of the protein at each step (see p 74 and p 76). Such behaviour would suggest that the enzyme might be attached to other bulky proteins such as

fragments of mitochondrial membranes. This is supported by centrifugation after ultrasonic treatment. It was shown (p 68) that most of the enzyme remained in suspension at 27,000 x g/20 mins. but that none of it remained in the supernatant after centrifugation at 105,000 xg for one hour. These results suggest that ultrasonic treatment yielded small particles which had enzyme activity, not soluble enzyme per se. and that solubilization, rather than precipitation, must be the next step in further attempts to purify this enzyme.

It is interesting that in the problems encountered in attempts to purify this enzyme, several similarities to the aromatic-N-deacylase of chicken kidney are also seen. (see p 156).

Substrate specificity of  $\epsilon$ -lysine acylase in birds.

During the present investigation one very unexpected result was obtained. It is shown in Table XXIX that the enzyme from chicken mitochondria deacylates  $\delta$ -N-acetyl ornithine. This is the first time that an enzyme of this group has been shown to be active on a compound which does not have a 6-carbon chain. It would seem, from the results compiled in Table XXIX that the chicken enzyme is specific for the acetate group, whereas enzymes prepared from other sources appear to be specific for the 6-carbon chain. The failure of the chicken enzyme to act on other lysine derivatives to any marked degree is not due to the size of the N-substituent. This is shown by the fact that the formyl derivative, which is the smallest N-substituent

tested, is negative. Berlinguet and Charbonneau (142) have discussed the effect of a series of N-substituted aspartic acid derivatives as inhibitors in the synthesis of citrulline by a particulate fraction of rat liver. These authors noted a similar lack of relationship between molecular size and inhibitory effect. In their case, however, the effect of the N-substituted derivatives could be related to the charge and reactivity of the molecule. In the present work, no such correlation can be made either with whole mitochondria where transport might be a factor, or in ruptured mitochondria where steric hindrance or difference in charge might prevent the substrate from forming an active ES complex. It would appear, therefore, that the specificity of the chicken enzyme, for an acetyl group, is probably a true specificity despite the crudeness of the present preparation.

In animals, on the other hand, no compound has shown significant activity unless the 6-carbon chain was present (9,5). L-lysine derivatives which have failed to show activity have all been compounds in which the substituent at the epsilon position was considerably larger than acetate. Some confusion is introduced by the variable levels of acylase I in various preparations but if the  $\epsilon$ -N-derivatives are considered alone, the following pattern can be seen:

- 1) Tosyl-, Benzoyl-, and carbobenzoxy- derivatives are not attacked by enzyme preparations from the rat or hog. (see Table XXIX).

- 2) formyl- and acetyl- derivatives are attacked (even when the lysine residue is involved in a bulky peptide, as in  $\alpha$ -N-glycyl- $\epsilon$ -N-acetyl-L-lysylglycine) (see Table XXIX).
- 3) Similarly Biocytin (5),  $\epsilon$ -glycyl-L-lysine, and  $\epsilon$ -N-acetyl-glycyl-L-lysine (9), - all of which have bulky groups at the epsilon position - are not attacked by the rat and hog enzymes respectively.
- 4) It has been reported that  $\epsilon$ -N-piperidyl-DL-lysine and  $\epsilon$ -N-cyclohexyl-DL-lysine will not replace lysine in the diet in the mouse (I43). It is concluded from this that there is no enzyme in the mouse able to split off these alkyl groups (I43).

These results indicate once more that the substrate specificity of  $\epsilon$ -lysine acylase in birds is quite different from that of the enzyme(s) found in animals. However, it would appear that the difference is not just a matter of relative activity with certain substrates. It would appear, on the basis of evidence available at present, that the chicken enzyme is specific for the acetyl group (and the length of the carbon chain may not be important) whereas the rat and hog enzymes appear to be specific for lysine derivatives in which the epsilon substituent is small in size.

Birds are unable to carry out the conversion of ornithine to citrulline (I44). It is, therefore, tempting to relate this fact to the finding that chicken kidney  $\epsilon$ -lysine acylase can deacylate  $\delta$ -N-acetyl-L-ornithine whereas animal prepa-

rations cannot (5,9). At the present time, however, such speculation would be premature. Since ornithine is the only compound tested in the present system which does not have 6 carbons, it cannot be stated that it is unique. It may be that the chicken kidney enzyme preparation is capable of deacylating many other compounds which have not yet been tested. Secondly, as discussed on p 156, there is a good probability that the enzyme preparations used in the present work also contained an enzyme capable of deacylating aromatic N-acetyl compounds. The substrate specificity of this second enzyme has not been tested on aliphatic compounds.

#### ε-lysine acylases from various sources as isozymes

In 1959 Markert and Møller proposed the theory of isozymes. According to these workers many enzymes exist in different molecular forms, with the same biological action, but with small differences in their physical characteristics (I45).

Isoenzymes were first discovered by differences in charge which made it possible to separate them electrophoretically (I45). They have also been distinguished by differences in the pH optimum of their action, their  $K_m$  values with a single substrate, their reaction with inhibitors, and their different immunological behavior (I46, I47, I48, I49). At the commencement of this work it was known that ε-lysine acylase isolated from different sources showed different pH optima, different subcellular distribution, and different

substrate specificity between bacteria and molds on the one hand and various animals and birds on the other. The question arose as to whether these differences reflected different enzymes or isozymes.

Although isozymes are usually considered to consist of multiple enzyme forms existing in the same tissue, this is not always apparent when the enzyme preparations are crude. In actual fact the proportions of isozymes which compose the "whole enzyme" can reveal the tissue of origin with sufficient precision to be a useful diagnostic tool. The present use of electrophoresis of lactic dehydrogenase of plasma to distinguish between heart and liver damage is an example (150).

Secondly, enzymes existing in different subcellular fractions of the same tissue have been shown to catalyze the same reaction in opposite directions. Boyd (128) has shown, for example, that for the glutamate-oxaloacetate transaminase of rat liver the mitochondrial fraction has a higher  $K_m$  value with  $\alpha$ -ketoglutarate as substrate than with L-aspartate as substrate. The enzyme from the soluble fraction of the same tissue shows the reverse relationship (128). Moreover, the pH-activity profile of the enzymes from the two cell fractions are also totally different. The mitochondrial enzyme shows virtually a plateau over a wide pH range whereas the soluble fraction shows quite a sharp peak around pH 8.5 (112).

It is only in the case of lactic dehydrogenase that

the chemistry and genetics of the relationship of isozymes to each other is beginning to be clarified. It has been shown that the five isozymes of this enzyme consist of hybrids of two distinctly different subunits (distinguished immunologically). It is therefore postulated that the five isozymes of lactic dehydrogenase are controlled by only two genes (I48). With other enzymes the picture is less clear and there is some evidence that one genetic locus may control a whole "family" of esterases (I51), and that the method of extraction of the enzyme affects the electrophoretic patterns obtained (I52).

The original definition of isoenzymes was of enzymes with the same function but slight differences in molecular form and, therefore, in some physical properties. In recent literature many workers refer to these enzymes as "multiple molecular forms" rather than as isoenzymes because the original term no longer has a clearly defined and limited meaning (I53). Without a sharp definition of terms it is hard to make an arbitrary decision as to whether  $\epsilon$ -lysine acylases from different sources are isoenzymes or different enzymes. Such decision will have to await the comparison of the electrophoretic patterns of pure enzymes from each source. In the meantime, all that can be said is that the differences between the  $\epsilon$ -lysine acylases found to date are not much greater than differences found in electrophoretically pure isoenzymes. An example of this is provided by the data of Moore and Angeletti (I46). These authors compare

isoenzyme fractions of acid phosphatase obtained from different organs and animals. They show that the individual isoenzymes (as indicated by specific electrophoretic bands) differ in their optimum pH (from 4.0 - 5.0), relative activity towards two substrates and many other properties. The proportions of each isoenzyme fraction found in each tissue also differ. The crude enzyme from each tissue would, therefore, show different properties according to which fractions predominated in that tissue.

In other cases it is possible that a single source may contain a single isoenzyme. For example, yeast alcohol dehydrogenase differs from horse alcohol dehydrogenase in its reaction rate with coenzyme analogues, in molecular weight, number of zinc atoms per molecule, and response to denaturing agents (154,155). In the matter of molecular weight, and number of zinc atoms per molecule, it would appear that one preparation might be simply a double unit of the other, yet these two enzymes are referred to in discussions of isoenzymes by some workers (155).

It must be asked whether the differences in  $\epsilon$ -lysine acylase from different sources is any greater than the differences which are encompassed by the term "isoenzyme" as used at present. Some of the data now available on  $\epsilon$ -lysine acylase from different sources is compiled in Table XXXI.

It can be seen from this table that there is no apparent common factor between the different enzyme preparations.

TABLE XXXI

Comparison of  $\epsilon$ -lysine acylase from different sources

Source of Enzyme	opt.pH	Km*	Relative activity( $\epsilon$ -N-acetyl-L-lysine = 100%)		
			$\epsilon$ -N-benzoyl-L-lysine	$\epsilon$ -N-chloroacetyl-L-lysine	$\epsilon$ -N-formyl-L-lysine
Rat	7.2(5)	-	-	161(5) 0 <sup>#</sup>	57%(5)
Hog	8.0(9)	4.6x 10 <sup>-3</sup> M	0(9)	52(9)	114(9)
Chicken USSN	9.0	5.2x 10 <sup>-2</sup> M	11	19	0
Achromobacter pestifer EA	4.8- 5.2(103)	-	200(103)	550(103)	-
Aspergillus oryzae	8.2- 8.4(7)	-	no value but benzoyl(7) >> acetyl	-	-

\* with  $\epsilon$ -N-acetyl-L-lysine as substrate. Detmnd at opt. pH of each enzyme respectively.

# See discussion in text p 134.

Numbers in brackets are references to work not in this thesis.

The differences in pH optima are very extreme. On these grounds it would seem that the bacterial enzyme must be distinct from the others. On the other hand, differences in substrate specificity would seem to place the mold enzyme and the bacterial enzyme in the same group. Further comparisons of substrate specificity can be made from Table XXX. In this table a comparison of the chicken, rat, and hog enzymes show a general similarity, although the chicken enzyme would appear to be more highly specific for the acetyl group than the other preparations. There are also differences in the reaction of the various enzyme preparations to inorganic ions (shown in Table XXXII). While the impurity of some preparations makes it difficult to draw positive conclusions, it would appear that the enzymes from bacteria and mold are primarily acyl enzymes, (ie. benzoyl lysine is their prime substrate).

The enzymes from rat and hog also appear to be acyl enzymes, but with  $\epsilon$ -N-acetyl-L-lysine as their most reactive substrate. The enzyme from chicken, on the other hand, would appear to be an acetyl enzyme because of its high specificity for acetyl derivatives. The substrate specificity of these enzymes has already been discussed and further comments are made in the next paragraph. It remains at the moment, to assess the interpretation of these differences in relation to the question of isoenzymes. As stated above, the matter cannot be decided unequivocally without comparison of the electrophoretic patterns of purified enzymes. However, on the basis of the available data, it would seem that there

TABLE XXIII

Effect of inorganic ions on  $\epsilon$ -lysine  
acylase from various sources.

ION.	Chicken(US <sub>SN</sub> )	Rat(5)	Hog(9)	Bacteria(103)	Molds(7)
Ca <sup>++</sup>	Inhib.	*prob. essent.	No effect	No effect	No effect
Mg <sup>++</sup>	Inhib.	*as Ca <sup>++</sup>	" "	" "	" "
Co <sup>++</sup>	Inhib.	*as Ca <sup>++</sup>	" "	" "	Inhib.
Mn <sup>++</sup>	Inhib. <u>or</u> stim.	No effect	" "	" "	No effect
Ni <sup>++</sup>	Inhib.	*as Ca <sup>++</sup>	" "	" "	Inhib.
Fe <sup>+++</sup>	No effect	-	" "	stim.	-
Cu <sup>++</sup>	V. inhib.	-	-	-	Inhib.

\* partly relieves inhibition due to Veronal ∴ probably one but  
not all of these ions required.

Numbers in brackets refer to work not covered in this thesis.

See references.

might be as many as three different enzymes - one in micro-organisms, one in animals, and one in birds.

Comparison of the chicken  $\epsilon$ -lysine acylase with chicken aromatic N-deacylase.

Reference has been made throughout this discussion to the similarity between the enzyme under consideration here and the aromatic N-deacylase of chicken kidney (E.C. No. 3.5.1.a). Aryl-N-deacylase activity has been shown to be primarily a function of the microsomes in rat liver (157), but it has also been found in intestine (158). This enzyme has been investigated primarily with respect to its ability to metabolize carcinogens (157,158), to acetylate serotonin (153,154) and sulfonamides (161, 162). These studies are not relevant to the present discussion except that from them came the discovery that chicken kidney contained an enzyme which deacetylated sulfonamides (162). Only one more recent paper on this enzyme from chicken kidney appears to have been published (138) and it is in this paper that a similarity is revealed between the properties of the enzyme which acts as aryl-N-deacylase and the enzyme which has been investigated in the present study. These similarities can be summarized as follows:

1) both enzymes are found in chicken kidney mitochondria and appear to be attached to other proteins (possibly to membrane fragments). This revealed by similar centrifugation patterns and similar behaviour with Triton-X 100.

- 2) both enzymes show similar pH optima in different buffers. For example, in glycine/NaOH buffer  $\epsilon$ -lysine acylase shows a pH optimum of 9.0, aryl-N-deacylase shows a pH optimum of 8.5. In view of the fact that different substrates were used and the pH optimum for several enzymes is known to change with the substrate used (26,112), this is quite close agreement.
- 3) the general physical characteristics of both enzymes are similar. Optimum temperature, temperature of inactivation, stability when stored frozen, stability as a lyophilized preparation (152), and stability as an aqueous suspension (162), are all similar for both enzymes.
- 4) there are differences in specificity for the N-substituent but in the case of the aryl-N-deacylase these are themselves markedly affected by other substituents on the aromatic ring. The reaction of the same N-substituents on a straight-chain compound would not, therefore, be expected to be the same as the corresponding aromatic compounds even if a single enzyme was concerned.
- 5) apparent  $K_m$  values obtained with chicken kidney aryl-N-deacylase vary from  $7.4 \times 10^{-3}$  to  $5.4 \times 10^{-2}$  depending on the substrate. It is interesting that the value of  $5.4 \times 10^{-2}$ , obtained with p-hexanamidobenzoic acid, indicates an unusually high value - similar to that obtained with  $\epsilon$ -N-acetyl-L-lysine in the present studies (see p 107 ).

There is no evidence that the chicken kidney aryl-N-deacylase has been tested on aliphatic compounds. Neither was

the present enzyme tested on any aromatic compounds. It cannot therefore be stated whether these two enzymes are a single enzyme. It must be concluded, however, that the aryl-N-deacylase would have been present in the enzyme preparations used in the work reported in this thesis. It would appear essential that the relative activity of each fraction towards aliphatic and aromatic N-acetyl compounds should be tested in all future attempts to purify either enzyme from chicken kidney mitochondria.

On the basis of the present evidence it cannot be concluded whether the enzyme studied in the present work is a variety of the  $\epsilon$ -lysine acylases found in animals, a further investigation of aryl-N-deacylase, or a third deacylating enzyme. The similarity of some of the physical characteristics of the two chicken kidney enzymes would suggest that the enzyme involved in the present study may have been either the aryl-N-deacylase or a mixture of two enzymes - one of which was the aryl-N-deacylase.

Summary of new work presented in this thesis.

$\epsilon$ -lysine acylase from various sources has been investigated. The enzyme shows distinct distribution patterns depending on the source - both as to organ distribution and as to subcellular distribution. There are also indications of a different substrate specificity related to source. The enzyme from chicken mitochondria was investigated in detail. No marked purification was obtained but some of its characteristics were examined. These were found to show resemblances not only to  $\epsilon$ -lysine acylase from animals but also to aryl-N-deacylase from chicken kidney.

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