BIOCHEMICAL STUDIES ON THE CATECHOLAMINE CONTAINING GRANULE OF THE ADRENAL GLAND

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INTRODUCTION

The existence of catecholamine containing granules in the adrenal medulla is a well established fact. Since independently Blaschko and Welch (1) and Hillarp and coworkers (2) showed that adrenaline and noradrenaline are stored in the adrenal medulla within specific granules, a large number of investigators have attempted to elucidate the mechanisms by which the storage and the release of these amines are taking place.

Because of the high concentration of catecholamines found in the adrenal medulla, it has been assumed that they are retained within the granules by some chemical means The observations of several groups of workers that ATP is always released concomitantly with catecholamines led them to assume that this nucleotide plays an important role in the storage and the release of catecholamines. Numerous evidences have been presented which agree with the possibility that ATP conjugates with the catecholamines to form a stable complex within the chromaffin granule.

The catecholamines being firmly bound within the granule, and released only under specific conditions, it seemed that a study on the release of adrenaline and noradrenaline might shed some light on the mechanism. There

exists a great number of substances, natural or synthetic, capable of exerting a cholinergic action on the adrenal medulla. The mechanism of action of these substances is direct or indirect, via hypoglycemia for example. Insulin belongs to this second group.

It was during these investigations that several groups of workers, independently, showed that the release of ATP and of catecholamines is also accompanied by the release of proteins. The investigations shedding some light on one aspect of the problem, created a new one: is the catecholamine containing granule completely destroyed during the liberation of catecholamines, or is the ghost or granular structure retained intact and reutilized?

When this work was started, recent investigations had shown that ethionine is a direct depletor of the liver ATP. It was thought that the problem of storage could be attacked through that angle, and an attempt was made to study the effect of ethionine on the possible release of catecholamines, via ATP, in several rat organs.

Since it is possible to obtain radioactive precursors of high specific activity, the labelling of granular ATP, catecholamines and proteins was tried in order to measure the half-life of these substances in the granules, and see wether they have the same turn-over rate. The attempts

with ethionine as well as with the labelling experiments were not successful. It was then decided that an indirect approach to the problem might lead to some interesting findings.

The depletion of catecholamines, ATP and proteins in chromaffin granules of the adrenal glands from guinea pigs pretreated with carbachol, insulin or reserpine was studied. The depletion as well as the repletion of the granules from the adrenal glands were carried out *in vivo*

The fate of the catecholamine containing granules was further investigated by testing the *in vitro* capability of the granules to pick up ^{14}C-adrenaline, after the adrenal glands had been depleted by an *in vivo* administration of either one of the three drugs cited above.

The results of this work are presented in the next pages as well as a discussion of the experimental evidences presently available from other groups of workers.

REVIEW OF THE LITERATURE

PART I. THE CATECHOLAMINE CONTAINING GRANULE

The studies pertinent to the presence of catecholamine containing granules within the adrenal gland have been extensive. Most of the work has been done on the ox adrenal medulla, but some data on other species are also available. In all the species studied, the presence of catecholamine containing granules in the adrenal glands has been detected. The existence of catecholamine storage granules as specific cell organelles of the adrenal gland is well established (1,3,4,5). A detailed account of the experimental evidences which led to their identification will be presented in section A of this part I. The second section B will be devoted to the presentation of the experimental results which permitted to elucidate the chemistry of the granule.

A) Evidences for the existence of catecholamine containing granules within the chromaffin cell :

The cells of the adrenal medulla yield a chromaffin reaction, first described by Henlé, in1865 (6). It was observed that in the presence of chromate, the adrenal medullary cells became brown or black. Numerous authors could confirm Henlé's findings using chromate or some other oxidizing agents to stain

the adrenal glands (7,8,9,10).

In 1918, Cramer reported that cells from rat or mouse adrenal glands, when treated with osmic acid, showed black isolated spots. Cramer's observation was the first to indicate that the catecholamines are localized in discrete packets within the chromaffin cells. This author called these packets "adrenaline granules" (11).

The observation of Cramer passed largely unnoticed until 1953, when two independent groups of workers, Blaschko and Welch, in England, (1), and Hillarp and coworkers, in Sweden, (2) produced conclusive evidences for the existence of subcellular granules which contained most of the adrenal medullary hormones: adrenaline and noradrenaline. Both groups utilized differential centrifugation technique as developed by Hogeboom and coworkers for the isolation of the cell organelles (12) These two independent reports opened a new era in the field of catecholamines. They therefore will be discussed in details now.

Blaschko and Welch prepared homogenates of bovine adrenal medulla in 0.3 M sucrose They measured the catecholamine content of the nuclei free homogenate and that of a sediment obtained by centrifugation of the nuclei free homogenate at 22,000 x g for 30 minutes. The catecholamine content of both extracts was determined by a biological method: the pressor activity of these two extracts was measured on the atropinized spinal cat. The amount of

adrenaline recovered in the 22,000 x g sediment averaged sixty two per cent of the total amount. According to these authors, in the bovine adrenal medulla, the bulk of the catecholamines is contained within cytoplasmic particles, the sedimentation properties of which resembled those of mitochondria (1).

In the work of Hillarp and coworkers, bovine adrenal medullae were homogenized either in isotonic sodium chloride, or in isotonic potassium chloride, or in 0 3 M sucrose. After the removal of cell debris at 600 x g centrifugation, the granular fraction was collected either by a 30 minute centrifugation at 6,800 x g, or by a 10 minute centrifugation at 24,000 x g, so that it was submitted to a total centrifugal gravity of about 200,000 x g. To determine catecholamines, the authors measured the intensity of the color developed during oxidation of the amines by iodine (13). By this method, Hillarp and coworkers were able to recover 70% of the total catecholamines in the sediment, while the remainder of the catecholamines was found in the supernatant In order to assess the purity of the preparation, the isolated granules were dyed with alanine and fuschin, a specific dye for the mitochondria Examination under the light microscope did not show any stained granules. This observation permitted Hillarp and his associates to conclude that in the bovine adrenal medulla, most of the catecholamines are contained within cytoplasmic granules which are histologically

distinct from mitochondria (2).

In a later study, Blaschko and coworkers reported that the granular fraction could be divided into two layers, the physical appearance of which was not similar. The layer sedimenting at the bottom of the centrifuge tube was brownish and densely packed, whereas the top layer was yellowish white and more fluffy in appearance. The examination of the catecholamines and of the respiratory enzyme activity revealed that the bottom layer comprised a large amount of the catecholamines, while most of the succinic dehydrogenase activity was found in the upper layer. Thus the specific activity of the catecholamines was high in the bottom layer, on the contrary the specific activity of the succinic dehydrogenase was high in the upper layer. The intravenous injection of the intact granules into the atropinized cat did not elicit an immediate pressor response in the animal which seems to indicate that the catecholamine containing granule must be destroyed before adrenaline can exert its physiological effect (14).

When the 11,000 x g sediment was layered on 1.5 M sucrose solution, a 60 minute centrifugation at 140,000 x g yielded a sediment rich in catecholamines; the top of the tubes showed a small layer which included about 10% to 12% of the total amines. The separation was probably due to a difference in the densities of the various granules which might not represent a homogenous preparation (15)

This observation was confirmed during investigations by sucrose density gradient centrifugation of the 11,000 x g sediment. The density gradient tube was sectioned into five fractions numbered from one to five starting at the top of the tube. The catecholamine rich granules were found mainly in the fraction four, while most of the mitochondrial enzymes were recovered in the fraction two. This was the confirmation that the granules are distinct cytoplasmic entities (16,17)

Thus the sucrose density gradient technique can be used to prepare pure samples of catecholamine containing granules, free of mitochondria, of lysosomes and of microsomes (18,19).

Under identical experimental conditions, Hillarp was able to recover 79% to 84% of the total catecholamines in layers which were situated between fractions three and five of the sucrose density gradient tube (20).

The biochemical evidences for the existence of catecholamine containing granules have been complemented by numerous histological data. Electron microscopic studies on slices of the adrenal medulla from rat (21), mouse, guinea pig, cat (22) and rabbit (23) showed densely osmophilic granules nearly 0.2 u in diameter. These granules appeared to be surrounded by a limiting membrane and were easily distinguished from other cell organelles. Identical electron microscopic evidences were obtained with granules isolated from the ox adrenal medulla (24).

It has been reported that the chromaffin cells could be differentiated in noradrenaline and adrenaline cells (25,26,27). It has also been claimed that adrenaline containing granules could be separated from noradrenaline containing granules (17,28)

B) Chemistry of the catecholamine containing granule :

Since the discovery that most of the catecholamines of the adrenal glands are concentrated within the granules, the chemical composition of these granules has been thoroughly investigated (24,29)

In 1954, Hillarp and his associates showed that the granules represent about 33% of the wet weight of the chromaffin cells and 40% to 42% of the total cytoplasmic proteins. The catechol cholamines contributed 4.3% of the wet weight, and 19% of the dry weight of the granule. On a dry weight basis, the lipids represented 24% of the granule. The phospholipids were the major component, but cholesterol was also detected in significant amount in the granule. These data were gathered on the granular fraction prepared by differential centrifugation. The possibility that numerous contaminant particles such as mitochondria, lysosomes and microsomes contributed for a large part to these results was not eliminated (30). This induced Hillarp and coworkers to revise their technique of isolation and led to a more precise determination of the chemical composition of the granule.

In a later study on isolated granules, obtained by the

sucrose density gradient technique, Hillarp published the chemical composition of the granules as such: water: 68.5%; proteins: 11.5%; catecholamines: 6.7%; adenosine phosphate(s): 4.5%; lipids: 7%. These data represent the percentages of the various components calculated on the dry weight of the granules (20,31). Although they include the major components of the solid content of the granule, significant amounts of magnesium, of calcium and of ribonucleic acid have been characterized in the catecholamine containing granules (5,32). The molar ratio of adenosine triphosphate to the cations has been found to be 1.21 (33).

1) Adenosine triphosphate (ATP)

Of great interest was the observation made by Hillarp and coworkers that in the ox adrenal medulla ATP is found in very large amounts (34). According to these authors, ATP is detected in the granules rich in catecholamines in a concentration of 500 milligrams per 100 grams of wet weight tissue. A slightly higher value, 505 milligrams per 100 grams of wet weight tissue was mentioned a little later by the same authors (35). It was thought that an error by defect due to the limitations of the method used for the ATP determinations might indicate a lower value than that actually present in the tissue. Further analysis demonstrated that ATP was the main phosphate ester in the granule, but traces of adenosine monophosphate (AMP) and significant amounts of adenosine diphosphate (ADP), up to 20% of ATP were also detected. However,

the possibility that ADP was an artefact due to the mode of preparation of the granules was suggested, on the basis that a significant ATPase activity had been noticed in the granules isolated from the ox adrenal medulla (20). Later studies could not confirm the presence of the ATPase in the isolated granules from ox adrenal medulla (17,36,37). More recently, the presence of ATPase in the granules of the ox adrenal medulla has been encountered (38).

The subcellular distribution of ATP in the ox adrenal medulla has been investigated. ATP is mainly concentrated within the catecholamine containing granules. Blaschko and coworkers noticed that their "bottom layer" containing the granules rich in catecholamines was also rich in ATP. Some of the nucleotide was found as well in the "top layer" which was poor in catecholamines. The molar ratio of adrenaline to ATP was equal to 6.7 for the bottom layer and to 9.8 for the top layer (39,40).

D'Iorio and Kada were the first to point out that in the rabbit the molar ratio of adrenaline/ATP varied from 3.16 to 4.78 with a mean of 3.81 (41). Subsequently, in the chicken adrenal gland a molar ratio of catecholamines to ATP was found to average 4 (42,43,44). In further work, these findings have been confirmed in the ox (45), fowl, goose, cat and goat adrenal medullary granules (46).

Based upon these findings, the suggestion has been made

that ATP might be involved in the retention of the catecholamines within the chromaffin granule (47).

2) Soluble proteins

In 1958, Hillarp remarked that the lysis of the granules by hypotonic solution led to a release of proteins from the granules along with the catecholamines. The released proteins comprised about 77% of the total proteins of the granules. On electrophoresis the protein preparation appeared to be homogenous and an isoelectric point of 4 was found. This indicated the acidic nature of the protein. This author utilized 50% ethanol to precipitate the soluble proteins from the granules (20,48).

Winkler and coworkers isolated the soluble proteins from the granules. They separated the granules from adrenal glands originating from horse, ox or pig. A starch electrophoresis of these proteins showed that the preparation from each species was not homogenous since several bands could be observed. Yet, in all the three species, there was one main component which migrated at the same speed. On the hypothesis that the soluble proteins might be essential to the functional integrity of the granules, these authors concluded that the main protein constituent, common to the three species might be the most interesting feature of their studies. This component might be directly involved in the function of the chromaffin granule (49).

Blaschko and Helle precipitated the soluble proteins

from the ox adrenal medullary granules and determined the molecular weight of the major constituent. It was found to be 25,000 (50,51). Smith and Kirshner purified further the soluble proteins of the chromaffin granules by passing the preparation through a Sephadex G-200 column. They were able to detect that the protein was composed of two subunits, having each a molecular weight of 40,000, which gave a molecular weight for the protein of 80,000 (52). This high molecular weight is closer to that communicated by several other independent workers (53,54).

More recently, Strieder and associates have reported that the soluble proteins of the granules isolated from the ox, human, horse and pig adrenal medullae exhibit similar amino acid composition (55).

conclusion of part I

There are definite evidences that the adrenal glands from various species contain large quantities of catecholamines which are concentrated within specific granules. These granules, which are visible under the electron microscope, are specific entities distinct from the mitochondria. Biochemically, they have been shown not to include any of the respiratory enzymes typical of the mitochondria. These catecholamine containing granules are limited by a membrane and appear to have a diameter of about 0.2 u.

The catecholamine containing granules can be obtained in the pure from, free of mitochondria, of lysosomes and of microsomes by sucrose density gradient centrifugation.

The chemical composition of the chromaffin granules is well established. This granule is especially rich in catecholamines and in adenosine triphosphate, the molar ratio of the total amines to ATP being around 4. Considerable amounts of lipids and of proteins have also been detected. Small but significant amounts of calcium, of magnesium and of ribonucleic acid have also been determined in the catecholamine containing granule.

The various attempts to isolate and to purify the soluble proteins of the granule have been partially successful. These proteins comprise a major component, the molecular weight of which has been reported to lie between 25,000 and 80,000. In the highest value, are included the molecular weights of the two subunits which have been found to constitute the protein. In one protein preparation, the isoelectric point has been observed to be 4, indicating the acidic nature of the protein. In various species, the amino acid composition of the soluble protein from the granule is identical.

There is, in general, good agreement on the chemical composition of the chromaffin granule. Yet, one point which might be of importance is still a subject of controversy. The presence of a magnesium-activated ATPase has been reported by a few authors, but others could not corroborate this finding.

At the present time, the mode of release of catecholamines from the granules is still controversial. The experimental evidences are not conclusive on whether the catecholamines are released from the granules in the free form or whether a complex of catecholamines-ATP-proteins is liberated from the granules and broken down outside of the granules. The conclusive evidence about the presence or the absence of ATPase in the granule might clarify this point. A more detailed discussion will be provided in the next part II of the review.

PART II. THE FATE OF THE CATECHOLAMINE CONTAINING GRANULE

Numerous experimental evidences are available showing that the catecholamine containing granules appear not to be destroyed during the stimulation of the adrenal gland, but that they remain as empty sacs within the chromaffin cell (23,56). The fate of the chromaffin granule after it has emptied its soluble content into the circulation is still a subject of controversy. This part of the review of the literature will be devoted to the experimental evidences provided by several groups of workers in order to elucidate the fate of the catecholamine containing granules. In the first section A, the experimental facts on the release of the granule content during stimulation of the adrenal glands will be described. The second section B will deal with the experimental results on the repletion of catecholamines in the chromaffin granules, following the stimulation of the adrenal gland by various agents.

A) Release of the soluble content from the catecholamine containing granules : 1) Release after the stimulation of the adrenal gland by cholinergic agents, insulin or reserpine

The release of catecholamines from the whole adrenal gland can be brought about by several mechanisms. Among the most common methods used are the direct stimulation, the reflex

stimulation and the stimulation by the action of a drug such as reserpine. Since the nerve supply to the adrenal medulla is cholinergic, substances such as acetylcholine, the natural mediator, (57), carbachol (58), choline and nicotine (59,60) can cause a release of catecholamines by the direct stimulation of the adrenal medulla. The catecholamines can also be liberated by a reflex stimulation which can be brought about by insulin via hypoglycemia (61,62,63). Finally a large number of drugs deplete catecholamines from the adrenal gland especially reserpine (64). The exact mechanism by which this depletion is brought about is not as yet elucidated, but numerous experimental evidences appear to point out to a damage of the amine storage by reserpine (65).

The depletion of catecholamines caused by either cholinergic drugs, or insulin, or reserpine is usualyy accompanied by a discharge of the soluble content of the granule (66,67,68).

In 1957, Butterworth and Mann observed that repeated injections of acetylcholine to the atropinised cat produced a severe depletion of catecholamines. The content of the adrenal gland was found to be decreased by 71% to 84% of its catecholamines, seventeen hours after the last intravenous injection of acetylcholine. A similar depletion for both noradrenaline and adrenaline occurred; no significant difference in the depletion of either of the amines could be detected. The noradrenaline as well

as the adrenaline were recovered in the venous effluent of the adrenal gland (57).

During the perfusion of the cat adrenal glands, Douglas and coworkers observed that perfusion with pure Locke's solution gave rise to a spontaneous release of catecholamines from the perfused gland (69). When the Locke's solution was supplemented with acetylcholine, at a concentration of 100 micrograms per milliliter of solution, the release of catecholamines was always increased above control level until it reached a rate of secretion of about 15 micrograms per minute per gland. This rate was attained during the first two minutes of perfusion. Then the rate returned to that of the non-stimulated gland. In the absence of calcium ions, there was no release of catecholamines, even in the presence of acetylcholine. The essential requirement of calcium ions for the release of catecholamines was further confirmed (70,71).

During the stimulation of the adrenal glands with acetylcholine, or by splanchnic nerve stimulation large amounts of AMP as well as ATP were excreted into the perfusate (68,72).

Banks, using Tyrode solution as the perfusion medium , studied the effect of carbachol perfusion on the isolated ox adrenal glands. This author found that repeated perfusion with carbachol caused a 63% depletion of the catecholamines. The amines thus released were recovered in the perfusate fluid. The secretory response as well as the spontaneous release of catecholamines were

dependent upon the presence of calcium ions in the perfusion solution. The release of catecholamines was accompanied by a simultaneous release of nucleotides which were identified as AMP, adenosine, inosine and hypoxanthine. The presence of ATP metabolites confirmed previous findings by Starjne (76). ATP was present in the perfusate only at the peak of the release of catecholamines (72,73).

An increase in the soluble protein content of the perfusate was remarked after carbachol perfusion, although in the absence of carbachol there was anspontaneous secretion of proteins from the ox adrenal medulla. These proteins were compared with soluble proteins isolated from the granules and were found identical according to an immunological method. The release of catecholamines from the perfused glandappears to be accompanied by a liberation of soluble proteins (74). This observation was recently confirmed during perfusion of the ox adrenal gland with acetylcholine (75).

In vivo, the release of proteins was also observed following stimulation of the splanchnic nerve in the calf. These proteins were identical to those in the granules and were designated as "chromogranins" (77). Independent groups of workers identified, after in vitro perfusion of the ox adrenal gland a major protein component: "chromogranin A" (78,79,81). The release of chromogranin A followed clesely in time that of the

catecholamines but there was a small lag period (78,79). Calcium ions were an essential requirement for the liberation of catecholamines which was also temperature dependent. A fall of 16°C in the temperature of the perfusion liquid brought about a decrease of 60% in the release (80).

Kirshner confirmed these findings and measured the ratio of catecholamines to the proteins in the perfusate. The release was calcium dependent. Moreover below a temperature of 13°C, no release could be obtained despite the presence of acetylcholine. Kirshner calculated that the ratio of the catecholamines to the proteins in the perfusate averaged 1.7 (81). This value was lower than that previously reported by Schneider which, in identical experimental conditions obtained a value of 4.8 (80). The major component of the proteins isolated from the perfusate was found, on electrophoresis, to migrate at the same speed as that which was isolated from the catecholamine containing granules (82).

Cannon and coworkers (61) and Houssay and coworkers (62) were the first workers to point out, in 1924, that insulin can produce a depletion of the catecholamine content of the adrenal glands. Cannon and his associates observed that in the cat, insulin would increase the heart rate, despite previous denervation of the heart. This increase in the heart rate was noticed at the time at which the blood glucose level was below normal. If adrenalectomy

was performed prior to the insulin injection no such a response was observed. From this observation, the authors concluded that hypoglycemia brings about a secretion of adrenaline from the adrenal glands (61).

Twenty years later, Vogt was unable to repeat Cannon's work, when injecting 0.12 units of insulin to the rats. Yet, when the dose of insulin was doubled, this author observed a significant release of catecholamines from the rat adrenal glands. She noted that the depletion of adrenaline was larger than that of noradrenaline in the adrenal gland (83).

It was not until the work of von Euler and Luft that the selective action of insulin was finally established. These authors injected into healthy human volunteers, male or female, 0.1 unit of insulin per kilogram of body weight. At this dosage of insulin, significant amounts of adrenaline could be recovered in the urine. The adrenaline,excreted was increased by about ten fold, whereas the noradrenaline was only moderately affected. Von Euler and Luft showed definitely that insulin has a selective effect in depleting the adrenal gland of adrenaline only (84).

This observation was corroborated by Dumer's work on the cat. The intravenous injection of insulin, 10 units per kilogram of body weight, elicited a severe depletion of adrenaline. Thirty five minutes after the injection, the suprarenal venous effluent contained adrenaline in a concentration ten times that of the

resting level, while the noradrenaline was unchanged (85). In a previous work, Duner had shown that the injection of glucose would reduce the secretion of adrenaline (86), so he claimed that the blood glucose level was the factor directly involved in the adrenal medullary secretion elicited by insulin.

Since the depletion of adrenaline due to insulin is caused by a reflex stimulation (29,64), the effect of insulin cannot be investigated on the isolated gland, but only in glands in the whole animal. It might explain the very few data concerning the study of the release of catecholamines, ATP and proteins under the effect of insulin.

It was recently reported that the protein content of the 26,000 x g sediment from rabbit adrenal glands was unchanged after pretreatment of the animals with insulin (65). This finding verified previous observations in the sheep, where insulin was found tocaus release only catecholamines and ATP but not the soluble proteins from the granules. The depletion of ATP was determined directly on the granules. The decrease in the ATP content of the granule could not be accounted for by an increase in the cytoplasmic ATP nor by an increase in the cytoplasmic or granular inorganic phosphate (66). In the rabbit, no significant change in the ATP content of the adrenal gland was observed after insulin treatment, despite the significant decrease in the catecholamine content of the adrenal gland (41). In the chicken, Hagen observed a concomitant decrease of catecholamine

of catecholamines and ATP, after insulin treatment (44), whereas in Schumann the experimental evidences are not conclusive (42).

It is interesting to note that since insulin is acting through a reflex stimulation, the depletion of catecholamines in the adrenal gland is in fact brought about by acetylcholine. Thus one would expect to obtain identical results, at least qualitatively with the two substances. But this is not the case, as can be judged when the results obtained with the cholinergic stimulation and those obtained with insulin are compared. The most striking difference is that with acetylcholine there is a release of the soluble proteins which is concomitant with the liberation of catecholamines from the gland (74,75,77,78,79,81), whereas with insulin stimulation there is a release of catecholamines which is not accompanied by the release of proteins (63,66). The discrepancy might be only apparent and inherent to the methods used rather than to a difference between insulin and cholinergic effects. In the experiments with acetylcholine, the release of the proteins was determined by measuring the outflow of proteins in the perfusate, whether the perfusion be in situ or in vitro; in the experiments with insulin the proteins were determined by measuring the content of the granule after stimulation of the adrenal gland.

The effect of reserpine has been extensively studied. It appears that reserpine is a very potent agent in the depletion of catecholamines (29,64,87).

Carlsson and Hillarp observed a complete disappearance of catecholamines from the adrenal glands of rabbits which had been given a single intravenous injection of 5 milligram of reserpine per kilogram of body weight. The complete depletion was observed as early as ten hours after the treatment (88). This finding was confirmed by Mirkin who could not detect any catecholamine in the rat adrenal glands five days after the animals had been injected with reserpine (89). Both adrenaline and noradrenaline are equally affected (90). In the rabbit, a severe depletion of both amines was also reported by Ludborg (91).

The reserpine induced depletion of catecholamines has been reported to be blocked by denervation (92), while several groups of workers claimed that the denervation of the gland resulted only in a reduction of the effect of reserpine (93,94). In the cat, it was reported that reserpine elicits a depletion of the catecholamines from the innervated gland only (95,96). Other authorshave observed that both innervated and denervated glands are stimulated by reserpine (97,98). In the latter case, the dosage of reserpine used to cause the depletion was twice as high as that used in the former report which seems to indicate that the response depends upon the dose.

In the rat, a single injection of reserpine was found to deplete the adrenal gland of its noradrenaline only. This effect was abolished by denervation (97). When a dose ten times

as high as the previous one was injected into the rat, both innervated and denervated glands were depleted of their catecholamines. Yet, in the denervated gland, the depletion was equal to 36% whereas in the innervated gland it was 76%. The nucleotide phosphatas were also decreased; the degree of depletion in the denervated and in the innervated glands paralleled that of the catecholamines (100). Other reports have stipulated that the effect of reserpine is not affected by the denervation of the animals prior to the treatment (89,90,101).

In the intact animal, reserpine appears to bring about a concomitant release of the catecholamines and of the nucleotides (43,102,103) but there are no experimental data concerning the release of the soluble proteins from the granules. Neither phospholipid nor cholesterol releases have been observed in experimental conditions where the catecholamines and ATP were liberated (79,104, 105). This finding is in favour of the hypothesis that the granule ghosts are kept within the chromaffin cell after catecholamine release in the extracellular space (23).

2) <u>Release of the soluble content of the catecholamine containing granule in vitro</u>

Although the removal of the adrenal gland is followed by an immediate release of catecholamines from the granules, the isolated granules at 0°C are stable in isotonic sucrose solution (20,106). The catecholamines are rapidly released from the isolated

granules by lowering the tonicity of the suspending medium and the degree of release is dependent upon the ionic strength of the medium (14,20,48,107).

The catecholamines can be released from the granules either by lowering the pH of the suspending medium below 6, or by increasing the temperature above 0°C (5,20,48,107,108). The freezing and thawing of the isolated granules can cause a rapid liberation of the catecholamines. A number of detergents at concentrations as low as 10mM can provoke a discharge of catecholamines from the isolated granules; small amounts of lysolecithine and of lecithinase A have a similar effect. In this case, it seems that there is a direct effect on the granule membrane which causes a leakage of the granular content into the medium (107).

Carbachol and acetylcholine were found to have no effect on the isolated granules (109). This is not surprising in view of the fact that acetylcholine has been shown to exert its effect on the plasma membrane of the chromaffin cell (110). On the other hand, reserpine, phenylamine and tyramine were found to release the catecholamines from the isolated granules. This release of catecholamines by reserpine and phenylamine was accompanied by a release of ATP, of calcium and of magnesium, while tyramine selectively depleted the granules of their catecholamines only (5).

Calcium ions (5) and magnesium ions (111) have been shown to release the catecholamines and ATP from the isolated granules.

A group of sulphydryl reagents has been found to liberate catecholamines and ATP from the granules isolated from the ox adrenal medulla (112,113). This release was accompanied by a simultaneous release of proteins. Glutathione and cysteine could prevent the action of the sulphydryl reagents but could not reverse it, indicating that the change in the granule membrane caused by the sulphydryl reagents was irreversible. In the presence of ATP and Mg^{++}, glutathione liberated catecholamines from the granules but not the proteins (113).

3) Mechanism of secretion

It is now well established that acetylcholine acts at the plasma membrane of the chromaffin cell (110). The exact mechanism by which the catecholamines are released from the adrenal gland into the general circulation is unknown, even more undisclosed is the actual function of the catecholamine containing granule.

Most of the hypotheses on the function of the chromaffin granules are based upon experimental data on the secretion of the adrenal glands. De Robertis and Vaz Ferreira have suggested that the catecholamines from the granules may be released directly into the extracellular space (23). This suggestion is based upon experimental evidences obtained mainly from the electron microscopic investigations. These authors observed a migration of the granules near the chromaffin cell membrane in the stimulated gland,

gland, while in the normal gland the granules were scattered at random in the cytoplasm. Moreover, some granules less dense than the others appeared near the cell membrane which suggested that their content had been emptied. However, similar low density granules were also observed in the cytoplasm away from the cell membrane. Other electron microscopic evidences in the rat adrenal gland have confirmed this observation (114). Some recent biochemical data appear to substantiate the hypothesis first proposed by De Robertis (68,79,115,116). According to this hypothesis, the stimulation of the adrenal medulla would result in the emptying of the granule content directly at the cell membrane, outside of the cell; this implies that the granule would be functionally equivalent to a secretory granule. It also indicates that the whole content of the granule would be emptied outside of the chromaffin cell. The fact that neither ATP nor inorganic phosphate are found in the cytoplasm following the stimulation of the gland (66) corroborates the hypothesis that the granular content is discharged outside of the chromaffin cell into the extracellular space. If this hypothesis corresponds to the actual mechanism of secretion, one would expect the granule to be completely destroyed during the secretory process and not used again for the storage of catecholamines.

A second theory first put forward by Blaschko and Welch in their first report (1) postulates that the function of the

granules is to store the catecholamines. This second theory is based upon the important finding that not all the catecholamine content of the adrenal medulla is found within the granules, but that significant amounts of the amines are recovered in the cytoplasmic sap of the chromaffin cell. This might represent an artefact of the preparation, since the isolated granules appear to be very labile. However, the possibility that an independent cytoplasmic pool of catecholamines is present in the chromaffin cell has not been eliminated. There are numerous experimental evidences pointing to the existence of a cytoplasmic catecholamine pool (117,118,119). The attractive feature of this theory is the simplicity of the explanation provided for the secretion of catecholamines after stimulation of the adrenal medulla. It also explains clearly the continuous leakage of the amines from the resting gland (64). According to this theory, the amines free or loosely bound within the cytoplasm would be in dynamic equilibrium with those stored within the granules. The agents stimulating the adrenal medulla would do so by increasing the permeability of the chromaffin cell membrane allowing large quantities of catecholamines to flow out of the gland. The process might be a simple diffusion or an active transport. After the equilibrium in the chromaffin cell has been upset by this lowering of the cytoplasmic catecholamine concentration, it would return to normal through a mechanism which would bring the

release of catecholamines from the granule into the cytoplasm. Thus the functional role of the granule would be one of storage. This second theory does not provide a satisfactory explanation for all the experimental evidences which have shown that the soluble proteins and ATP are found in the perfusate of the isolated gland, following secretion of the chromaffin cells.

A third theory based mainly upon the electron microscopic studies suggests that the whole intact granule is as such excreted into the extracellular space and only there empties its content into the extracellular space (120,121). An important drawback of this theory is the fact that it does not explain the experimental finding that neither lipid, the major component of the granule membrane, nor cholesterol can be found in the perfusate during the stimulation of the adrenal gland (79,105).

Whatever the theory considered, it is always implied that the granule expels its content, during the stimulation of the adrenal medulla. However, it is not clear whether the release of catecholamines as free bases occurs first; the rest of the granule content would then diffuse freely out of the damaged granule. The alternative might be that the catecholamines are secreted out of the granules in a complex formed of catecholamines-ATP-proteins. It would be only outside of the granules that the complex would be dissociated to give the free amines.

B) Storage of catecholamines within the catecholamine containing granule : 1) Repletion of catecholamines following the stimulation of the adrenal gland

The depletion of catecholamines and ATP from the adrenal gland after its stimulation is a rapid process. But the repletion of the adrenal gland is a very slow process.

As early as 1929, Crowden observed that in the cat adrenal gland, catecholamine depletion brought about by cold exposure was still significant three days after the exposure. Denervation did not appear to affect the rate of repletion of the gland (122).

In another series of experiments, on the atropinized cat, the depletion of catecholamines in the adrenal gland was induced by repeated injections of acetylcholine. It took six to seven days to reach the normal values of total catecholamines in the depleted gland. Upon analysis of noradrenaline and of adrenaline at the various stages of repletion, it was observed that the noradrenaline level was above normal whereas the adrenaline level was much lower than the normal values. It required one month about for the adrenaline and the noradrenaline to return to their normal level (125).

It was found that in the rat adrenal gland depleted of catecholamines by a single injection of insulin, the replacement of adrenaline was complete six days after the injection (86,123).

In the rabbit, under identical conditions, 50% of the adrenaline depleted from the adrenal glands had reappeared three days after the injection (124).

Vivares and coworkers showed that in the rabbit, four days after the injection of insulin, the total catecholamine content of the adrenal glands had returned to normal; twelve days after the reserpine treatment, the adrenal gland had not yet recovered its normal catecholamine content (65). It is well known that noradrenaline is a direct precursor of adrenaline (126), and the fact that the concentration of noradrenaline appears to rise above normal level during repletion (123,127) indicates that the determination of total catecholamines in not very significant as an index of repletion of adrenaline and of noradrenaline in the adrenal gland.

The reappearance of noradrenaline at an earlier stage of repletion than that of adrenaline has been reported by several groups of workers (101,123,127,128). After reserpine treatment, an excess of noradrenaline over normal level was observed in the rat adrenal gland as early as seven days after treatment (129). Yet, Eranko and Hopsu did not detect such an excess of noradrenaline in the rat adrenal gland; they found that the time courses of adrenaline and of noradrenaline repletion were identical for the two amines (130). Kirpekar and coworkers observed that after reserpine treatment, the noradrenaline repletion in

the rat adrenal gland was slightly faster than that of adrenaline. But they did not find an excess of noradrenaline in the gland. ATP was found to follow a time course repletion identical to that of the total catecholamines which reached a normal level by the seventh day after the treatment (131).

2) Uptake of catecholamines by the isolated catecholamine containing granules.

Many observations appear to point out that the catecholamine containing granule is surrounded by a membrane (1,2,14,22, 23). According to Kirchner it is a semipermeable membrane (132), still other workers claim that it is permeable to exogenous catecholamines under specific experimental conditions (31,133).

In his first attempt to investigate whether granules isolated from the ox adrenal medulla could pick up catecholamines, Hillarp incubated the pure granules in a medium supplemented with ^{3}H-adrenaline, at 0°C. In these conditions, significant amounts of radioactivity could be detected in the granules. Yet, this author did not consider it to be of any physiological significance since the ^{3}H-adrenaline used for the incubation was contaminated by about 20% with decomposition products (31). In a later study, this author claims that the granules from ox adrenal medulla could pick up adrenaline up to 30%-40% of the amount of adrenaline and noradrenaline initially present in the gland (134). ATP and magnesium have been found to increase

significantly the uptake of catecholamines by the isolated chromaffin granules (135,136,137).

Kirshner further investigated the effect of the temperature on the uptake of ^{14}C-adrenaline by the granules isolated from the ox adrenal medulla. The uptake was temperature dependent. At 37°C the rate of uptake was twenty fold that observed at 0°C (135), whereas it was about fifteen fold at 30°C (136). The presence of ATP in the incubation medium caused a three fold increase in the uptake. Although magnesium alone had no effect, when added in the presence of ATP it resulted in a three to five fold increase of the uptake at 37°C, at 30°C and at 0°C as well. Reserpine and ethylene diamine tetraacetic acid (EDTA) were inhibitors. The inhibition by EDTA was complete but could be reversed by the addition of magnesium ions. The inhibition due to reserpine was about 50% and irreversible even after dialysis of the suspension (135,136). As pointed out by Kirshner, the fact that ATP is a requirement, and that the uptake is temperature dependent appears to imply that the mechanism of uptake isenergy dependent. The uptake at 0°C would be due mainly to a simple diffusion, while at 37°C an active transport might be involved. The granules used by Kirshner were isolated from normal glands and thus can be considered as functional (135,136).

A secondary effect of the temperature which might be of importance in interpreting the results and inherent to the

method appears to need some remark at this point. The isolated granules even in isotonic medium are not very stable at 37°C (64,138). Thus an increase in the temperature of incubation might lead to a larger destruction of the granules. The radioactivity found in the granular fraction might not be inside of the granule but loosely bound to damaged granular membrane. The gentle lysis of the granule performed by Kirshner might have eliminated for the greatest part this artefact of the method, but the possibility is not completely eliminated.

More recently, a group of workers reported that the homogenate from normal rabbit adrenal glands can concentrate ^{14}C-adrenaline at 0°C. The uptake by the homogenate was dependent upon the concentration of adrenaline in the incubation medium. In the granules from reserpinized or insulin treated rabbit adrenal gland, the uptake of ^{14}C-adrenaline was decreased. ATP and magnesium stimulated the uptake (65).

Glutathione was found to decrease the uptake of ^{14}C-adrenaline by granules isolated from the ox adrenal medulla. ATP and magnesium ions were essential for the effect of glutathione (113).

The uptake of exogenous adrenaline might represent an exchange between endogenous and exogenous adrenalines. ATP and magnesium have been found to increase the release of the catecholamines from the isolated granules of ox adrenal gland (139,140).

If the uptake of adrenaline represents an exchange, it is not impossible that the addition of ATP and of magnesium to the medium, by lowering the concentration of the endogenous adrenaline results in a larger uptake of exogenous adrenaline. The energy dependence of the uptake would be apparent only. This problem might be solved by a determination of the relative specific activities of the radioactive adrenaline outside and inside of the granule, at the completion of the uptake. The fact that glutathione reduces the uptake of ^{14}C-adrenaline by reducing the release of this amine from the granule, might be considered as an indirect evidence that the uptake, *in vitro*, is partially due to an exchange between the adrenaline from the granules and the adrenaline from the extracellular fluid.

conclusion of part II

There is a general agreement that the release of the catecholamines from the granules of the adrenal gland is usually accompanied by a concomitant release of the nucleotide phosphates. This liberation has been observed during the stimulation of the adrenal gland *in situ* as well as in the *in vitro* stimulation of the isolated organ, and this in various species. Although ATP is a major constituant of the secreted nucleotides, its metabolites have also been found in perfusates. It is not as yet known

whether the breakdown of ATP corresponds to a truly physiological phenomenon inherent to the catecholamine secretion or whether it represents an artefact of the techniques.

The mechanism of the release of catecholamines is still a hypothetical subject. But numerous experimental evidences appear to favour the phenomenon of exocytosis as reflecting the physiological secretion of catecholamines closely. Numerous discrepancies on the release of the soluble proteins from the granules hamper somewhat the theory of exocytosis. Moreover, this theory does not account for the important experimental evidence that not all the catecholamines are concentrated in the chromaffin granules, but that a significant amount of the amines is recovered from the cytoplasmic sap, in the adrenal gland. From the data available for and against the two main theories - exocytosis and cytoplasmic pool - it appears that each theory complements the other. The actual mechanism of secretion of the catecholamines might well be a combination of both hypotheses.

The repletion of catecholamines and of ATP in the depleted adrenal gland is a slow process. This is probably due to the slow rate of synthesis of the neurohormones, especially of adrenaline (141). This replacement of adrenaline has been reported to be accompanied by an increase in the uptake of radioactive adrenaline by the granules.

The *in vitro* uptake of radioactive amines by the isolated granules is dependent upon the temperature as well as upon the presence of ATP and of magnesium ions in the medium. The concentration of exogenous adrenaline appears to influence the amount of radioactive material concentrated by the granules.

The uptake of radioactive adrenaline might reflect an exchange between exogenous and endogenous adrenaline, thus implying that the granular membrane is permeable to exogenous adrenaline. In this case, the possibility that the granules from the stimulated glands can be reutilized after they have emptied their content is not remote. For this reason, several series of experiments were undertaken to check the validity of this hypothesis. The depletion of catecholamines from the guinea pig adrenal glands was brought about by carbachol, insulin or reserpine. The repletion and the *in vitro* uptake of catecholamines were investigated together with the repletion of ATP. The results of this work will be presented in the next experimental part of this thesis.

EXPERIMENTAL RESULTS

PART I. THE EFFECT OF A SINGLE INTRAPERITONEAL INJECTION OF DL-ETHIONINE ON THE CONTENT OF CATECHOLAMINES AND ATP IN THE RAT ADRENAL GLAND

I. Materials and Methods

A) Materials

All the animals used in these experiments were Sprague-Dawley rats, of either sex.

DL-Ethionine was purchased from Nutritional Biochemicals Corporation. 750 milligrams of ethionine were dissolved into 25 milliliters of distilled water by adding a few drops of 1 N NaOH. The final pH of the solution was 6.4; the final concentration of ethionine was 25%.

dl-Adrenaline bitartrate and dl-noradrenaline bitartrate were obtained from Winthrop lab. Each of the two amines was dissolved into 0.05 N perchloric acid to give a final concentration of 1 microgram of the free base per one milliliter of solution.

Adenosine triphosphate disodium salt, from Sigma Chemicals Co., was dissolved in 5% trichloroacetic acid (TCA)

to a final concentration of 1 milligram per milliliter.

Firefly lanterns, stock No FFT, were purchased from Sigma Chemicals Co. . One hundred milligrams were homogenized in 10 milliliters of a medium containing : 0.3 M Na_2SO_4; 0.025 M Na_2HAsO_4. The pH of this medium was 7.6 (142). The homogenate was then centrifuged at 600 x g for ten minutes and the supernatant was used as the source of enzyme.

Disodium ethylene diamine tetraacetate (EDTA) and sodium metabisulfite were obtained from Fisher Scientific Co. .

Isotonic sodium chloride contained 0.85 gram of the salt per 100 milliliters of solution. When used for injecting to the control animals the pH of the solution was adjusted to 8.4.

The following reagents used for the determination of catecholamines were prepared in water which had been doubly distilled in glass.

- Aluminum oxide, from Woelm, Neutral activity Grade 1, was further purified as described by Anton and Sayre (143).

- Perchloric acid (70%), manufactured by Baker and Adamson, was used in two dilutions: 0.4 N and 0.05 N.

- Phosphate buffer (0.5 M, pH 7) was prepared by titration of a solution of sodium dihydrogene phosphate with 1 N NaOH.

- Acetic acid (1.6N) was prepared by mixing 0.5 milliliter of glacial acetic acid and 5.0 milliliters of water. This

solution was prepared just before use.

- Potassium ferricyanide at a concentration of 0.25% was prepared just before use, and was used within the hour of its preparation.

- Ascorbic acid, from Fisher Scientific Co. , was dissolved in water - 10 milligrams of ascorbic acid per 0.1 ml -. The solution was made alkaline by the addition of 5 milliliters of 10 N NaOH. This solution was used within one hour of its preparation.

B) Methods

In all the experiments, the animals weighed between 150 grams and 170 grams.

1) Preparation of animals

Two groups of animals were employed in each series of experiments. The first group consisted of control rats which received a single intraperitoneal injection of isotonic sodium chloride (pH 8.4), the dosage being 3 milliliters per 100 grams of body weight. The second group included ethionine treated rats which received 3 milliliters of ethionine solution per 100 grams of body weight. All the animals were fasted 17 hours prior to the injection.

Three series of experiments were performed. In the first and in the second series, female rats were used. They were sacrificed three hours and six hours after receiving a single injection.

In the third series of experiments, male rats were used. They were sacrificed six hours after a single intraperitoneal injection. In all cases, the animals were killed by guillotine decapitation.

2) Preparation of tissue homogenate

The adrenal glands, liver and brain were removed, washed in cold isotonic saline, blotted on a filter paper and weighed. Each tissue was then immersed in ice-cold 5% trichloroacetic acid and homogenized in an all glass homogenizer, for 3 to 5 minutes. A 10% homogenate was prepared except for the adrenal glands where 5 millilitera of 5% trichloroacetic acid were used per pair of glands. The homogenate was then transfered to a polyethylene centrifuge tube and centrifuged in a Servall, refrigerated centrifuge (Model 88-3, rotor SM-24) for 10 minutes at 30,000 x g. The clear supernatant was collected and divided into two fractions. These fractions were placed immediately in the deep-freeze, pending further determinations.

3) Determination of catecholamines

One fraction was used for the determination of catecholamines as described by Anton and Sayre (143), as modified by Missala (144) who introduced the batch procedure, and Lussier-Proulx (145) who used water driven magnetic stirrer instead of the mechanical stirrer.

4) Determination of ATP

The second aliquot was used for ATP determination as described by Strehler and Totter (162). The following modifications were made to adapt this method to our experimental conditions. The sample to be tested was prepared in the following manner: 0.3 milliliter of the incubation medium was placed in a cuvette of 1 centimeter light path; 0,5 milliliter of the enzyme extract was added. After mixing, 0.2 milliliter of the unknown was added. Readings were taken exactly 20 seconds after the addition of the unknown sample. A blank was made by replacing the unknown sample by 0.2 milliliters of distilled water. The standard consisted of a solution of ATP treated exactly like the unknown sample. A plot of ATP concentrations (0.5 micrograms to 2 micrograms per milliliter) versus intensity of light yielded a straight line. Since our measurements were always within that range of ATP concentrations, the method was considered to be suitable to yield consistent results.

Discussion

The method used for catecholamine determination permitted to identify noradrenaline and adrenaline. It is a differential method. At pH 2, adrenaline alone is measured while at pH 7 both adrenaline and noradrenaline are determined. The procedure itself, although tedious, is reliable as long as the experimental conditions are respected and carefully repeated.

A large difference in the relative concentrations of both amines will introduce a greated risk of error than when the relative concentrations are close. This factor might be partly responsible for the high standard deviation values obtained in some of the experiments. This is the only drawback of the method. At the present time, it is the most accurate method available and it is reliable at concentrations as low as 0.01 microgram of amine per 2 milliliters.

II. Results

Two series of experiments were performed in order to study the effect of ethionine on the content of catecholamines and ATP in various organs of the rat. In the first series of experiments, the effect of a single injection of ethionine was studied in some organs from female rats, while male rats were utilized in the second series of experiments. For each series of experiments, the individual data, their mean value and the standard deviation from the mean are given for each group. The values of P were calculated by the Student t test. The group comparison included three hour-treated group versus control group, and six hour-treated group versus control group.

A) <u>The effect of a single injection of ethionine on the catecholamine and ATP content of the female rat brain, liver and adrenal glands</u> :

In this series of experiments, two groups of rats were employed. The animals of the first group were killed three hours after the injection, those from the second group were killed six hours after the injection.

In Table I, the data recorded show the effect of ethionine on the ATP content of the liver. The data are expressed as micrograms of ATP per gram of wet weight tissue. Three hours after a single intraperitoneal injection of ethionine, there is a marked decrease in the ATP content of the liver.

TABLE I

The Effect of a Single Injection of DL-Ethionine on the ATP Content of the Female Rat Liver

Rat =	Control	Ethionine-treated 3 hours	Ethionine-treated 6 hours
	ATP micrograms per gram of wet weight tissue		
1	398.81	226.02	140.64
2	344.24	232.04	175.03
3	400.23	202.20	187.52
4	462.56	191.15	178.73
5	425.00	220.61	183.78
6	339.62	208.61	185.00
7	438.31		
8	379.13		
9	300.34		
10	344.16		
11	355.50		
Mean	380.71	213.48*	175.08**
± S.D.	49.14	15.57	7.13

*$p < 0.005$; **$p < 0.001$ for control versus treated groups.

This decrease is still more pronounced six hours after the administration of ethionine. While three hours after the injection the level of ATP in the treated animal has fallen to 56% that of the control, it is only 46% of that control level six hours after the injection. In both treated groups, the difference is statistically significant.

The results presented in Table II show the effect of ethionine on the ATP and catecholamine content of the female rat brain. There is no change in the ATP and catecholamine content of the brain although the effect of ethionine was allowed to proceed for six hours.

In Tables IIIa and IIIb, the results obtained for the adrenal glands are presented. In Table IIIa, page 49, the results are expressed as micrograms of substance per pair of adrenal glands, while in Table IIIb, they are expressed as micrograms of substance per gram of wet weight tissue. The ethionine treatment has no effect either on the ATP level or on the catecholamine level of the female rat adrenal glands. Six hours after treatment, there is a very slight decrease of the ATP and of the catecholamine content of the treated animals; however, this difference is not statistically significant. The wet weights of the adrenal glands from the treated and from the control animals were not significantly different, thus the results when expressed per gram of wet weight tissue reflect the same finding.

TABLE XI

The Effect of a Single Injection of DL-Ethionine on the ATP and Catecholamine Content of the Female Rat Brain

Rat	Control			Ethionine-treated 6 hours		
	micrograms per gram of wet weight tissue					
	NA*	A**	ATP	NA*	A**	ATP
1	0.158	0.067	180.00	0.171	0.084	115.25
2	0.158	0.102	146.00	0.172	0.018	127.35
3	0.187	0.092	140.00	0.137	0.098	160.04
4	0.254	0.015	146.20	0.291	0.039	188.89
5	0.155	0.075	209.40	0.177	0.114	202.12
6	0.185	0.079	138.00	0.241	0.009	219.68
Mean	0.182	0.071	159.83	0.198	0.075	168.50
± S.D.	0.014	0.010	11.65	0.022	0.010	17.03

* NA : noradrenaline; ** A : adrenaline

TABLE IIIa

The Effect of a Single Injection of DL-Ethionine on the ATP and Catecholamine Content of the Female Rat Adrenal Glands

Rat	Control				Ethionine-treated 3 hours				Ethionine-treated 6 hours			
	AG* mg	micrograms per pair of glands			AG* mg	micrograms per pair of glands			AG* mg	micrograms per pair of glands		
		NA**	A***	ATP		NA**	A***	ATP		NA**	A***	ATP
1	29	2.00	9.65	10.25	38	5.30	8.62	13.51	41	4.57	4.92	8.50
2	40	3.30	11.67	14.42	39	3.87	11.29	16.58	41	1.30	11.02	5.25
3	47	3.52	7.87	8.70	38	0.00	15.88	14.12	30	2.32	8.40	13.25
4	33	5.25	6.65	19.00	35	3.89	10.83	11.51	32	4.42	8.32	8.09
5	36	3.74	9.75	14.14	38	1.57	10.71	15.01	41	3.30	10.12	13.25
6	34	2.47	12.67	15.53	39	0.00	14.34	14.37	35	3.00	14.10	10.50
7	34	2.44	14.14	13.05	38	0.35	16.32	14.16				
8	30	1.43	13.36	11.34								
9	41	2.48	13.31	13.05								
10	36	1.86	14.03	15.19								
11	38	3.22	13.28	15.54								
Mean	36	2.88	11.27	13.47	38	2.14'	12.54'	14.18'	36	3.15'	9.48'	9.79"
± S.D.	5	1.07	3.02	3.22	1	2.18	2.98	1.53	5	1.25	3.09	3.15

* AG : adrenal glands; ** NA : noradrenaline; *** A : adrenaline

' $P > 0.05$, "$P < 0.05$ for control versus treated groups.

TABLE IIIb

The Effect of a Single Injection of DL-Ethionine on the ATP and Catecholamine Content of the Female Rat Adrenal Glands

Rat	Control			Ethionine-treated 3 hours			Ethionine-treated 6 hours		
	micrograms per gram of wet weight tissue								
	NA*	A**	ATP	NA*	A**	ATP	NA*	A**	ATP
1	70.40	317.75	313.40	139.47	221.57	355.52	111.50	120.70	207.30
2	82.50	286.70	360.50	99.23	289.48	425.12	31.70	269.40	128.00
3	74.90	170.00	142.50	00.00	417.89	371.57	77.50	280.00	441.60
4	159.09	140.90	575.70	111.14	309.42	328.85	150.80	260.10	250.00
5	102.83	259.82	388.78	41.31	281.84	395.00	80.50	232.30	323.00
6	72.64	372.64	456.76	00.00	367.69	368.46	85.50	400.20	300.00
7	71.76	415.88	383.82	9.21	429.47	372.63			
8	47.66	445.33	378.00						
9	60.48	324.63	318.29						
10	51.66	389.72	421.94						
11	84.73	349.47	408.94						
Mean	79.87	315.71	377.13	57.19'	331.05'	373.87'	72.90'	260.00'	304.00'
± S.D.	30.45	96.19	105.70	58.51	76.56	30.20	44.30	89.50	88.84

* NA : noradrenaline; ** A : adrenaline

' $P > 0.05$ for control versus treated groups

B) The effect of a single injection of ethionine on the catecholamine content of the male rat adrenal glands :

Since no change in either the ATP or the catecholamine content of the female rat adrenal glands could be detected after treatment of the animals with ethionine, it became of interest to see whether the male rat would exhibit an identical behaviour. The data obtained in this series of experiments are given in Table IV, page 52. The results are expressed as micrograms of substance per gram of wet weight tissue, or as micrograms of substance per pair of adrenal glands. There is no change in the catecholamine level, six hours after the administration of ethionine. The slight increase in the catecholamine level of the adrenal glands from the treated animals is not statistically significant.

TABLE IV

The Effect of a Single Injection of DL-Ethionine on the Catecholamine Content of the Male Rat Adrenal Glands

Rat	Control					Ethionine-treated 6 hours				
	AG* mg	micrograms per pair of glands		micrograms per gram of wet weight tissue		AG*	micrograms per pair of glands		micrograms per gram of wet weight tissue	
		NA**	A***	NA**	A***		NA**	A***	NA**	A***
1	38	1.56	11.32	43.33	367.22	37	2.67	9.72	70.26	303.75
2	37	3.71	12.07	92.75	301.75	39	1.84	10.01	57.50	312.81
3	36	1.38	11.36	38.33	315.55	38	4.57	14.18	108.00	354.50
4	38	3.84	12.61	96.00	315.25	36	5.45	12.86	136.25	320.00
5	39	2.38	13.17	62.63	346.57	38	4.90	14.17	122.50	354.25
6	35	3.21	12.71	81.75	320.65					
Mean		2.68	12.20	69.13	327.83		3.88'	12.17'	99.06'	329.06'
± S.D.		1.06	0.88	24.88	24.26		1.54	2.18	33.85	23.81

* AG : adrenal glands; ** NA : noradrenaline; ***A : adrenaline
' $P > 0.05$ for control versus treated groups

III. Discussion

Our observation that ethionine brings about a marked depletion of the liver ATP is in agreement with the previous findings (146,147,148,149). It has been shown that there is a rapid fall of the ATP concentration in the liver of female rats which have been treated with ethionine. This effect of ethionine is counteracted by the administration of methionine. The depletion of liver ATP appears to occur because of an accumulation of S-adenosyl ethionine which is unreactive. The ATP is thus blocked, and its indirect depletion observed (150,151).

The fact that in the adrenal medulla the secretion of catecholamines is always accompanied by an equivalent secretion of ATP (102,103,152) led us to investigate the effect of ethionine administration on the ATP and catecholamine content of the adrenal gland. Since ATP seems to play a role in the storage of amines in the chromaffin granules, it would be expected that the ATP depletion caused by ethionine would result in a release of the catecholamines from the granules. A depletion of ATP in the adrenal glands might also reduce the concentration of S-adenosyl methionine which is necessary as a methyl donor for the synthesis of adrenaline from its immediate precursor noradrenaline. The rate of adrenaline synthesis would be decreased and this decrease would be reflected on the content of the adrenal gland. However, it was believed

that within the six hour period of treatment no significant effect could have been seen on the rate of synthesis of adrenaline. The failure of ethionine to induce the depletion of ATP in the adrenal glands could be explained on the assumption that ethionine cannot penetrate into the granules where the largest concentration of ATP is found (34). An alternative explanation could be that the ATP of the chromaffin granules has a very slow rate of turn over. Many authors believe that the ATP of the chromaffin granules is closely involved in binding the catecholamines within the granules (39,117). The rate of turnover of the catecholamines is very slow (124,141), therefore it is tempting to assume that the turnover rate of ATP might be comparable to that of the biogenic amines, hence the failure of ethionine to deplete the ATP from the adrenal glands.

In the brain, the failure of ethionine to bring about any any change in the ATP level could be accounted for on the concept of the blood-brain barrier (153).

The attempt to use ethionine in order to deplete the ATP in the adrenal glands, which in turn was expected to result in the depletion of catecholamines has failed. This failure led us to attack the problem under a different angle. Since the storage of adrenaline and of noradrenaline in the granules of the adrenal glands is linked apparently to the presence of ATP, it was thought that the simultaneous investigation of the rate of turnover of ATP and of the rate of turnover of the granular proteins might throw

some light on the problem. The study of the rate of turnover was undertaken and use of radioactive precursors of a high specific activity was made for this investigation. The results of this work are presented in the next Part II of this thesis.

PART II. THE IN VIVO LABELLING OF ATP, CATECHOLAMINES AND PROTEINS IN THE GRANULES OF THE GUINEA PIG ADRENAL MEDULLA

I. Materials and Methods

A) Materials

All the animals used in these experiments were male guin guinea pigs from Canadian Breeding Laboratory, Montréal. They weighed between 350 grams and 400 grams.

$Na_3{}^{32}PO_4$ was purchased from the Atomic Energy of Canada.

DL-3(3,4-dihydroxyphenyl)alanine-2-^{14}C, (DOPA), specific activity: 3.2 millicuries per millimole; L-leucine-4,5-^{3}H, specific activity: 23.5 curies per millimole; adenine-2,8-^{3}H, specific activity: 3.6 curies per millimole, were bought from Nuclear Chicago Co.

Insulin, pork "protamine and iletin" by Eli Lilly Co. was kindly donated by Dr. R. L. Singhal of the Department of Pharmacology. The concentration of the solution was 40 units of insulin per milliliter of solution.

The sucrose solution was 0.3 M, and buffered with 0.1 M Tris-HCl, pH 7.4 .

The scintillation liquid for radioactivity measurements was prepared according to Kirshner et al. (154). Ten milliliters of the scintillation liquid were used for each determination.

B) Methods

1) Preparation of the animals

In this series of experiments, the animals were divided into three main groups: group 1 for the labelling of ATP, group 2 for the labelling of proteins, and group 3 for the labelling of catecholamines. All the animals were treated with two units of insulin, seventeen hours prior to the injection of the radioactive precursor.

Group 1: The animals from this group were divided into two subgroups A and B. The animals from subgroup A received, daily, 2 microcuries of ^{32}P-Phosphate per guinea pig; those from subgroup B were injected with ^{3}H-adenine, daily, at a dosage of 100 microcuries per animal.

Group 2: The guinea pigs of this group were injected with ^{3}H-leucine, daily, at a dosage of 100 microcuries per animal.

Group 3: The animals of this group were injected with 1.37 microcuries of ^{14}C-Dopa, daily.

Except for the difference in the solutions injected, the animals of all the groups were submitted to the same treatment. For eight consecutive days, they were given daily a single intraperitoneal injection of the radioactive precursor. On the ninth day, one animal of each group was sacrificed. For the following nine days one animal of each group was sacrificed daily. Thus for ten consecutive days, the fate of the radioactive precursor

was studied.

2) Homogenisation and preparation of the granular fraction

After the animal had been sacrificed, the adrenal glands were removed, washed thoroughly in isotonic sodium chloride and homogenised per pair in one milliliter of 0.3 M sucrose buffer, pH 7.4. The homogenate was then centrifuged at 600 x g to remove the nuclei and cell debris. The pellet was discarded and the supernatant was centrifuged at 11,000 x g for fifteen minutes, in order to sediment the catecholamine containing fraction. The sediment was washed twice with an aliquot of sucrose buffer. Following the last washing, the sediment was resuspended in one milliliter of 5% trichloroacetic acid. After centrifugation, the supernatant was collected.

This method yielded an extract which was used for radioactivity measurements, and ATP and catecholamine determinations when desired. For the measurement of the protein radioactivity, the sediment was resuspended in 1 N NaOH and allowed to dissolve overnight at room temperature. This solution was used for protein determination as well as for radioactivity measurements. The protein content was measured by the method of Lowry (155).

5) Measurement of the radioactivity content of the extract

The radioactivity content of the samples was measured on a 0.2 milliliter aliquot of the extract using a Nuclear Chicago Mark I liquid scintillation counter. Ten milliliters of cold scintillation liquid were placed into a glass scintillation vial. To each vial 0.2 milliliter of the sample to be counted was added. The vial was placed in the refrigerated chamber so that the liquid could equilibrate at the temperature of the counter before the measurements were made. Each vial was counted for ten minutes and the number of counts per minute obtained directly. A correction factor was introduced for the efficiency of the counter.

6) Efficiency of the counter

The efficiency of the counter was measured by the method of internal standards. The following values were obtained:
^{32}P : 92%; ^{14}C : 70%; ^{3}H : 10%.

All the other methods used in this series of experiments have been described in Part I of this thesis.

II. Results and Discussion

The attempts to label *in vivo* the ATP, catecholamines and the proteins of the granular fraction of the adrenal glands were not successful. In no case was it possible to detect any significant amount of radioactivity. A sample of the type of results obtained in these series of experiments is given below. These data were obtained for the radioactivity content of ^{32}P in the samples.

- TABLE V -

Days after the last injection	Radioactivity in the sample c/min	Radioactivity in the glands c/min
1	25	50
2	19	38
3	22	44
4	16	32
5	16	32
6	23	46
7	15	30
8	21	42
9	24	46

It is seen that the readings are about equal to the background of the counter (20 c/min). There is no change in the values from one day to the other so that this method could not be considered consistent in spite of the sensitivity of the counter and of the high specific activity of the radioactive precursors employed. Since the adrenal glands represents a very

small percentage of the total body weight, and since most of the injected compounds would be also concentrated by other organs, it was believed that too small a fraction of the injected precursor was penetrating in the adrenal gland. It had been previously reported that the rate of incorporation of ^{32}P by the adrenal glands is very slow (156).

It was at this point of the proceedings that it was decided to take an indirect approach to the problem. For some time it has been known, that several substances bring about a marked depletion of the catecholamines of the adrenal glands (29). It was decided to make use of such substances and to investigate the rate of replation of the catecholamines together with that of ATP, after such a depletion. Three substances were used: carbachol, a synthetic choline ester which has a direct action on the depletion of the catecholamines from the adrenal glands, insuline which acts via the hypoglycemic stimulation, and reserpine which is a potent depletor of the catecholamines and of ATP of the adrenal glands. The description of this work is presented in the next Part III of this thesis.

PART III. CATECHOLAMINE AND ATP DEPLETION OF THE GRANULAR FRACTION FOLLOWING DEPLETION OF CATECHOLAMINES BY INSULIN CARBACHOL AND RESERPINE

I. Materials and Methods

A) Materials

Carbachol (carbamylcholine chloride, C.P.) was obtained from Merck Research Laboratories Inc. . The aqueous solution contained 100 micrograms per milliliter.

Reserpine, from National Biochemicals Co. was dissolved in 25% propylene glycol to yield a final concentration of 2 milligrams of reserpine per milliliter.

All the other reagents used in this Part III have been described in the previous parts of this work (page 43 & ff. and page 60).

B) Methods

1) Carbachol treatment

0.3 milliliter of the solution of carbachol (100 µg/ml) was injected into the animal, either subcutaneously or intraperitoneally. It was found that either route had the same effect. An average of 70% of the treated guinea pigs survived the treatment. The animals were sacrificed by decapitation 17 hours after the carbachol injection.

2) Insulin treatment

The guinea pigs treated with insulin were fasted overnight. They were then given subcutaneously two units of insulin. They underwent shock within three to four hours following the injection. They recovered seven to eight hours after the injection. The survival was about 60% to 70% of the animals. In this group, the guinea pigs were killed twenty four hours after the insulin injection.

3) Reserpine treatment

0.5 milliliter of the reserpine solution (2 mg/ml) were injected into the animals via the intraperitoneal route. The animals were sacrificed twenty four hours after the treatment.

4) Sucrose density gradient method for the isolation of the catecholamine containing vesicles

After the animal had been killed by decapitation, the adrenal glands were rapidly removed, cleaned from the surrounding fatty tissue, and washed in physiological saline. After their weight had been determined, the glands were homogenized in the sucrose buffer - five milliliters - in an all glass homogenizer. The homogenate was then transferred into a twelve milliliter polyethylene centrifuge tube and centrifuged for five minutes at 600 x g in order to remove the nuclei and cell debris. The supernatant thus obtained was centrifuged at 11,000 x g for 20 minutes. The pellet which was obtained after this centrifugation was used for further

investigation, while the supernatant was discarded. The pellet was gently homogenized in 0.3 milliliter of sucrose buffer and the pestle of the homogenizer was washed with 0.5 milliliter of the same solution.

The resuspended pellet was gently layered over a linear sucrose density gradient contained in a centrifuge tube. The density gradient varied from 2.0 M sucrose at the bottom to 0.3M sucrose at the top of the tube. The tube was then loaded in a precooled swinging bucket rotor SW-39, and was centrifuged in a refrigerated preparative Spinco centrifuge, Model L, for thirty minutes at 100,000 x g. At the end of the centrifugation, the tube was gently lifted out from the rotor.

Four layers could be observed in the tube. The first layer, labelled fraction I, was on the top of the tube; it was opaque, containing lipid like material. The second layer, fraction II, was dense containing granular like material. The third layer, fraction III, had the same dense appearance and was very thick. There was a small sediment at the bottom of the tube which was labelled fraction IV.

Each fraction was carefully removed, by lowering the tip of a Pasteur pipette in the fraction and sucking very gently. Each fraction was then transfered into individual precooled polyethylene tubes which contained 5% trichloroacetic acid. From this extract, a 1 milliliter aliquot was removed for the estimation

of catecholamine and ATP content.

7) Preparation of the 11,000 x g sediment

After the adrenal glands had been quickly removed from the guinea pig, they were washed in saline, blotted on a filter paper and weighed. The glands were then homogenized in sucrose buffer. After five minute centrifugation at 600 x g to remove the cell debris and the nuclei, the supernatant was centrifuged for fifteen minutes at 11,000 x g. The pellet thus obtained, labelled 11,000 x g sediment, was dissolved into five milliliters of 5% trichloroacetic acid, after having been washed once in sucrose buffer. The clear supernatant was collected by centrifugation of the trichloroacetic acid extract and was labelled granular fraction extract. One milliliter aliquot was used for the catecholamine determination, while the remaining four milliliters were used for ATP measurements.

Since it had been found that the 11,000 x g sediment represented approximately the fraction III obtained by the sucrose density gradient technique, it was used for most of the experiments. The sucrose density gradient technique is a time-consuming technique, and in the case of the catecholamine containing granules, this factor is of importance. The granules from the gland of treated animals are more fragile than those from the gland of normal animals. The leakage of the various substances from the granules might be significantly increased

by manipulations. It was found, by experience, that the data obtained when using the sucrose density gradient method were affected by the long standing of the granules in non-physiological media. For this reason, after we had been able to show that the 11,000 x g sediment is a good approximation of the granular fraction obtained by the density gradient method, we shifted to that technique of preparation to diminish the risk of experimental errors.

II. Results

Three series of experiments were performed. In the first series, the depletion of catecholamines was studied on the whole adrenal glands, using either carbachol, insulin, or reserpine as the depleting agent. In the second series of experiments, the depletion of ATP and catecholamines was studied on the granules which had been isolated from the adrenal glands. In the third series of experiments, the repletion of catecholamines and ATP following the depletion by either one of the agents cited above was investigated. For all of these experiments, individual experimental results are given. The total catecholamine values have been calculated by making the sum of individual noradrenaline and adrenaline values. The mean and standard deviation from the mean, as well as the P values resulting from a group comparison test (Student t test) are presented under the experimental data.

A) <u>The depletion of catecholamines in the adrenal glands following a single injection of either carbachol, insulin or reserpine to male guinea pig</u> :

The effect of carbachol on the adrenaline and noradrenaline content of the adrenal glands was studied. The results of these experiments are given in Table VIa, page 69. The results are expressed as micrograms of substances per gram

of wet weight tissue. The weights of the glands were similar in both the treated and the control animals. Seventeen hours after a single subcutaneous injection of carbachol, there is a decrease in the total catecholamine content of the adrenal glands. This decrease, of about 25%, is significant ($P < 0.05$). In the carbachol treated guinea pig, there is a 20% increase in the noradrenaline content of the adrenal gland and this is statistically significant ($P < 0.05$). On the other hand, there is a 37% decrease in the adrenaline content which is also significant ($P < 0.05$). Thus carbachol does affect the release of catecholamines from the adrenal glands.

In Table VIb, page 70, are presented the results of the effect of insulin on the catecholamine content of the guinea pig adrenal glands. The results are expressed as micrograms of substances per gram of wet weight tissue. Twenty four hours after a single subcutaneous injection of insulin, there is a 23% decrease in the total catecholamine content of the adrenal gland, which is significant ($P < 0.05$). Both the noradrenaline and the adrenaline contents are decreased; the depletion of adrenaline (22%) is larger than that of noradrenaline (12%). Moreover, the change in the noradrenaline content of the adrenal glands from treated animals is not statistically significant ($P > 0.05$). This might be due to the very large variations between individual data as probably reflected

TABLE VIa

The Effect of a Single Injection of Carbachol on the Catecholamine Content of the Male Guinea Pig Adrenal Glands

Animal	Control			Treated		
	micrograms per gram of wet weight tissue					
	NA*	A**	CA***	NA*	A***	CA***
1	62.72	279.06	341.78	51.25	60.00	111.25
2	49.35	243.85	293.20	63.19	252.00	315.19
3	37.25	194.54	231.79	60.00	145.02	205.62
4	42.85	305.37	348.13	61.36	221.59	282.95
5	50.00	296.95	346.15	66.66	219.64	286.30
6	52.23	294.75	347.01	51.72	178.44	230.60
Mean	49.06	268.78	318.01	59.03	179.44	238.65
± S.D.	8.66	42.40	47.23	6.25	69.30	74.13
P' <				0.05	0.025	0.05

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines
' P : P values obtained by group comparison of control versus treated groups

TABLE VIb

The Effect of a Single Injection of Insulin on the Catecholamine Content of the Male Guinea Pig Adrenal Glands

Animal	Control			Treated		
	micrograms per gram of wet weight tissue					
	NA*	A**	CA***	NA*	A**	CA***
1	62.72	279.06	341.78	78.57	239.68	318.25
2	49.35	243.85	293.20	33.54	184.81	218.35
3	37.25	194.54	231.79	45.71	223.21	268.92
4	42.58	305.37	348.13	19.41	164.11	183.52
5	50.00	296.15	346.15	40.07	198.52	238.59
6	52.23	294.75	347.01			
Mean	49.06	268.78	318.01	43.46	202.06	245.52
± S.D.	8.66	42.40	47.23	21.90	30.05	51.15
P' <				0.60	00.01	0.025

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines
' P : P values obtained by group comparison of control versus treated groups

TABLE VIc

The Effect of a Single Injection of Reserpine on the Catecholamine Content of the Male Guinea Pig Adrenal Glands

Animal	Control			Treated		
	micrograms per gram of wet weight tissue					
	NA*	A**	CA***	NA*	A**	CA***
1	62.72	279.06	341.78	6.48	218.30	225.78
2	49.35	243.85	293.20	3.32	115.98	121.30
3	37.25	194.56	231.79	37.92	247.05	284.97
4	42.85	305.37	348.13	34.16	86.59	110.75
5	50.00	296.15	346.15	28.81	92.01	110.82
6	52.23	294.75	347.01			
Mean	49.06	268.70	318.01	22.53	151.98	170.72
± S.D.	8.66	42.40	47.23	15.53	75.17	80.17
P' <				0.005	0.01	0.005

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines
' P : P values obtained by group comparison of control versus treated groups

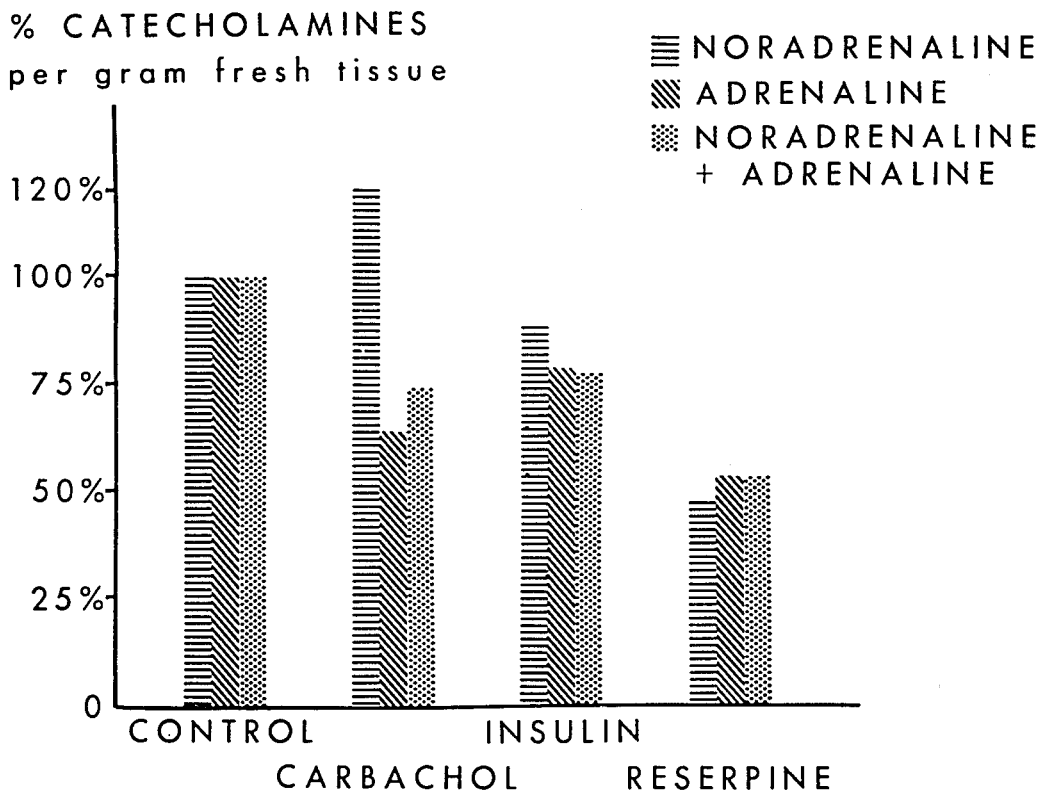

Figure 1
The depletion of catecholamines in the adrenal glands of treated guinea pigs

in the high value of the standard deviation, for this group of animals.

Table VIc, page 71, shows the catecholamine content of the adrenal glands from normal and reserpinized animals. The results are expressed as micrograms of substances per gram of wet weight tissue. The depletion of total catecholamines in the adrenal glands of the guinea pigs treated with reserpine is large, about 47%, twenty four hours after a single intraperitoneal injection of reserpine. The difference between controls and treated animals is significant ($P < 0.05$). The decrease of the noradrenaline content (46%) is larger than that of the adrenaline content (53%) in this group of animals. The P values are low in spite of large standard deviations, which indicates that the reserpine treatment has profound effect in depleting the catecholamines of the adrenal glands.

These findings are summarised in Figure 1, page 72. The values of noradrenaline, adrenaline and catecholamine found in the adrenal glands of the controls are chosen as 100%. The values from carbachol, insulin, and reserpine treated animals have been calculated as the percentage of the control values. The noradrenaline content of the adrenal glands is differently affected by the three agents. In the carbachol treated group, the noradrenaline content is increased (120%), while in both the insulin and the reserpine treated

groups there is a decrease of the noradrenaline content (88% and 46% of the control value respectively). On the other hand, qualitatively the effect of either of the three drugs on the content of adrenaline in the adrenal glands is similar. The most marked depletion is brought about by the action of reserpine (53%). The total catecholamine content of the adrenal glands is decreased by the three drugs, but since, in the carbachol treated group, the noradrenaline content is increased, this drug appears to have a smaller effect on the depletion.

B) The protein, ATP and catecholamine content of the granular fraction of the adrenal glands from normal, carbachol-, insulin-, or reserpine-treated male guinea pigs :

1) The protein content

The effect of either of the three drugs on the protein content of the adrenal glands are presented in Tables VIIa, VIIb, VIIc and VIId. The wet weight of the adrenal glands in milligrams per pair has been given as a sample of the weight of the glands in the guinea-pig. The variations from one animal to the other are small.

For the protein content, the results are expressed as milligrams of protein per fraction. As was mentionned in methods, four fractions were isolated by the sucrose density gradient method. In Table VIIa, page 76, the distribution of

the proteins in the four fractions is given for the control group. The amount of proteins varies from one fraction to the other, fraction III having the highest concentration of proteins. In the next Table VIIb, page 77, the data obtained with the carbachol treated animals are given. The protein distribution in the four fractions is identical to that observed in the controls. There is no difference in the protein content of each fraction when compared to that of the same fraction in the control group. In Table VIIc, page 78, the data obtained with the insulin treated guinea pigs are presented. As noted previously for the carbachol treated group, both the distribution and the individual protein content of each fraction are similar to that of the controls. In Table VIId, page 79, the results obtained with reserpine treated guinea pigs are given. There is no change in either the distribution or the content of protein in each fraction when compared to the controls.

A comprehensive summary of these experimental findings is provided in Figure 2, page 80. This figure summarizes the effect of carbachol, insulin or reserpine on the distribution of proteins in the four fractions obtained by the sucrose density method. The percentage of each fraction is calculated relatively to the total content of the four fractions chosen as 100%. The distribution of proteins is not affected by either

TABLE VIIa

The Protein Content of the Sucrose Density Gradient Fractions of the 11,000 x g Sediment from the Adrenal Glands of Normal Male Guinea Pigs

weight of the glands mg	Fraction I	Fraction II	Fraction III	Fraction IV
	milligrams of protein per fraction			
126	0.082	0.152	1.950	0.152
124	0.052	0.336	1.730	0.163
126	0.086	0.430	2 280	0.163
130	0.072	0.225	2.170	0.173
134	0.065	0.420	1.592	0.152
Mean128	0.071	0.312	2.016	0.160
S.D. 4	0.010	0.121	0.214	0.0009

TABLE VIIb

The Protein Content of the Sucrose Density Gradient Fractions of the 11,000 x g Sediment from the Adrenal Glands of Carbachol Treated Male Guinea Pigs

weight of the glands mg	Fraction I	Fraction II	Fraction III	Fraction IV
	milligrams of protein per fraction			
144	0.068	0.445	2.200	0.304
140	0.086	0.467	2.460	0.173
116	0.076	0.430	2.000	0.206
130	0.086	0.300	2.250	0.315
120	0.082	0.410	2.060	0.217
Mean 130	0.079	0.410	2.194	0.243
±S.D. 12	0.000	0.064	0.179	0.062

TABLE VIIc

The Protein Content of the Sucrose Density Gradient Fractions of the 11,000 x g Sediment from the Adrenal Glands of Insulin Treated Male Guinea Pigs

weight of the glands mg	Fraction I	Fraction II	Fraction III	Fraction IV
	milligrams of proteins per fraction			
126	0.058	0.406	1.913	0.250
158	0.063	0.467	2.005	0.271
160	0.078	0.652	2.173	0.195
170	0.082	0.652	3.040	0.250
136	0.073	0.541	2.403	0.225
Mean 146	0.070	0.541	2.403	0.225
±S.D. 17	0.000	0.109	0.497	0.022

TABLE VIId

The Protein Content of the Sucrose Density Gradient Fractions of the 11,000 x g Sediment from the Adrenal Glands of Reserpine Treated Male Guinea Pigs

weight of the glands mg	Fraction I	Fraction II	Fraction III	Fraction IV
	milligrams of protein per fraction			
131	0.097	0.543	2.065	0.140
122	0.091	0.391	2.665	0.119
136	0.106	0.445	2.226	0.141
120	0.100	0.467	1.768	0.173
144	0.108	0.423	2.021	0.137
Mean 130	0.100	0.453	2.151	0.188
±S.E. 10	0.000	0.044	0.325	0.094

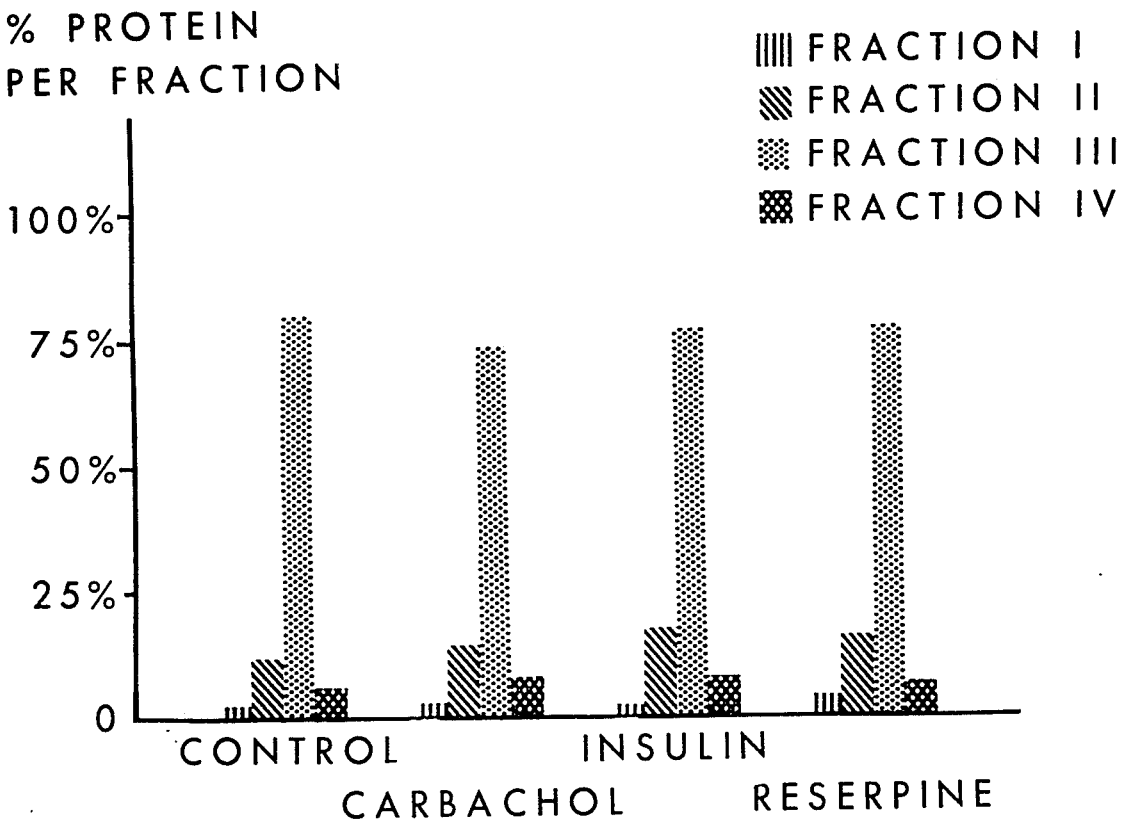

Figure 2

The protein content of the sucrose density gradient fractions of the 11,000 x g sediment

of the three drugs. The small differences observed are due probably to the imprecision of the method used for the collection of the various fractions.

2) The catecholamine content

The content of noradrenaline in the four fractions of the control, carbachol-, insulin-, or reserpine-treated groups is given in Tables VIIIa, VIIIb, VIIIc and VIIId. The concentration of the measured amines is expressed as micrograms per fraction.

In Table VIIIa, page 83, the distribution and the content of adrenaline and noradrenaline in the four fractions are given for the control group. There is a large amount of both amines concentrated in the fraction III. There is also a significant amount of both amines present in fraction IV.

The distribution and the content of noradrenaline and adrenaline in the four fractions of the carbachol treated group are presented in Table VIIIb, page 84. The distribution of adrenaline and noradrenaline is similar to that observed in the controls. There is a large amount of both amines in fraction III and a small amount of both amines is found in fraction IV. Both fractions I and II contain negligible amounts of catecholamines. The actual amounts of adrenaline and of noradrenaline are low in both fractions

III and IV of the carbachol treated group as compared to that of the control group. This decrease in the concentration of both amines is significant ($P < 0.05$) for adrenaline only.

Table VIIIc, page 85, shows the results obtained with the insulin treatment. The concentration of adrenaline in fractions III and IV is decreased, as previously noted for the carbachol treated group.

In Table VIIId, page 86, the data obtained for the reserpine treated group are presented. There is no change in the distribution of noradrenaline and adrenaline in the four fractions. In fraction III, only the decrease in the concentration of adrenaline is statistically significant as compared to that of the control group ($P < 0.05$). This decrease, however, is not larger than that brought about by insulin, and much smaller than that brought about by carbachol. The decrease in the adrenaline content of the fraction IV is also statistically significant ($P < 0.05$).

The data presented above are summarized in Figures 3 and 4. In Figure 3, page 87, the effect of the three depleting agents on the distribution of catecholamines in the four fractions is shown. For each fraction, the concentration of catecholamines is expressed as the percentage of the total concentration of the four fractions. In the control group as

TABLE VIIIa

The Catecholamine Content of the Sucrose Density Gradient Fractions of the 11,000 x g Sediment from the Adrenal Glands of Normal Male Guinea Pigs

weight of the glands mg	Fraction I		Fraction II		Fraction III		Fraction IV	
	micrograms of catecholamine per fraction							
	NA*	A**	NA*	A**	NA*	A**	NA*	A**
126	0.004	0.102	0.084	0.124	1.076	10.880	0.728	4.480
124	0.056	0.081	0.064	0.040	1.704	9.424	0.752	4.000
126	0.048	0.101	0.039	0.164	0.284	9.750	0.142	1.644
130	0.077	0.028	0.039	0.234	2.270	9.244	0.510	3.750
134	0.088	0.000	0.148	0.220	0.416	7.633	0.100	1.422
Mean 128	0.051	0.123	0.074	0.157	1.150	9.374	0.445	3.052
±S.D. 4	-	-	-	-	0.943	1.164	0.304	1.411

* NA : noradrenaline; ** A : adrenaline

TABLE VIIIb

The Catecholamine Content of the Sucrose Density Gradient Fractions of the 11,000 x g Sediment from the Adrenal Glands of Carbachol Treated Male Guinea Pigs

weight of the glands mg	Fraction I		Fraction II		Fraction III		Fraction IV	
	micrograms of catecholamine per fraction							
	NA*	A**	NA*	A**	NA*	A**	NA*	A**
144	0.006	0.020	0.048	0.026	0.668	4.648	0.052	0.144
149	0.016	0.004	0.068	0.068	1.224	3.196	0.344	1.180
116	0.016	0.020	0.020	0.060	0.676	1.844	0.360	1.708
130	0.052	0.000	0.096	0.050	0.742	4.728	0.550	0.784
120	0.024	0.008	0.056	0.084	1.370	1.880	0.390	1.068
Mean 130	0.022	0.010	0.057	0.057	0.930	3.250	0.339	0.976
±S.D. 12	-	-	-	-	0.334	1.412	0.182	0.537
P' <					0.60	0.001	0.50	0.005

* NA : noradrenaline; ** A : adrenaline
' P : P values obtained by group comparison of control versus treated groups

TABLE VIIIc

The Catecholamine Content of the Sucrose Density Gradient Fractions of the 11,000 x g Sediment from the Adrenal Glands of Insulin Treated Male Guinea Pigs

Weight of the glands mg	Fraction I		Fraction II		Fraction III		Fraction IV	
	micrograms of catecholamine per fraction							
	NA*	A**	NA*	A**	NA*	A**	NA*	A**
126	0.068	0.192	0.084	0.236	0.936	7.188	0.472	2.363
158	0.078	0.134	0.100	0.180	3.120	5.140	0.372	2.060
140	0.000	0.200	0.000	0.102	1.140	8.800	0.130	2.068
170	0.032	0.148	0.068	0.280	0.944	6.468	0.096	2.224
136	0.044	0.200	0.054	0.264	1.496	6.408	0.376	2.056
Mean 146	0.044	0.174	0.063	0.208	1.527	6.788	0.289	2.228
±S.D. 17	-	-	-	-	0.918	1.379	0.165	0.487
P' <					0.50	0.0005	0.30	0.50

* NA : noradrenaline; ** A : adrenaline
' P : P values obtained by group comparison of control versus treated groups

TABLE VIIId

The Catecholamine Content of the Sucrose Density Gradient Fractions of the 11,000 x g Sediment from the Adrenal Glands of Reserpine Treated Male Guinea Pigs

weight of the glands mg	Fraction I		Fraction II		Fraction III		Fraction IV	
	micrograms of catecholamine per fraction							
	NA*	A**	NA*	A*	NA*	A**	NA*	A**
131	0.000	0.028	0.056	0.100	0.564	6.000	0.360	1.880
122	0.000	0.026	0.088	0.040	0.108	5.400	0.026	1.708
136	0.024	0.160	0.023	0.132	0.506	8.320	0.280	2.160
120	0.004	0.860	0.012	0.220	0.120	8.360	0.240	1.620
144	0.000	0.000	0.088	0.128	0.156	5.060	0.000	1.682
Mean 130	0.012	0.050	0.037	0.118	0.496	6.628	0.225	1.809
±S.D. 10	-	-	-	-	0.427	1.589	0.145	0.218
P' <					0.10	0.005	0.10	0.05

* NA : noradrenaline; ** A : adrenaline

' P : P values obtained by group comparison of control versus treated groups

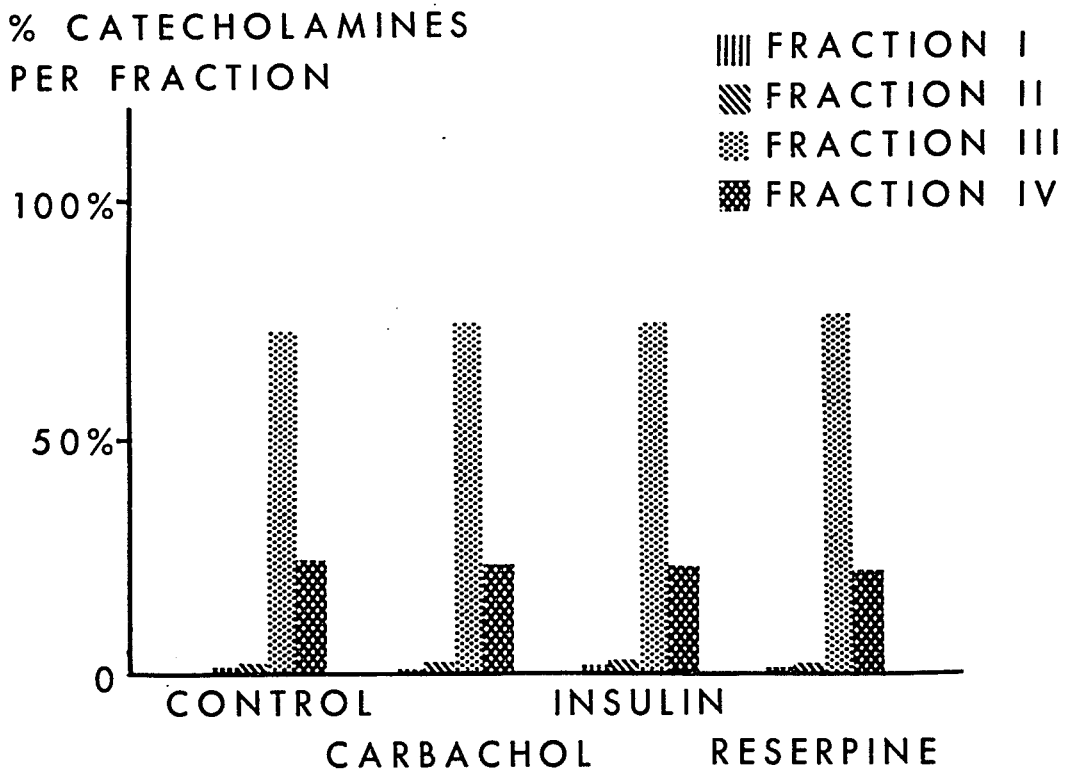

Figure 3

The catecholamine content of the sucrose density gradient fractions of the 11,000 x g sediment

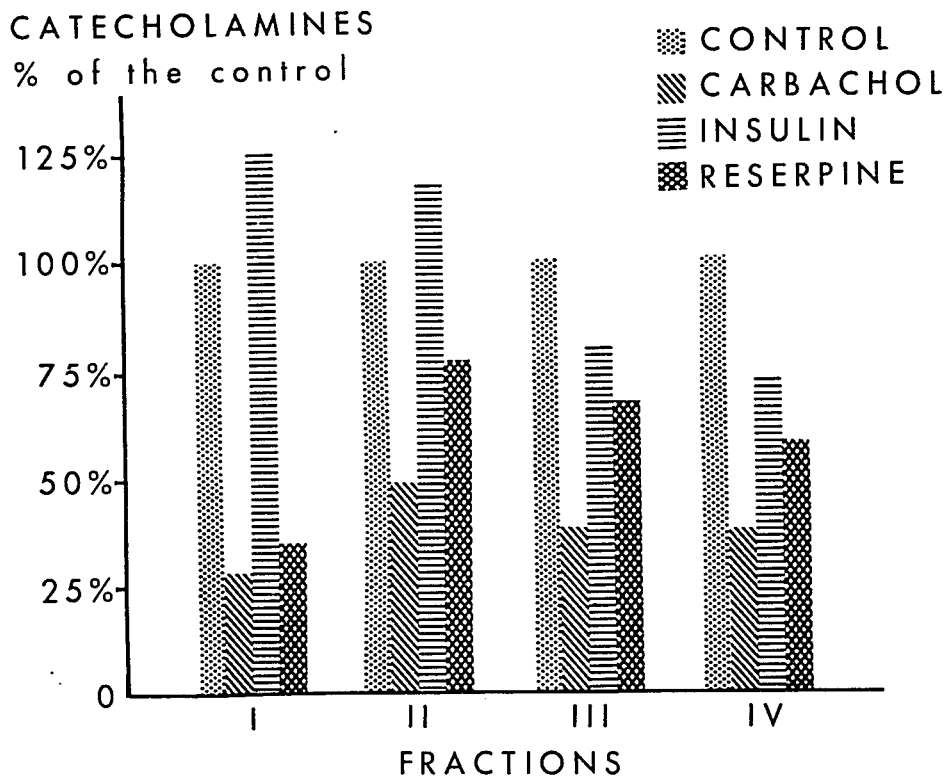

Figure 4

The effect of carbachol, insulin, or reserpine on the catecholamine content of the various sucrose density gradient fractions

well as in the carbachol-, insulin-, and reserpine-treated groups, the pattern of the distribution of catecholamines in the various fractions is the same. There is between 72% and 75% of the total catecholamines in the fraction III, while in fractions I, II, and IV the percentage is low.

In Figure 4, page 88, the content of catecholamines in each of the fractions as affected by the three drugs is shown. The results obtained for each of the four fractions in the control group are taken as 100%. For the three treated groups, the results for each of the four fractions are expressed as the percentage of the corresponding one of the control group. Although we have presented the values for all the fractions, the experimental data obtained for fractions I and II are too low to be of any significance. In fraction III, the effect of carbachol is very profound, the catecholamine content of this fraction being 38% that of the control. Insulin and reserpine appear to affect the catecholamine content in a similar manner, the decrease being 21% and 33% respectively. The same pattern is observed in fraction IV, a finding which is not inconsistent with the possibility that this fraction represents a cluster of granules.

3) <u>The ATP content</u>

The ATP content could be measured only in fraction

III, in the other fractions it was not detectable with the method used. The experimental results obtained with the normal and treated groups are given in Table IX, page 91. The results are expressed as micrograms of ATP per fraction. The content of ATP in fraction III is decreased after the treatment of guinea pigs with either of the three drugs. The decrease is statistically significant for all the three groups when compared to the control group ($P < 0.05$). On the other hand, the extent of the depletion does not differ from one treated group to the other.

The results presented in this section B show that the catecholamines and ATP are found in large amounts in the fraction III which contains the catecholamine containing vesicles. For this reason, the experimental results obtained from fraction III have been expressed as micrograms of substances per milligram of proteins (Tables Xa and Xb).

In Table Xa, page 92, the effect of carbachol, insulin and reserpine treatment on the depletion of catecholamines in fraction III is shown. For each group, the protein and catecholamine contents of the fraction III are given (columns 1 and 2). It is from these data that the concentration of catecholamines in micrograms per milligram of proteins was calculated (column 3). The concentration of catecholamines in the control group is significantly higher than that of any of each treated group ($P < 0.05$). When expressed

TABLE IX

The ATP Content of the Sucrose Density Gradient Fraction III of the 11,000 x g Sediment from the Adrenal Glands of Normal, Carbachol, Insulin, or Reserpine Treated Guinea Pigs

Animal	Control	Carbachol Treated	Insulin Treated	Reserpine Treated
	micrograms of ATP per fraction III			
1	15.32	4.00	6.44	5.08
2	13.32	5.32	2.27	4.20
3	9.87	4.64	7.76	6.44
4	11.48	5.08	3.84	4.40
5	12.31	3.32	5.08	5.76
Mean	12.44	4.47	5.08	5.18
±S.D.	2.02	0.81	2.15	0.93
P' <		0.001	0.001	0.001

' P : P values obtained by group comparison of control versus treated groups

T A B L E Xa

The Catecholamine Content of the Sucrose Density Gradient Fraction III of the 11,000 x g Sediment from the Adrenal Glands of Normal, Carbachol, Insulin, or Reserpine Treated Guinea Pigs

Animal	Control			Carbachol Treated			Insulin Treated			Reserpine Treated		
	P*	CA**	CA***	P*	CA**	CA***	P*	CA**	CA***	P*	CA**	CA***
1	1.95	11.96	6.13	2.20	5.32	2.42	1.91	8.12	4.25	2.06	5.64	3.19
2	1.73	11.12	6.43	2.46	4.33	1.76	2.01	8.16	4.08	2.67	5.51	2.07
3	2.28	10.03	4.40	2.00	2.52	1.26	2.17	8.94	4.12	2.23	8.32	3.74
4	2.17	11.51	5.30	2.25	5.47	2.43	3.04	7.31	2.40	1.77	8.48	4.76
4 5	1.59	8.05	5.06	2.06	3.25	1.58	2.40	7.90	3.30	2.02	5.22	2.58
Mean	2.08	10.54	5.46	2.19	4.18	1.89	2.40	8.09	3.63	2.15	6.62	3.27
±S.D.	0.22	1.56	0.82	0.18	1.28	0.52	0.50	0.59	0.78	0.33	1.63	1.05
P'					0.01	0.01		0.01	0.01		0.01	0.01

* P : protein , milligrams per fraction III; ** CA : total catecholamines µg per fraction III
*** CA : total catecholamines, micrograms per milligram of protein
' P : P values obtained by group comparison of control versus treated groups

TABLE Xb

The ATP Content of the Sucrose Density Gradient Fraction III of the 11,000 x g Sediment from the Adrenal Glands of Normal, Carbachol, Insulin, or Reserpine Treated Guinea Pigs

Animal	Control			Carbachol Treated			Insulin Treated			Reserpine Treated		
	P*	ATP**	ATP***	P*	ATP**	ATP***	P*	ATP**	ATP***	P*	ATP**	ATP***
1	1.95	15.32	7.86	2.20	4.00	1.82	1.91	6.44	3.37	2.07	5.08	2.46
2	1.73	13.32	7.70	2.46	5.32	2.16	2.02	2.27	1.14	2.67	4.20	1.57
3	2.28	9.67	4.33	2.09	4.64	2.32	2.17	7.76	3.57	2.23	6.46	2.90
4	2.17	11.48	5.29	2.25	5.08	2.26	3.04	3.84	1.26	1.77	4.40	2.49
5	1.59	12.31	7.73	2.06	3.32	1.61	2.40	5.08	2.14	2.08	5.76	2.85
Mean	2.02	12.44	6.58	2.19	4.47	2.03	2.40	5.08	2.30	2.15	5.18	2.45
±S.D.	0.21	2.02	0.74	0.18	0.81	0.30	0.50	2.15	1.14	0.33	0.93	0.53
P'					0.01	0.01		0.01	0.01		0.01	0.01

* P : protein, milligrams per fraction III; ** ATP : ATP, micrograms per fraction III
*** ATP : ATP, micrograms per milligram of protein
' P : P values obtained by group comparison of control versus treated groups

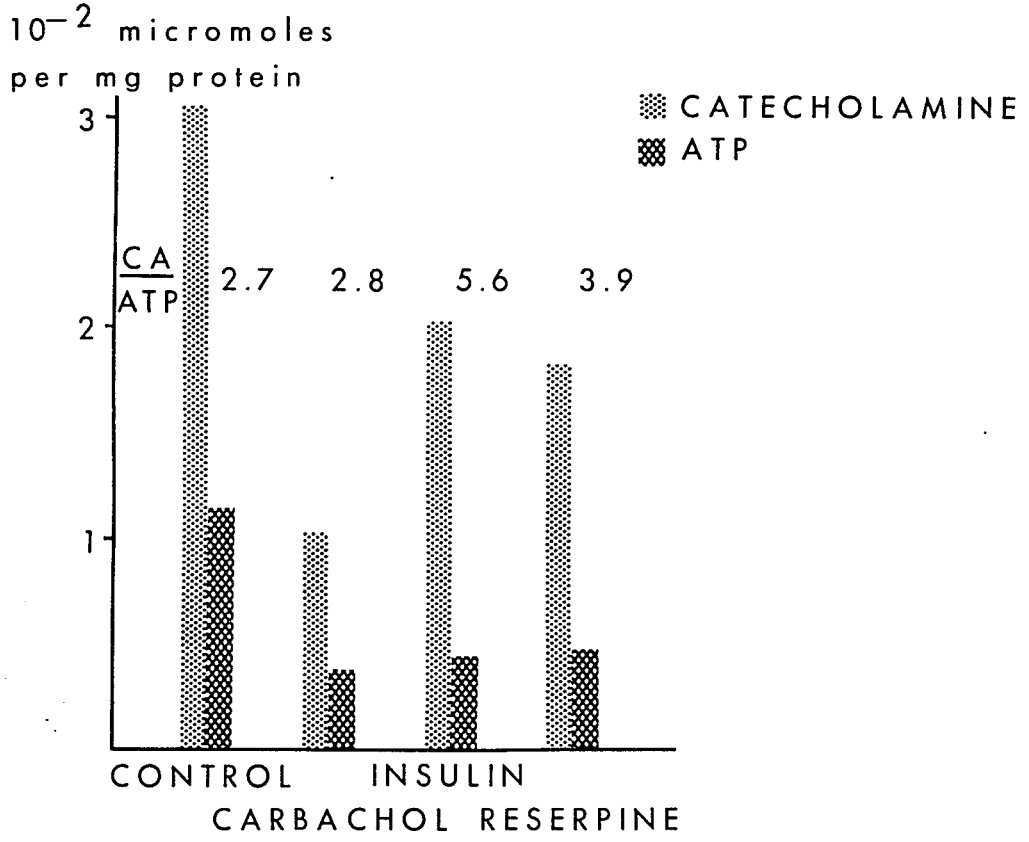

Figure 5

The effect of carbachol, insulin, or reserpine on the catecholamine and ATP content of the sucrose density gradient fraction III

under this form, it is found that the catecholamine content for the carbachol treated group is 34.4%, for the insulin treated group 66.4% and for the reserpine treated group 59.8% that of the control group.

On the basis of per milligram of proteins, the depletion of ATP is identical for all the treated groups (Table Xb, page 93). In the carbachol treated group the ATP content is 30.8%, in the insulin treated group it is 34.8%, and in the reserpine treated group it is 37.1% that of the control group.

These findings are summarized in Figure 5, page 94, where they are expressed as micromoles of substances per milligram of proteins. When presented under this form, the results show that the depletion of catecholamines is closely related to the depletion of ATP. The values of the molar ratio: CA/ATP are for all groups situated around 3, except for the insulin group which has the value equal to 5.6.

C) <u>The repletion of catecholamines and ATP by the adrenal glands of male guinea pigs pretreated with carbachol, insulin, or reserpine</u> :

In these series of experiments, the determinations of catecholamines and ATP were made on the 11,000 x g sediment since it has been found that it closely resembles the fraction

III obtained by sucrose density gradient technique. From then on, we shall refer to this 11,000 x g sediment as the granular fraction of the adrenal glands.

1) The repletion of catecholamines and ATP following the depletion of these substances by a single injection of carbachol to the male guinea pigs

The content of catecholamines and ATP in the granular fraction of the adrenal glands from animals pretreated with carbachol was determined two days, four days and eight days after a single injection of carbachol. These results are expressed as micrograms of substances per gram of wet weight tissue (Tables XIa and XIb).

Two days after the injection of carbachol, there is an increase (25%) in the noradrenaline content of the granular fraction (Table XIa, page 98). On the other hand, the adrenaline content is markedly decreased (42%). Both the increase in the noradrenaline and the decrease in the adrenaline contents are statistically significant. When the two values are combined to yield the total catecholamine content of the granular fraction, the change is not significant ($P \geq 0.05$). The ATP content of the granular fraction is also significantly decreased.

Four days after the injection of carbachol, the

noradrenaline content is still high (Table XIb, page 99), but not as high as that found two days after the injection. The adrenaline content has not changed and the total catecholamine content is significantly decreased when compared to that of the controls. The ATP level, though lower than in the control is increased above the level reached two days after the injection.

Eight days after the injection, the content of noradrenaline is still above normal level (Table XIb, page 99), but decreased compared to that of the fourth day. The adrenaline has not yet reached its normal value but is increased when compared to that of the fourth day. The ATP level has returned to its normal value.

A group comparison between control and carbachol treated groups on one hand, and between the various treated groups on the other hand has been computed in Table XIc, page 100.

When the carbachol treated groups are compared to the control group, it is observed that the difference in the catecholamine level is significant only on the fourth day following the injection. Two days after the injection, the high level of noradrenaline masks the decrease in the adrenaline, while on the eighth day, the high level of adrenaline masks

TABLE XIa

Repletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs Pretreated with Carbachol

Animal	Control				Carbachol Treated 2nd day			
	micrograms per gram of wet weight tissue							
	NA*	A**	CA***	ATP	NA*	A**	CA***	ATP
1	25.00	115.76	140.76	119.02	59.46	99.71	159.17	89.29
2	18.87	146.93	165.80	128.82	79.38	91.67	161.05	71.25
3	20.75	164.15	184.80	136.67	69.08	64.28	133.26	73.53
4	23.86	126.17	150.56	115.05	57.62	48.77	106.39	61.48
5	41.66	128.33	169.99	131.11	71.85	73.80	144.65	76.01
6	32.44	135.63	168.07	143.88	75.88	78.35	154.23	54.28
Mean	27.09	136.25	163.33	129.29	68.67	79.43	143.30	71.00
±S.D.	8.52	17.12	15.53	10.66	8.75	19.69	20.75	12.14

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines

TABLE XIb

Repletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs Pretreated with Carbachol

Animal	Carbachol Treated 4th day				Carbachol Treated 8th day			
	micrograms per gram of wet weight tissue							
	NA*	A**	CA***	ATP	NA*	A**	CA***	ATP
1	53.27	71.41	134.68	85.04	25.14	123.75	148.89	115.70
2	59.35	68.52	127.88	83.24	40.23	113.48	153.71	142.05
3	52.38	74.28	126.66	85.03	29.49	101.22	130.71	135.14
4	34.50	71.92	106.41	107.50	49.75	107.63	157.37	112.50
5	54.77	97.67	152.45	106.21	55.98	123.63	179.61	126.68
6	37.94	77.78	115.72	97.97	31.60	97.87	129.48	149.28
Mean	48.70	76.93	127.30	94.16	38.70	111.26	149.96	130.22
S.D.	10.02	10.63	15.86	11.17	12.18	11.02	18.65	14.59

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines

TABLE XIc

Repletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs Pretreated with Carbachol

GROUP COMPARISON

Groups Compared	P values			
	Noradrenaline	Adrenaline	Total Catecholamines	ATP
Carbachol vs Control 2nd day	< 0.001	< 0.001	> 0.10	< 0.001
Carbachol vs Control 4th day	< 0.001	< 0.001	< 0.001	< 0.001
Carbachol vs Control 8th day	> 0.10	< 0.020	> 0.20	> 0.25
Carbachol vs Carbachol 4th day / 2nd day	< 0.005	> 0.80	> 0.20	< 0.01
Carbachol vs Carbachol 8th day / 4th day	< 0.02	< 0.001	< 0.05	< 0.001

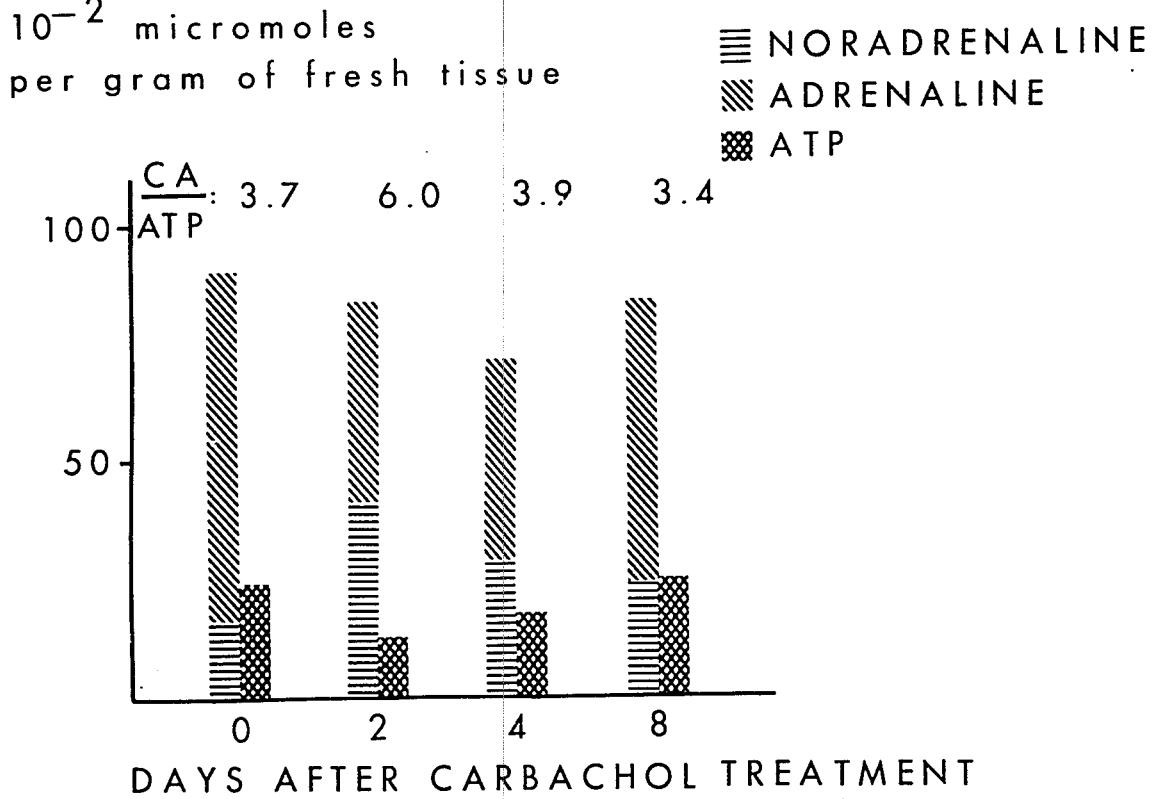

Figure 6

The ATP and catecholamine repletion in the granular fraction of the adrenal glands of carbachol treated guinea pigs

the change in the noradrenaline. The level of ATP is significantly lower on the second and fourth days, but has returned to normal on the eighth day.

The ATP is significantly increased between the second and the fourth days, and between the fourth and the eighth days. For the catecholamines, the decrease in the noradrenaline content is significant between the second and the fourth days only, while the increase of both adrenaline and total catecholamine levels is significant between the fourth and the eighth days only.

In Figure 6, page 101, the repletion of catecholamines and ATP has been expressed on a molar basis, per gram of wet weight tissue. The results thus expressed show that the synthesis of ATP follows closely the synthesis of adrenaline, but does not seem to be linked to the synthesis of noradrenaline.

2) <u>The repletion of catecholamines and ATP following the depletion of these substances by a single injection of insulin to the male guinea pigs</u> :

In Tables XIIa and XIIb, are presented the experimental data expressed as micrograms of substances per gram of wet weight tissue, obtained with insulin treated animals.

Two days after the injection, there is a slight increase in the noradrenaline level which is not significant

(Table XIIa, page 104). But the decrease in adrenaline is large so that the total catecholamine content is significantly decreased ($P < 0.05$). The level of ATP is 44% lower than that of the control.

Four days after the injection, the noradrenaline level is still well above normal (Table XIIb, page 105). The level of adrenaline is still below normal, but its increase from the second day is such that the total catecholamine value has returned to its normal value. The ATP level is still below normal.

Eight days after the injection, the levels of noradrenaline, adrenaline and ATP have returned to their normal values (Table XIIb, page 105). The slight increase in the ATP and adrenaline levels is not significant.

The P values obtained with the Student t test are presented in Table XIIc, page 106.

Two days after the treatment, the adrenaline, ATP and total catecholamine contents are significantly decreased ($P < 0.05$). Four days following the treatment the adrenaline and ATP levels are significantly decreased ($P < 0.05$), the noradrenaline level is significantly increased ($P < 0.05$), while the total catecholamine level is not significantly changed ($P > 0.05$). There is no statistical difference between the treated group and the control group, eight days after the

TABLE XIIa

Depletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs Pretreated with Insulin

Animal	Control				Insulin Treated 2nd day			
	micrograms per gram of wet weight tissue							
	NA*	A**	CA***	ATP	NA*	A**	CA***	ATP
1	25.00	115.76	140.76	119.02	27.27	93.64	132.91	52.31
2	18.87	146.93	165.80	128.82	24.12	81.14	105.26	46.24
3	20.75	164.15	184.80	136.67	29.56	101.58	130.15	62.74
4	23.86	126.70	150.56	115.05	48.22	87.58	135.80	65.88
5	41.66	128.33	169.99	131.11	50.00	104.30	154.30	123.50
6	32.44	135.63	168.07	143.08	42.36	86.22	128.59	82.04
Mean	27.09	136.25	163.33	129.29	36.92	92.75	131.17	72.46
±S.D.	8.52	17.13	15.53	10.86	11.31	9.21	15.73	27.66

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines

TABLE XIIb

Repletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs Pretreated with Insulin

Animal	Insulin Treated 4th day				Insulin Treated 8th day			
	micrograms per gram of wet weight tissue							
	NA*	A**	CA***	ATP	NA*	A**	CA***	ATP
1	51.87	109.20	161.07	99.20	31.08	147.17	178.25	132.31
2	32.80	130.12	162.93	81.25	31.38	148.92	180.31	139.15
3	40.40	139.86	179.47	116.13	29.29	133.50	162.80	152.19
4	52.33	92.54	144.87	79.79	33.97	138.18	172.15	129.16
5	75.52	86.03	161.55	113.57	20.41	149.45	169.87	137.70
6	56.57	88.88	165.44	110.41	17.18	173.86	191.04	119.72
Mean	51.58	107.77	162.60	100.04	27.22	148.51	175.74	135.18
±S.D.	14.58	22.77	11.12	16.20	6.77	13.98	9.75	10.95

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines

TABLE XIIc

Repletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs

Pretreated with Insulin

GROUP COMPARISON

Groups Compared	P values			
	Noradrenaline	Adrenaline	Total Catecholamines	ATP
Insulin vs Control 2nd day	> 0.2	< 0.001	< 0.01	< 0.001
Insulin vs Control 4th day	< 0.01	< 0.05	> 0.60	< 0.005
Insulin vs Control 8th day	-	> 0.20	> 0.20	-
Insulin vs Insulin 4th day 2nd day	> 0.10	> 0.20	< 0.05	> 0.10
Insulin vs Insulin 8th day 4th day	< 0.005	< 0.005	> 0.10	< 0.005

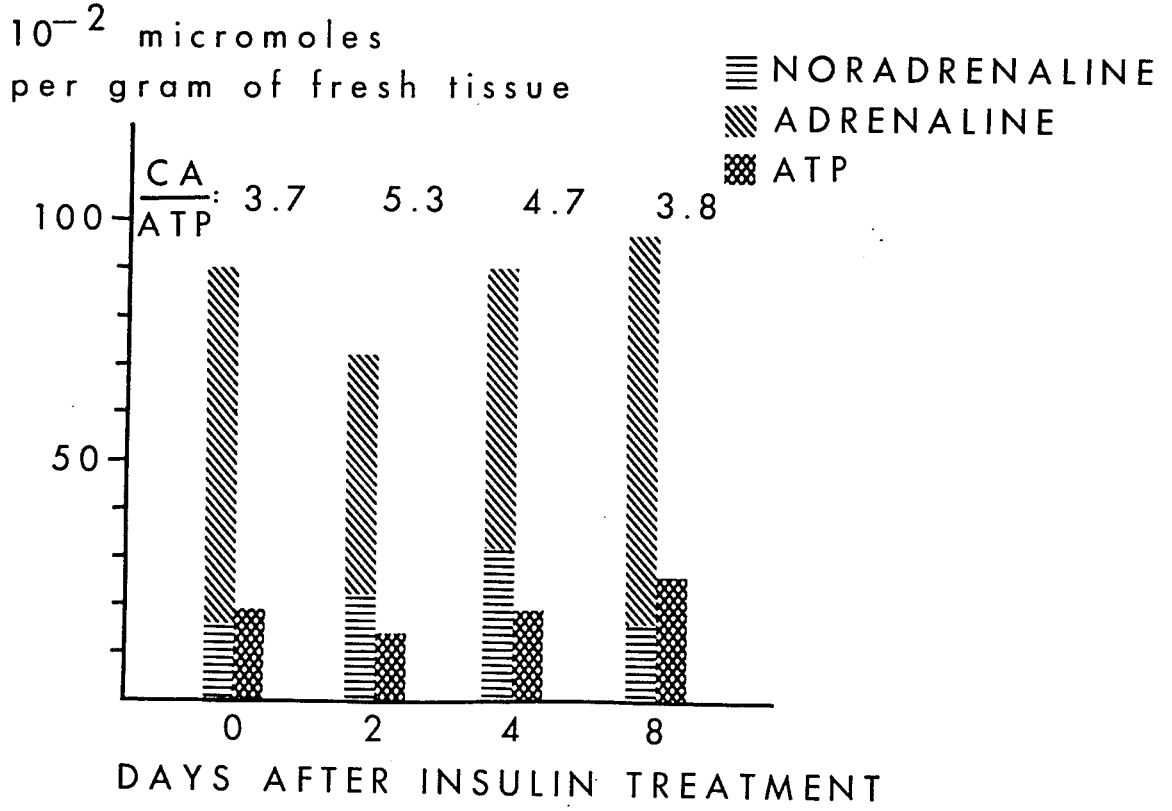

Figure 7

The ATP and catecholamine repletion in the granular fraction of the adrenal glands of insulin treated guinea pigs

treatment with insulin ($P \geq 0.05$).

There is no significant difference between group 2 (two days after the injection) and group 4 (four days after the injection), except for the total catecholamine content which is increased in the latter group. On the other hand, between group 4 and group 8 (eight days after the injection) the levels of ATP and adrenaline are both significantly increased. The level of noradrenaline is significantly decreased ($P < 0.05$). Because of the inverse variations in the adrenaline and noradrenaline levels, the total catecholamine level does not exhibit any significant change between the fourth and the eighth days ($P \geq 0.05$).

The molar concentration of the catecholamine and ATP (in micromoles of substances per gram of wet weight tissue) have been calculated and the results obtained are presented in Figure 7, page 107. As was previously observed for the carbachol treated group, the synthesis of noradrenaline does not appear to be related to the synthesis of ATP which is closely linked to that of adrenaline. The values of the molar ratio CA/ATP returns to normal only when the adrenaline level has reached its normal value. Moreover, an overshooting of adrenaline synthesis is closely paralleled by an overshooting of ATP synthesis (group 8).

3) The repletion of catecholamines and ATP following the depletion of these substances by a single injection of reserpine to the male guinea pigs :

The experimental data obtained with the reserpinized guinea pigs are presented in Tables XIIIa and XIIIb. The results are expressed in micrograms of substances per gram of wet weight tissue.

Two days after reserpine treatment, there is a marked decrease in the noradrenaline, adrenaline and ATP contents of the granular fraction (Table XIIIa, page 111). The total catecholamine content is also significantly decreased.

Four days after the treatment, there is a further decrease in both the ATP and the adrenaline levels (Table XIIIb, page 112). But, although the noradrenaline level is still below normal, it is increased when compared to that of the second day. The total catecholamine content has not changed.

On the eighth day, all values are below the normal values, but the adrenaline and ATP levels are much higher than they were on the fourth day (Table XIIIb, page 112).

The statistical treatment of these data is summarized in Table XIIIc, page 113.

In all the treated groups, even eight days after the treatment with reserpine, there is a significant decrease

in the adrenaline, total catecholamines and ATP levels as compared to the controls ($P < 0.05$). The noradrenaline content, on the other hand is not significantly changed on the eighth day after reserpine treatment ($P \geq 0.05$).

While the decrease in both the adrenaline and the noradrenaline levels is significant between the second and the fouth days following the treatment, the total catecholamine as well as the ATP contents have not significantly changed during these two days. On the other hand, the increase in the adrenaline, total catecholamine and ATP is significant between the fourth day and the eighth day following the injection of reserpine. The noradrenaline level has not changed significantly during that period.

The molar concentrations of all these substances, as affected by the reserpine treatment, are presented in Figure 8, page 114. The reserpine treatment decreases the total catecholamine content by decreasing both the adrenaline and the noradrenaline contents. The repletion of both amines is slow and out of phase. While the repletion of noradrenaline starts between the second and the fourth days after the treatment, that of adrenaline is delayed until the fourth day. The synthesis of ATP is also delayed until the fourth day after the treatment. Because the synthesis of adrenaline as well as that of ATP are delayed, the molar

TABLE XIIIa

Repletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs Pretreated with Reserpine

Animal	Control				Reserpine Treated 2nd day			
	NA*	A**	CA***	ATP	NA*	A**	CA***	ATP
1	25.00	115.76	140.76	119.02	6.00	83.00	89.00	76.25
2	18.87	146.93	165.80	128.82	4.62	78.70	83.32	57.87
3	20.75	164.15	184.80	136.67	5.66	80.66	86.32	75.87
4	23.86	126.70	150.56	115.05	5.36	76.33	81.68	78.12
5	41.66	128.33	169.99	131.11	7.87	86.51	94.44	79.62
6	32.44	135.63	168.07	143.08	6.25	73.55	79.80	66.58
Mean	27.09	136.25	163.33	129.29	5.95	79.86	85.11	72.40
±S.D.	8.52	17.13	15.53	10.86	1.09	4.67	5.73	8.45

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines

micrograms per gram of wet weight tissue

TABLE XIIIb

Repletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs Pretreated with Reserpine

Animal	Reserpine Treated 4th day				Reserpine Treated 8th day			
	micrograms per gram of wet weight tissue							
	NA*	A**	CA***	ATP	NA*	A**	CA***	ATP
1	18.38	83.45	101.83	72.42	8.46	125.38	133.84	96.15
2	13.66	66.08	79.94	52.84	14.68	121.83	135.91	71.30
3	6.00	50.33	56.33	64.66	14.00	110.33	124.33	86.10
4	23.92	75.00	98.92	63.39	33.18	90.00	123.18	94.33
5	9.23	81.53	90.76	75.76	15.57	117.02	132.55	88.40
6	18.47	61.23	79.70	69.38	17.24	113.10	130.34	80.34
Mean	16.64	69.60	84.55	66.40	17.70	112.94	130.10	86.10
±S.D.	4.96	12.78	16.64	8.10	9.25	12.51	5.30	9.29

* NA : noradrenaline; ** A : adrenaline; *** CA : total catecholamines

TABLE XIIIc

Repletion of Catecholamines and ATP by the Adrenal Glands of Male Guinea Pigs Pretreated with Reserpine

GROUP COMPARISON

Groups Compared	P values			
	Noradrenaline	Adrenaline	Total Catecholamines	ATP
Reserpine vs Control 2nd day	< 0.001	< 0.001	< 0.001	< 0.001
Reserpine vs Control 4th day	< 0.05	< 0.001	< 0.001	< 0.001
Reserpine vs Control 8th day	> 0.10	< 0.025	< 0.005	< 0.001
Reserpine vs Reserpine 4th day / 2nd day	< 0.001	< 0.01	-	> 0.25
Reserpine vs Reserpine 8th day / 4th day	> 0.80	< 0.001	< 0.001	< 0.005

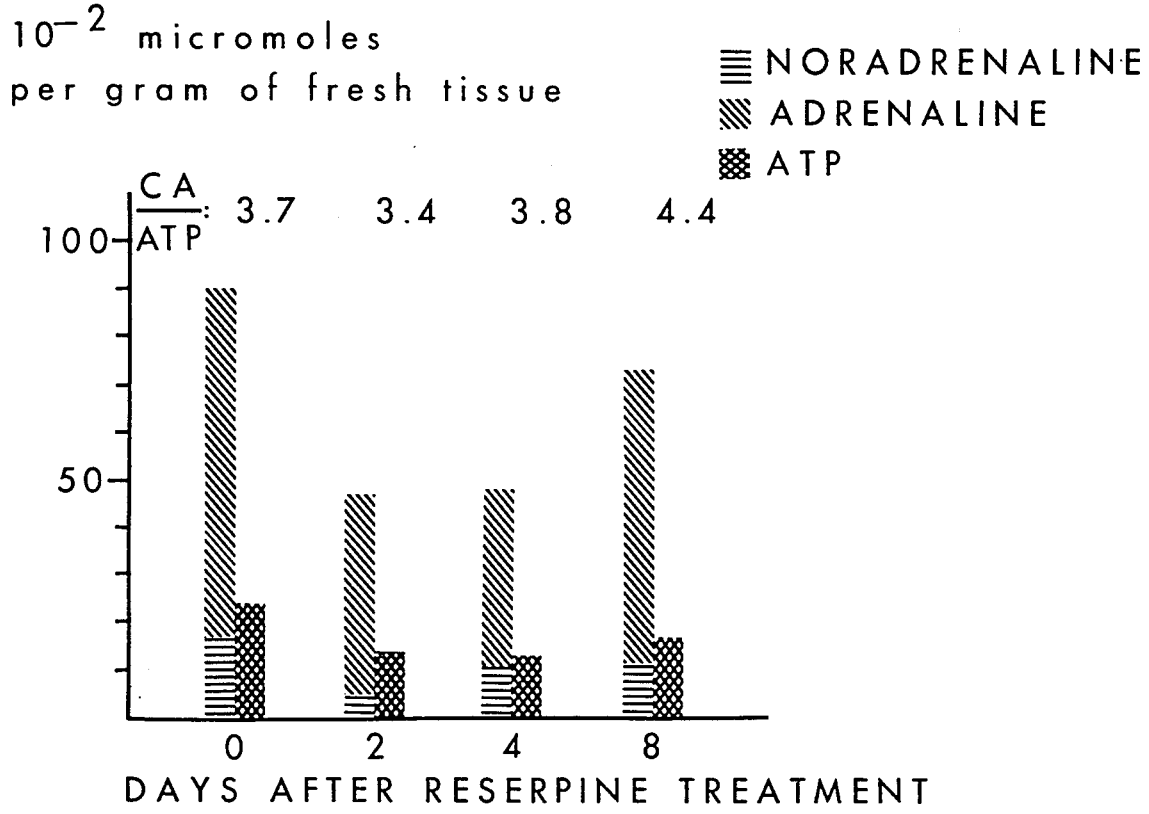

Figure 8

The ATP and catecholamine repletion in the granular fraction of the adrenal glands of reserpine treated guinea pigs

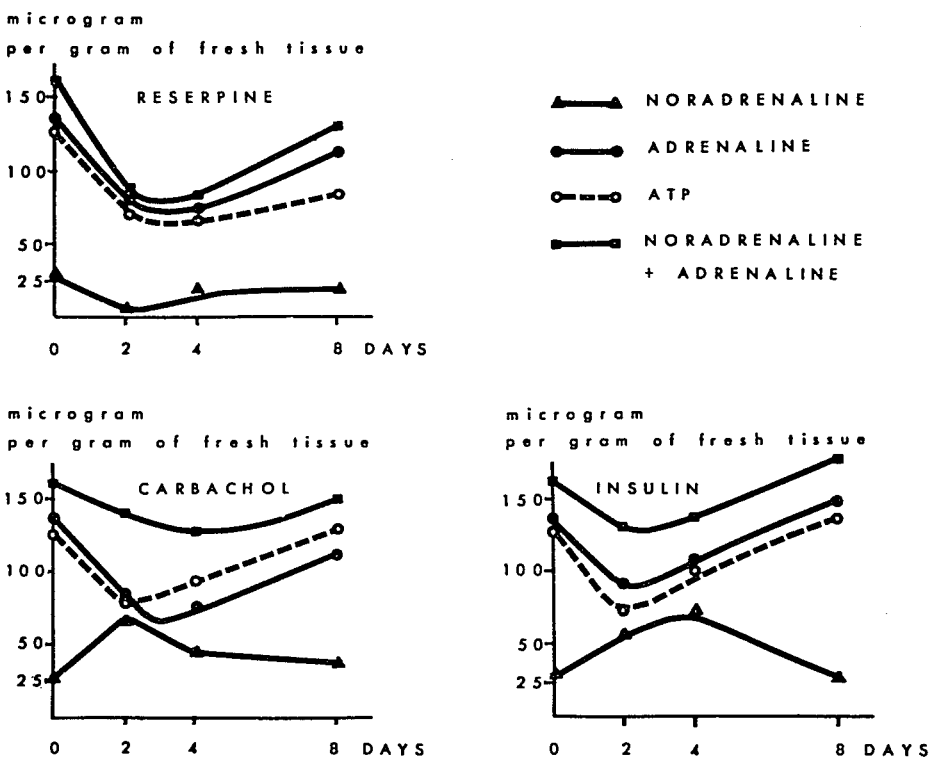

Figure 9

The rate of ATP & catecholamine repletion in the granular fraction of the guinea pig adrenal gland

ratio CA/ATP is increased and does not reach normal value even on the eighth day after the treatment.

The time course effect of carbachol, insulin and reserpine on the content of the catecholamines and ATP is given in Figure 9, page 115. The results are expressed in micrograms of substances per gram of wet weight tissue as a function of the period of treatment. Each point of the curves represents the mean value of the experimental data.

In the carbachol treated group, the noradrenaline first increases, reaches a maximum around the second day then decreases to reach almost normal values in the eighth day. The adrenaline and catecholamine concentrations decrease to reach a minimum at the fourth day then rises to subnormal values on the eighth day. The level of ATP passes through a minimum on the second day then increases to reach normal values on the eighth day.

In the insulin treated group, the level of noradrenaline increases to attain a maximum on the fourth day, it then decreases to normal level on the eighth day. The adrenaline, catecholamine and ATP levels pass through a minimum around the second day then reaches normal values on the eighth day.

In the reserpine treated group, for all the substances there is first a decrease with a minimum between the

second and the fourth days. Although the levels of the substances rise, they never reach normal values even on the eighth day.

In all of the three groups the rate of disappearance of adrenaline is related to that of ATP. The negative slopes of the curves are approximatively identical. In the carbachol treated group, the rate of appearance of ATP is closely related to that of adrenaline, as shown by an apparent identical slope for the ascending part of the curves. There is no such relationship between the rates in either of the other two groups.

III. Discussion

Since the chromaffin granules have been identified as the catecholamine containing granules, numerous experimental evidences have been presented on the release of the catecholamine content of the granules during the stimulation of the adrenal gland (29,64). Conclusive evidence has shown that ATP is released together with the catecholamines. The amount of released nucleotides and catecholamines is in a molar ratio of 1 to 4, identical to the ratio found in the granules. This led to suppose that ATP plays a role in the storage of the catecholamines within the granules (34,39,47). At the same time, electron microscopic evidences have been presented showing that the granule appears to empty its content during the stimulation of the adrenal gland, leaving "ghost" granules in the cell (23,114).This finding is in contradiction with the observation that the depletion of the adrenal gland in vivo is not accompanied by any change in the protein content of the chromaffin granules (66,157). Recently, a series of independent observations have pointed towards a release of the soluble proteins from ox, rabbit or cat adrenal glands perfused with a solution containing a depleting agent such as carbachol or acetylcholine.

In view of these recent findings, it was thought that further investigation of the fate of the chromaffin granule after

the *in vivo* depletion of the adrenal glands might be of interest. Since no attempt had been made to study the repletion of the chromaffin granules from the adrenal glands of animals which had been pretreated with acetylcholine like agents, it was believed that this approach to the problem might shed some light on the controversial subject of the fate of the catecholamine containing granules.

For many years, it has been known that there exist several agents capable of inducing the release of catecholamines from the adrenal glands, *in vivo* (158). Among these agents, the most widely employed are acetylcholine, insulin, and reserpine. Acetylcholine induces the release of catecholamines from the adrenal medulla by increasing the permeability of the plasma membrane to calcium ions (159,160,161). The rapid inactivation of acetylcholine in the animal, by the action of acetylcholinesterase, requires that the animal be given repeated injections of acetylcholine to produce a significant stimulation of the adrenal gland. Under these experimental conditions the animals have to be treated with atropine and this introduces a new variable in the system. Recently, it has been observed that carbachol can successfully replace acetylcholine as a stimulating agent for the depletion of catecholamines from the adrenal glands (58). This synthetic choline ester which essentially behaves like acetylcholine is not hydrolyzed by acetylcholinesterase which makes it a long lasting stimulating agent (162).

Reserpine is a depletor of the catecholamines in all the tissues. Its mode of action has not as yet been elucidated completely (87).

Insulin has been shown by Cannon and coworkers (61) and independantly by Houssay and coworkers (62) to have a very profound effect on the secretion of the adrenal glands indirectly via hypoglycemia. Further investigations by von Euler and Luft (64) showed that the hypoglycemic stimulation of the adrenal glands has a selective effect upon the medullary secretion in that adrenaline alone is secreted. The mechanism of the secretory effect of insulin is not fully understood but appears to be a reflex stimulation of the adrenal gland (29,64).

These three agents which bring about a depletion of the catecholamines of the adrenal glands appear to act according to different mechanisms and for this reason were chosen as tools to study the fate of the catecholamine containing granules.

A) <u>The depletion of catecholamines in the adrenal gland following a single injection of either carbachol, insulin or reserpine to the male guinea pig</u> :

A single injection of carbachol produces a marked decrease in the total catecholamines of the whole adrenal glands. The decrease is in fact brought about on adrenaline since the noradrenaline content of the whole gland is increased. Although the data have not been reported here, preliminary experiments have

shown that either one, four or eight hours after a single injection of carbachol to the animal, the catecholamine content of the adrenal gland remains unchanged. For this reason, the seventeen hour period was chosen as the optimum time at which carbachol exerts its effect. A comparison with other works was limited since the effect of carbachol had been studied only during perfusion of the glands. In these conditions, the effect of carbachol was observed five to ten minutes after the start of the perfusion (72,73). This latent period would probably be greatly enhanced in the in vivo experiments. Since the effect of carbachol is observed after seventeen hours, the finding that the noradrenaline content is not decreased might imply that the choline ester has a selective effect by bringing about the depletion of adrenaline only. In this respect, carbachol would not act exactly like acetylcholine which is known, as shown by Butterworth and Mann, to release both adrenaline and noradrenaline of the cat adrenal glands (57,125). Since the cat adrenal glands contain large amount of noradrenaline (163), this difference might reflect a species difference only.

Twenty four hours after a single injection of insulin, the concentration of adrenaline in the whole adrenal gland has decreased by about 25% of its normal value. It appears that the peak of insulin effect occurs between three and eight hours after the injection period during which the animals appear to suffer

from a hypoglycemic coma. Since they start to recover eight hours after the insulin injection it seems that the depletion of adrenaline starts at this time. These findings are in good agreement with the previous observation of Dumer who found the peak of insulin effect to be twenty minutes after the intravenous injection of insulin to the cat (86). The results presented in this thesis are also in agreement with the previous finding that insulin depletes selectively adrenaline from the adrenal glands (84).

The depletion brought about by reserpine affects the noradrenaline as well as the adrenaline concentrations of the whole adrenal gland. The concentrations of both amines are decreased by about 50% twenty four hours after the reserpine injection. The noradrenaline level is lower than that of the adrenaline which is not inconsistent with the observation that reserpine acts partly by preventing the entrance of dopamine in the adrenal gland; dopamine is an essential precursor of the catecholamine synthesis (135,136). Our findings are in agreement with previous in vivo studies which showed also that the depletion of catecholamines brought about by reserpine is quite large and affects both biogenic amines (65,101,103).

The increase in the noradrenaline content of the whole adrenal glands after carbachol treatment might indicate that carbachol does not interfer with the synthesis of catecholamines

in the adrenal gland. In the case of insulin, there is a large decrease in the adrenaline content but no change in the noradrenaline content. This shows that insulin has a selective effect in depleting the whole gland of its adrenaline only; it is not impossible that insulin indirectly inhibits the synthesis of catecholamines since no increase in the noradrenaline level is observed after insulin treatment. By bringing down the glucose level insulin decreases the energy available to the cell which results in a lower metabolic rate. Finally, reserpine affects the synthesis of catecholamines by lowering the intracellular concentration of dopamine, precursor of noradrenaline.

Since there is a significant depletion of the catecholamines of the whole adrenal gland after the treatment of guinea pigs with carbachol, insulin or reserpine, it became of interest at this point of the proceedings to investigate changes in the isolated granules as a result of the same treatment. The enzyme for the final step of adrenaline synthesis is found in the cytoplasmic sap (164,165) and it might be expected that in a period of changes in the concentrations of noradrenaline and of adrenaline significant amounts of both amines might be found outside of the granules. It was for this reason that the studies to be discussed now have been undertaken.

B) The protein, ATP and catecholamine content of the granular fraction of the adrenal glands from normal, carbachol-, insulin-, or reserpine-treated male guinea pigs :

The sucrose density gradient separation yielded four fractions which is in agreement with previous results obtained with the ox adrenal glands (16,17,20). These four fractions are identical to those observed by the above cited authors, fraction I containing lipid like material, fraction II containing the mitochondrial fraction, fraction III the catecholamine containing vesicles and fraction IV a sediment rich in catecholamines.

1) The protein content

The protein content of the four fractions is concentrated mainly in the fraction III, which is in agreement with the previous findings that this fraction corresponds to the catecholamine containing vesicles (16,17,20). In the sediment rich in catecholamines, the amount of proteins is negligible, while it is relatively high in the mitochondrial fraction.

The distribution of proteins is not affected by treatment with carbachol, insulin or reserpine. Our observation confirms the data previously reported by Burack and coworkers who could not detect any change in the protein content of the granules from the fowl adrenal gland after reserpine treatment (157). Similarly, Carlsson and coworkers working with the granules of sheep adrenal glands could not observe any change in the granular proteins, after

the animals had been treated with insulin (66). It appears thus that the hypothesis that the granular membrane is kept intact after the granule has emptied its content is strengthened by our observation. On the other hand, the possibility that the depletion results in a partial emptying of the granular content is not yet excluded, which would mean that the granule being only partly depleted is still functional.

As mentioned in the introduction of this discussion, a change in the protein content of the granules after perfusion of the adrenal glands was reported by independent groups of workers. Kirshner and coworkers reported changes in the protein content of the granules following the perfusion of ox adrenal glands with acetylcholine (78,81,82). The proteins were evaluated by measuring the enzymic activity of dopamine-β-hydroxylase and its variations as a function of the acetylcholine perfusion. This method would detect much smaller changes than the classical method of protein determination used in this thesis, since the enzymic method is more sensitive. These authors claim that their technique permits them to follow changes taking place in the granular membrane since this enzyme is firmly bound to the membrane. Their claims do not appear to be consistent with the fact that the unbinding of dopamine-β- hydroxylase was brought about by simple lysis of the granules in ice-cold distilled water. Schneider presented evidence on the changes of the protein content of the granules

from ox adrenal medulla perfused with carbachol, phenylethylamine or ethylene diamine. The changes in a specific protein of the granule was measured by an immunological method, which is a very sensitive method of protein measurement. The total proteins in the perfusate were measured by the microbiuret method. (80). The author claims that there is an increase in the release of the proteins in the perfusate, after stimulation of the adrenal gland, but does not present experimental data. A critical evaluation of the finding is hampered by this lack of experimental data. It seems that in the in vitro experiments, the methods used to measure the proteins of the granules are more sensitive than those used in the present work. Moreover, in the in vitro experiments the change in the granular protein content was indirectly determined by measuring the amount of proteins present in the perfusate; in our work the change in the protein content of the granule was measured by determining directly the content of the granule. This might explain the apparent discrepancies between in vivo and in vitro results. It was believed that the destruction of the membrane during the release of the granule content would bring about changes in the protein content which would be detectable with the Lowry method. Thus our negative results are not incompatible with the hypothesis that the granular membrane is not significantly altered by the in vivo release of the granule content after stimulation of the adrenal gland.

2) The catecholamine content

The distribution of the catecholamines in the four fractions is identical to that of the proteins with the peak of the concentration in fraction III. The contents of noradrenaline and of adrenaline are high in this fraction, the molar ratio of noradrenaline to adrenaline averages 0.13. In the adult guinea pig, the adrenaline content of the adrenal gland is much higher than that of noradrenaline which shows that in this respect guinea pigs and rats can be compared, whereas cats and guinea pigs can not be compared (163). The total catecholamine content of fraction IV is high, with the concentration of noradrenaline slightly superior to that of adrenaline when compared to the fraction III since the ratio noradrenaline/adrenaline is 0.15. This is in agreement with previous findings of Fortier and coworkers who reported for the fractions of ox adrenal medulla respective ratios of 0.37 and 0.53 for fractions III and IV. (17). In this investigation the difference in the value of the ratios average 15%, while these authors found about 30% difference in the values of the ratios.

Carbachol, insulin or reserpine do not affect the distribution of catecholamines in the four fractions. The percentage of total catecholamines concentrated in the fraction III is about 74% and that in fraction IV 23%.

On the other hand, the catecholamine content of the granular fraction is very much decreased after treatment of the

guinea pigs with the depleting agents. Surprisingly enough, the largest decrease is observed in the granules of the adrenal gland from guinea pigs which have been pretreated with carbachol; 38% only of the normal catecholamine value is found in the granules. On the other hand, in the chromaffin granules from animals which have been pretreated with insulin 79% of the normal catecholamine value is found, whereas a value of 67% is obtained for the guinea pigs pretreated with reserpine. The treatment of the guinea pig with carbachol results in a depletion of the stimulated adrenal gland which is much more obvious in the isolated granules than in the whole glands. This implies that considerable amounts of the catecholamines are kept in the cytoplasm after the depletion. This finding might be an artefact of the method. Because of pretreatment with carbachol, the granular membrane might be partially damaged, which would result in the leakage of the amines from the granules. However, in view of the finding that not such difference is observed with the other two drugs, one might eliminate this explanation. The presence of large amounts of free or loosely bound catecholamines in the cytoplasmic sap might be explained by the action of carbachol at the level of the granular membrane, where it would produce the release of ATP and of catecholamines within the chromaffin cell. Yet in view of the well-established fact that acetylcholine acts on the outerpart of the plasma membrane (110), and since carbachol is an analogue of acetylcholine, the hypothesis just outlined does not

seem to be valid. On the other hand, it is not impossible that the depletion produced by carbachol brings a large decrease in the catecholamine content of the adrenal gland. This drop in the concentration of the amines would in turn stimulate the synthesis of the catecholamines. The noradrenaline and adrenaline found in the cytoplasmic sap would be newly synthesized amines which had not yet reached the storage granules. Although the immediate precursor of adrenaline - noradrenaline - is synthesized in the granules, it might have to be released in the cell sap to be further methylated into adrenaline, since the enzyme responsible for the methylation, N-methyl transferase, is found in that part of the cell. The amount of noradrenaline found in the cytoplasm is larger than that of adrenaline which supports this hypothesis and confirms Hillarp's findings of a cytoplasmic pool of catecholamines in the adrenal glands (166).

3) The ATP content

The ATP content of the granule is very much decreased by either carbachol, insulin or reserpine treatment, and in a manner which is almost identical to that observed for the total catecholamines. The molar ratio of catecholamines/ATP has an average value of 2.7 for the granule from the adrenal glands of normal guinea pigs. This value is lower than that usually reported for other species (39,40,45,46), however it has previously been observed that such a low value might be found *in vivo* (47).

In the carbachol treated animals the molar ratio of catecholamine/ATP for the granular fraction of the adrenal gland is near normal, while it is slightly elevated in the granules of the adrenal gland from reserpine treated guinea pigs. This molar ratio is very high in the chromaffin granules from animals which have been treated with insulin. The explanation for this finding might reside in the fact that the ratio noradrenaline/adrenaline is higher in the granules from insulin treated animal, than in those from either reserpine or carbachol treated animals. It would suggest that the ATP level is more associated with the adrenaline than with the noradrenaline level.

C) <u>The repletion of catecholamines and ATP in the adrenal glands of male guinea pigs pretreated with carbachol, insulin or reserpine</u> :

The repletion of the catecholamines and ATP in the granular fraction of the adrenal glands from carbachol treated guinea pigs might be considered complete eight days after the injection. On the eighth day, the levels of ATP, total catecholamines and noradrenaline have returned to normal but the adrenaline level has not yet reached the normal concentration of the resting gland.

The repletion starts as early as two days after the injection of carbachol as shown by an increased noradrenaline level. This increase suggests that the synthesis of catecholamines is not impaired by carbachol. The onset of the synthesis of adrenaline and

of ATP appears to take place between the second and the third day following the treatment to reach normal values on the eighth day. The rates of repletion of ATP and of adrenaline are closely related; on the other hand the rate at which the noradrenaline concentration returns to normal is slower. It is of interest to notice that noradrenaline is stored within the granules before being used for the synthesis of adrenaline, since all the data presented here were obtained with the granular fraction. However, since the rate of disappearance of noradrenaline is not similar to the rate of appearance of adrenaline, apparently there is a significant amount of the unmethylated amine free in the cytoplasm. The ratio catecholamine/ATP has its highest value two days after the treatment, period at which the concentration of noradrenaline reaches its maximum. This observation confirms that ATP and adrenaline syntheses are related, but that ATP and noradrenaline syntheses are not closely linked. The high value of the ratio is due to a low level of ATP because on that second day, the amount of total catecholamines is lower than normal. In the cat, Butterworth and Mann found that the level of total catecholamines also reaches its normal value eight days after treatment of the animal with acetylcholine (125). However, these authors noted that the relative amount of the two amines was reversed, since the noradrenaline level was well above its normal value while the adrenaline level was well below its normal value. Since in the resting

adrenal gland of the cat, the level of noradrenaline is much higher than that observed in the guinea pig adrenal gland, the differences observed between the findings of Butterworth and Mann and our own findings might be due to species difference.

The effect of insulin on the replation of catecholamines and ATP in the guinea pig adrenal glands appears to be identical to that of carbachol since eight days after the insulin treatment the content of catecholamines and ATP in the granular fraction has returned to normal. In this case, however, a delay in the onset of adrenaline synthesis is observed. The concentration of noradrenaline reaches its maximum value only four days after the treatment of the animal. This delay in the noradrenaline synthesis is then reflected in the rate of appearance of adrenaline which is not as closely related to the rate of ATP synthesis as it is seen for the granules from the adrenal glands of carbachol treated guinea pigs. Our observation is in contradiction with the findings of Viveros and coworkers who studied the effect of insulin administration, 40 units per kilogram of body weight, in the rabbit.(65). These authors observed a return to normal of the catecholamine level four days only after the intravenous injection of insulin. These studies were conducted in the total homogenate of the adrenal glands and if, as according to Hillarp and coworkers (167,168,169) newly synthesized amines are kept free in the cytoplasm, the discrepancies between this observation and ours is only apparent.

In the rabbit, Udenfriend and his associates observed that eight days were required to obtain normal adrenaline level in the adrenal glands stimulated by insulin (124), which is in agreement with the results reported here.

The long lasting effect of reserpine is well illustrated since even eight days after a single injection, the normal levels of catecholamines and ATP in the chromaffin granules are not yet reached. The low level of noradrenaline suggests that this delay in the repletion of catecholamines and ATP is partly due to the fact that the precursor dopamine, essential for the synthesis of the amines is not available. In the rat it has been found that the repletion of catecholamines required 21 days after treatment with reserpine, one milligram per kilogram of body weight, daily for three days (90). At higher dosage of reserpine, 10 milligrams per kilogram of body weight, once, the catecholamines were found to have returned to their normal level, as early as seven days after the treatment (131). In the rabbit, whatever the dosage of reserpine used - one milligram per kilogram body weight, five or ten milligrams per kilogram body weight - the repletion of the catecholamines in the adrenal glands appears to be almost complete eight days after the treatment (63,99). In all the cases and also observed in this work, the repletion of noradrenaline is slightly faster than that of adrenaline.

The rates of appearance of adrenaline, ATP and total

catecholamines as well as the rate of disappearance of noradrenalins are linear. This implies that they do not change with time and confirms previous observation (131).

The repletion of ATP in the catecholamine containing granules is not complete until eight days after the treatment of the guinea pigs with either of the depleting agents. It thus seems that in the granules the half-life of this nucleotide is low. Our failure to obtain labelled ATP following the injection of radioactive precursors is not surprising in view of this finding. It has been reported that the incorporation of ^{32}P in the granule ATP is very slow (156) and our observation is in agreement with these previous findings. It also explains why a substance such as ethionine cannot affect the level of ATP in the chromaffin granules. Since ethionine depletes the organ of its ATP by trapping the free ATP in S-adenosyl ethionine (149), in the granules where the turnover of ATP seems to be very slow, such a compound would not have any effect.

Since the changes in the protein content of the chromaffin granules are not noticeable, labelled amino acids possessing a high specific activity will not be reliable tools for the measurements of the protein changes, if any. The observation that, upon stimulation of the adrenal glands, the proteins remain unchanged whereas the catecholamines and ATP are released, is not inconsistent with the hypothesis that the granules are kept intact histologically,

but damaged biochemically; by this is meant that the granules have emptied their ATP and catecholamine content, but retained their structure. The empty sacs might stay within the chromaffin cell (23) and be slowly destroyed. If such a mechanism takes place, the approach to the problem taken up till now will not provide an answer to the problem. For this reason, in the next series of experiments, we investigated the capability of the granules to pick up ^{14}C-adrenaline , after they had been depleted of their content by the action of carbachol, insulin or reserpine in the whole animal. The capability of the granules to pick up adrenaline was studied as a function of the time elapsed after the treatment of the animals. Since eight days after the injection of reserpine, the granular fraction cannot yet be considered normal, an additional time period of eight days was added so that the uptake of ^{14}C-adrenaline by the granules was investigated up to sixteen days after a single injection of either of the three drugs to the guinea pigs. This subject will now be discussed in part IV of this thesis.

PART IV. THE IN VITRO UPTAKE OF DL-ADRENALINE-^{14}C BY THE GRANULAR FRACTION OF ADRENAL GLANDS FROM GUINEA PIGS NON TREATED; OR TREATED WITH CARBACHOL, INSULIN OR RESERPINE

I. Materials and Methods

A) Materials

dl-Adrenaline (carbinol-^{14}C) dl-bitartrate was bought from Nuclear Chicago, U.S.A. . The specific activity was 9.6 millicuries per millimole.

Magnesium chloride from Fisher Scientific Co, was of the reagent grade.

Adenosine triphosphate disodium salt was purchased from Sigma Chemicals Co. .

Scintillation liquid for radioactivity measurements was prepared according to Bray (170). It contained the following substances : naphtalene (6%), PPO (2,5-diphenyloxazole) (0.4%), dimethyl-POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) (0.02%), methyl hydrate (10%), and propylene glycol (2%), in dioxane.

The incubation medium consisted of two milliliters of the homogenate of the adrenal glands (100 milligrams) freed of the nuclei and cell debris by centrifugation at 600 x g for ten minutes. The glands were homogenized in buffered isotonic sucrose.

To these two milliliters, ten micromoles of ATP and 0.045 micromoles of ^{14}C-adrenaline (6×10^5 c/min) were added to give a final volume of 2.5 milliliters.

B) Methods

1) Incubation

The incubation of the medium described in Materials was carried out at 37°C, in a Dubnoff water bath, for ten minutes. The reaction was stopped by chilling of the tubes into ice and immediate centrifugation.

2) Preparation of the sucrose density gradient and determination of the radioactivity content of the samples

The method of the sucrose density gradient was described in the previous part III, page 63. At the end of the run, the tube was punched at the bottom and fractions of five drops of liquid were collected into a scintillation vial containing ten milliliters of the Bray solution. The number of scintillation vials used for each density gradient tube averaged twenty four. The radioactivity content was then measured as previously described page 59.

3) Determination of the catecholamine content of the samples

The determination of catecholamines was carried out in the following manner: the sucrose density gradient tube was punched at the bottom and fractions of five drops were collected into a tube containing 5% trichloroacetic acid. After centrifugation, the clear supernatant was made to two milliliter volume with 5% trichloroacetic

acid. The catecholamines were then determined as previously described on page 42.

4) Determination of the radioactivity content of the 11,000 x g sediment "granular fraction"

The pellet obtained after centrifugation at 11,000 x g was washed twice with buffered isotonic sucrose. The pellet was then treated with three milliliters of 0.05 N perchloric acid, carefully suspended in this acid and centrifuged again. The clear supernatant was collected and one milliliter aliquot placed into the scintillation vial containing ten milliliters of the Bray solution. The determination of the radioactivity of this sample was performed as previously described page 59.

As mentioned earlier, the sucrose density gradient method was used to ascertain that the radioactivity content of the sample was found at the same level as fraction III, that is to say at the level of the catecholamine containing granules. Once this had been ascertained the method was discontinued and use was made of the 11,000 x g sediment instead. The sucrose gradient technique was applied to each of three normal controls and to one carbachol treated animal. In figure 10, page 139, and in figure 11, page 140, showing the distribution of the radioactivity and of the catecholamines in the sucrose density gradient tube, it can be seen that the peak of radioactivity corresponds to that of the catecholamines in the fraction III. In all the cases studied, the radioactivity

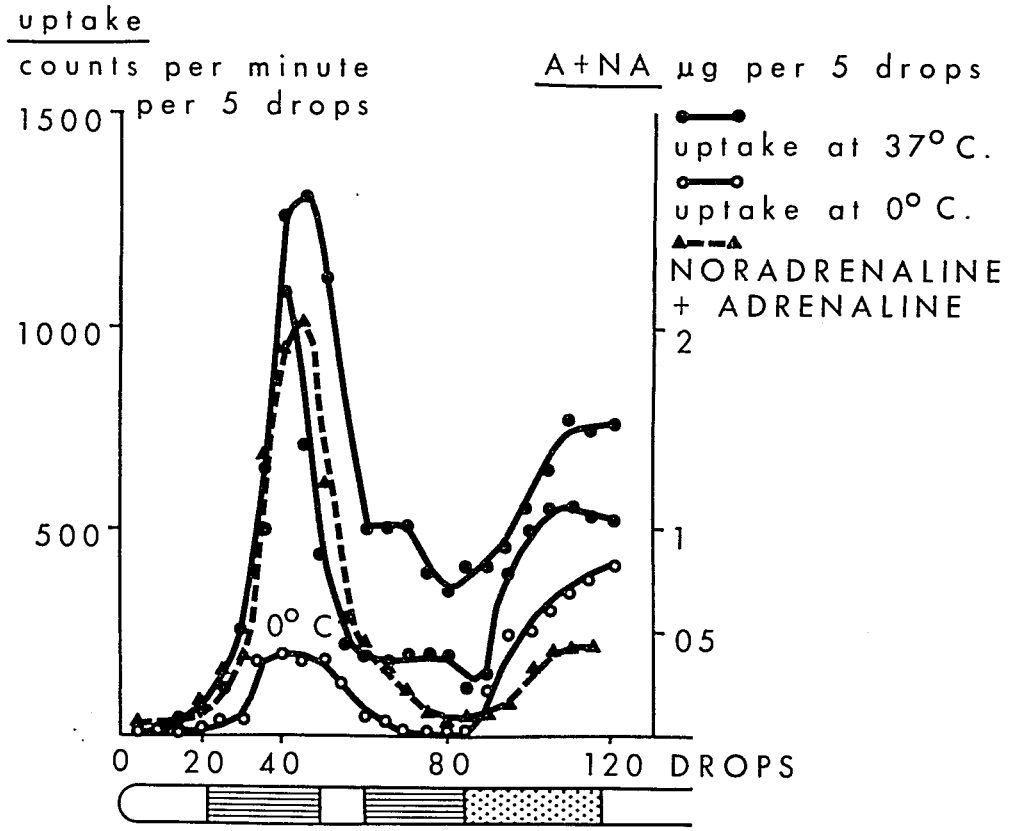

Figure 10

The distribution of catecholamines and radioactive material in the sucrose density gradient of the 11,000 x g sediment from normal guinea pig adrenal glands

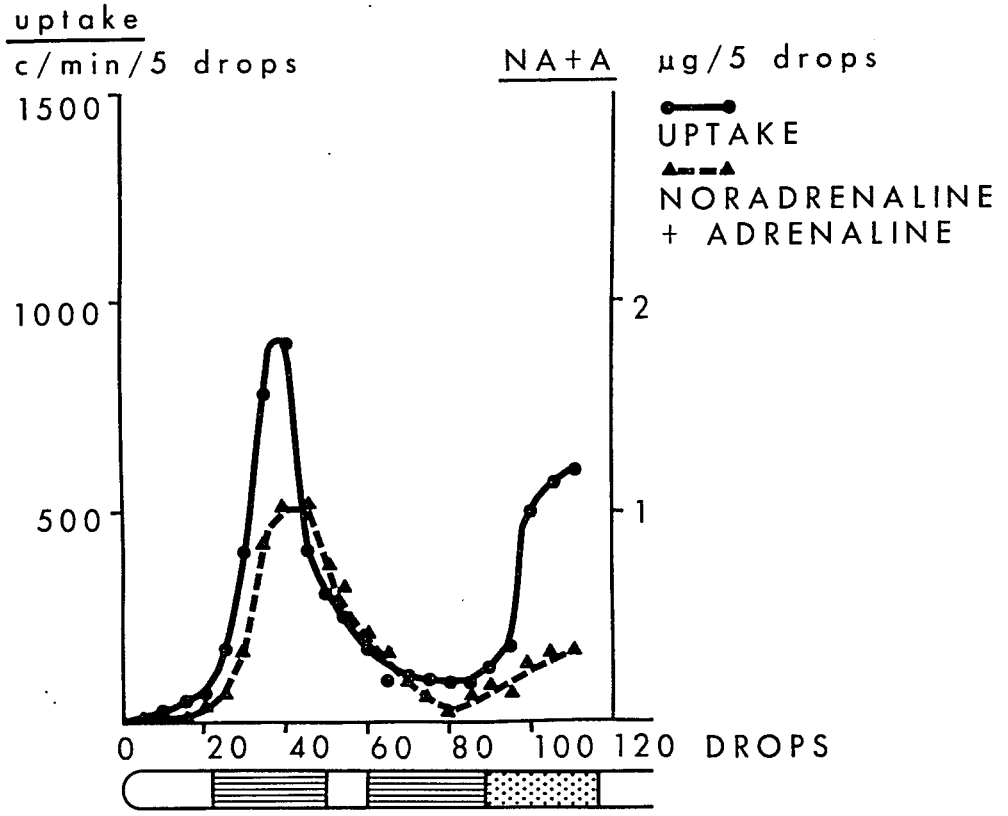

Figure 11

The distribution of catecholamines and radioactive material in the sucrose density gradient of the 11,000 x g sediment from the adrenal glands of carbachol treated guinea pigs

peak is superimposed on the peak of catecholamines which allows us to assume that the radioactivity uptake is associated with the catecholamine containg granules. These findings permitted us to discontinue the sucrose density gradient method and the 11,000 x g sediment was used as such.

II. Results

Two series of experiments are reported in this part. In the first series, the effect of a single injection of carbachol, insulin or reserpine on the uptake of ^{14}C-adrenaline by the adrenal glands was tested. The second series of experiments was devoted to the study of the time-course of the uptake, after a single injection of either of the three drugs.

A) <u>The effect of a single injection of carbachol, insulin or reserpine on the uptake of ^{14}C-adrenaline by the granular fraction from the guinea pig adrenal glands</u> :

In order to test whether there is an effect of carbachol, insulin or reserpine on the uptake of ^{14}C-adrenaline by the granular fraction, a single injection of either of these three drugs was given to male guinea pigs. The animals treated with carbachol were sacrificed seventeen hours after the injection, while the animals treated with insulin or with reserpine were killed twenty four hours following the injection. The results obtained with the granular fraction from the adrenal glands of treated as well as normal guinea pigs are presented in Table XIV, page 143. They are expressed as counts per minute per 100 milligram of wet weight tissue.

Seventeen hours after the injection of carbachol, there is a decrease in the uptake of ^{14}C-adrenaline by the granular fraction of the adrenal glands. This decrease of about 52% is

TABLE XIV

The Uptake of dl-^{14}C-Adrenaline by the Granular Fraction of the Adrenal Glands from Normal, Carbachol, Insulin, or Reserpine Treated Male Guinea Pigs

Animal	Radioactivity content of the granular fraction c/min per 100 mg of wet weight tissue			
	Control	Carbachol Treated	Insulin Treated	Reserpine Treated
1	11,583	7,419	5,511	9,762
2	12,180	8,154	5,025	7,086
3	12,681	8,793	5,943	7,116
4	17,742	6,093	5,451	5,607
5	12,882	6,875	5,199	6,099
6	18,087	-	-	-
Mean	14,192	7,467	5,425	7,134
±S.D.	2,919	1,057	349	1,605
P'<		0.001	0.001	0.001

' P : P values obtained by group comparison of control versus treated groups

statistically significant ($P < 0.05$).

In the insulin treated guinea pigs, there is also a significant decrease in the uptake of radioactive adrenaline by the granular fraction of the adrenal glands. Twenty four hours after the injection, only 48% of the normal uptake is observed in the insulin treated group.

The values obtained for reserpinized guinea pigs are within the same range as those exhibited by the two previous groups. There is a 50% decrease in the uptake of ^{14}C-adrenaline by the granular fraction of the adrenal glands, which is statistically significant ($P < 0.05$).

B) <u>The time-course of ^{14}C-adrenaline uptake by the granular fraction of the adrenal glands from male guinea pigs pretreated with carbachol, insulin or reserpine</u> :

In this series of experiments, the uptake of ^{14}C-adrenaline by the granular fraction of the adrenal glands was investigated two, four, eight and sixteen days after the treatment of the animals. The experimental results are presented in Tables XVa, XVb, and XVc, expressed as counts per minute per 100 milligrams of wet weight tissue. The mean and standard deviation from the mean as well as the P values obtained by a group comparison between control and treated groups are also given.

The results obtained with the adrenal glands from animals which had been pretreated with carbachol are presented in Table XVa,

on page 146. Two days after the carbachol injection, the uptake of ^{14}C-adrenaline by the granules from the adrenal glands is decreased by 60% of the normal uptake. On the fourth day, there is a slight increase in the uptake which is still well below the normal value. It is only between eight and sixteen days after the carbachol treatment that the uptake of ^{14}C-adrenaline by the chromaffin granules returns almost to its normal value.

In Table XVb, page 147, the results obtained with the granules of the adrenal glands from insulin treated animals are given. The time-course of the ^{14}C-adrenaline uptake exhibits the same variations as those observed with the carbachol treated group. On the second day after the insulin injection, there is a marked decrease in the uptake of radioactive adrenaline by the chromaffin granules. This decreased uptake reaches a minimum on that second day and from then on, the uptake of adrenaline increases to reach almost normal value between the eighth and the sixteenth days after the insulin treatment.

In Table XVc, page 148, the results from the reserpine group are presented. The minimum uptake of ^{14}C-adrenaline by the granular fraction is also found on the second day following the treatment of the animals. On the sixteenth day after the reserpine treatment, the uptake has not yet reached its normal value. This lower value however is not statistically significant ($P > 0.05$).

A plot of the concentrations of radioactivity in the

TABLE XVa

The Time Course of dl-^{14}C-Adrenaline Uptake by the Granular Fraction of the Adrenal Glands from Male Guinea Pigs Pretreated with Carbachol

Animal	Radioactivity content of the granular fraction c/min per 100 mg of wet weight tissue				
	Control	Carbachol Treated			
		2nd day	4th day	8th day	15th day
1	13,578	5,919	6,947	10,488	11,768
2	14,763	5,747	6,519	8,079	12,309
3	12,233	5,065	6,567	7,636	11,021
4	11,964	4,971	6,152	9,784	11,465
5	12,461	5,274	6,513	9,286	13,354
6	-	-	-	-	12,736
Mean	12,941	5,395	6,539	9,654	12,108
±S.D.	1,051	418	281	1,183	859
P[1]		0.001	0.001	0.005	0.25

[1] P : P values obtained by group comparison of control versus treated groups

TABLE XVb

The Time Course of dl-^{14}C-Adrenaline Uptake by the Granular Fraction of the Adrenal Glands from Male Guinea Pigs Pretreated with Insulin

Animal	Radioactivity content of the granular fraction c/min per 100 mg of wet weight tissue				
	Control	Insulin Treated			
		2nd day	4th day	8th day	16th day
1	13,578	7,134	6,892	8,396	11,392
2	14,763	5,929	8,272	9,369	11,766
3	12,233	5,348	8,934	9,961	13,328
4	11,964	5,916	7,297	7,981	12,764
5	12,461	6,710	6,625	8,254	13,878
Mean	12,941	6,217	7,605	8,794	12,625
±S.D.	1,051	704	972	836	1,041
P'		0.001	0.001	0.001	-

' P : P values obtained by group comparison of control versus treated groups

TABLE XVc

The Time Course of dl-^{14}C-Adrenaline Uptake by the Granular Fraction of the Adrenal Glands from Male Guinea Pigs Pretreated with Reserpine

Animal	Radioactivity content of the granular fraction c/min per 100 mg of wet weight tissue				
	Control	Reserpine Treated			
		2nd day	4th day	8th day	16th day
1	13,578	5,662	7,147	9,561	13,021
2	14,763	6,139	7,630	7,968	11,171
3	12,233	4,921	8,433	10,041	10,958
4	11,964	5,181	6,151	8,487	12,618
5	12,461	5,831	7,221	7,864	12,081
Mean	12,941	5,546	7,316	8,788	11,969
±S.D.	1,051	492	827	967	894
P'		0.001	0.001	0.001	0.20

' P : P values obtained by group comparison of control versus treated groups

granular fraction as a function of the time after the treatment is presented for the three treated groups. Each point of the curves represents the mean of the experimental values. The three curves obtained with the three treated groups are presented in Figure 12, on page 150. The rate of increase in the ^{14}C-adrenaline uptake is identical for the chromaffin granules of either of the treated groups. Extrapolation to reach normal values of the uptake yields a time of twenty four days for the complete return to a normal uptake. The rate of adrenaline uptake decreases with time.

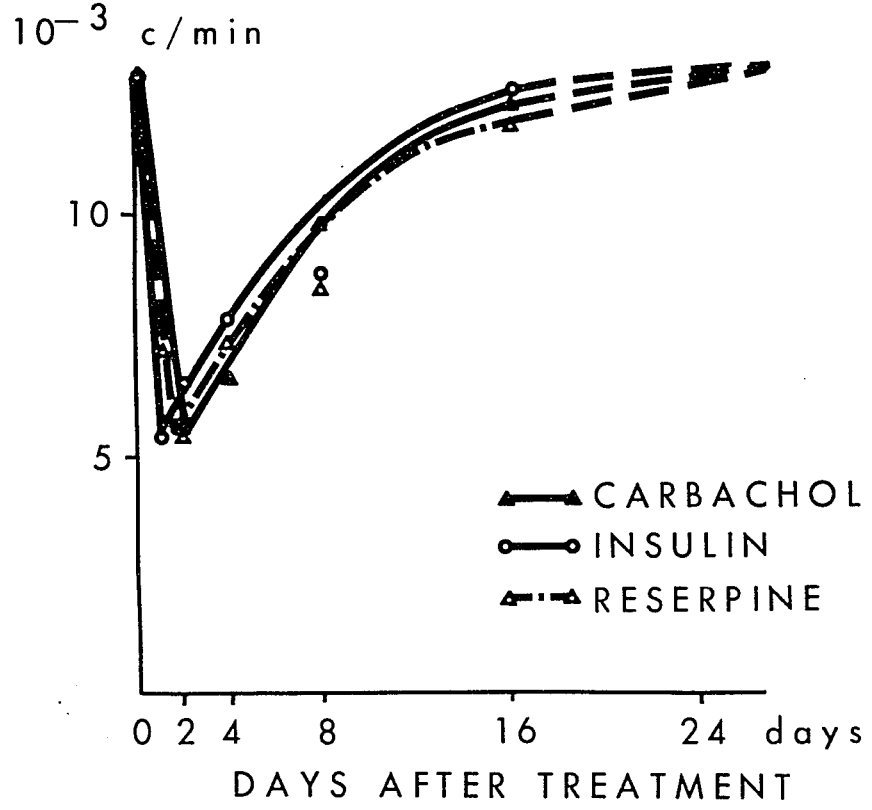

Figure 12

The rate of dl-^{14}C-adrenaline uptake by the granular fraction of the adrenal glands from carbachol, insulin or reserpine treated guinea pigs

III. Discussion

Few experimental evidences have been presented concerning the *in vitro* uptake of radioactive catecholamines by the adrenal glands, whether the glands be in their rested state or depleted. The exact mechanism of the uptake is not elucidated as yet, but there are increasing experimental evidences that this energy-dependent uptake might be an active transport of the exogenous catecholamines into the granules. Such an uptake is not observed at 0°C, and appears to be temperature dependent (135,136,137). If the function of the granule is secretory, the membrane must be playing an important role in the accomplishing of this function. The possibility to bring indirect evidences, in order to show whether the membrane of the chromaffin granule is intact after catecholamine depletion was investigated. It was believed that a knowledge of the state of the granules might indicate whether they are used again or discarded after having emptied their content. The use of ^{14}C-adrenaline as the factor to test the permeability of the membrane presented itself easily since adrenaline is such an important constituent of the catecholamine containing granules in the guinea pig adrenal glands.

Carbachol, the first depleting agent to be tested, brings about a decrease in the capability of the adrenal gland granules to pick up ^{14}C-adrenaline, as soon as seventeen hours after the

treatment of the guinea pigs. This capability is further decreased two days after the treatment, and on that day, the adrenaline content of the granule is also very low. The fact that the uptake is decreased might imply that the granules, if intact, since the protein content is unchanged, are not functional. The uptake of ^{14}C-adrenaline then increases slowly to reach almost normal value sixteen days after the treatment.

When the animals were pretreated with insulin, twenty four hours after the injection the granules have retained only 52% of their capability to pick up ^{14}C-adrenaline. This is the lowest value observed since on the second day after the injection the capacity of the granules to pick up adrenaline is 62% that of the normal uptake. It is not until the sixteenth day that the full capability of the granules to concentrate exogenous adrenaline has almost returned to normal.

Reserpine quite surprisingly appears to have on the uptake an effect identical to that of carbachol. The capability of the chromaffin granules from reserpinized animals to pick up radioactive adrenaline is very low on the second day after the injection, to increase and reach almost normal uptake on the sixteenth day.

In the rabbit, Viveros and coworkers observed that the normal uptake of ^{14}C-adrenaline was reached four days only after insulin injection, and eight days after reserpine injection.(65). However, these authors measured the ^{14}C-adrenaline content in the

total homogenate, and it is difficult to assess whether the results presented by the authors reflect the actual uptake by the isolated granules. Since several workers have reported that significant amounts of catecholamines are found outside of the chromaffin granule (1,106), the findings of Viveros and coworkers might reflect the rate of repletion of adrenaline in the whole adrenal gland but not that in the granule. The uptake of ^{3}H-noradrenaline has been reported to be normal two days after an injection of reserpine to the rabbit (171). No experimental data were presented to support that claim so it is difficult to examine why such discrepancies between these observations and ours exist. They seem to point out that more investigation is necessary before an hypothesis on the fate of the granule can be presented which is valid for all the species.

The adrenal medullary granules from guinea pigs which have been pretreated with carbachol, insulin or reserpine behave in an identical manner <u>in vitro</u>, in that the rate of uptake of ^{14}C-adrenaline is similar (Figure 12 on page 150). The normal uptake appears to be reached only twenty four days after the beginning of the treatment. This would yield a half-life of 12 days for the catecholamine containing granules. Since it has been observed that in the guinea pig adrenal gland, the half-life of adrenaline is about eight days, it means that the turnover of the chromaffin granules has a slower rate than that of adrenaline. In

that case, it is possible that a certain percentage of the granules is reutilized and this might explain the lag period between the two half-lives. Further investigations would be of interest to clear that point. It seems that a concomitant study on the rate of uptake of noradrenaline might shed some light on the subject. However, since *in vivo*, reserpine only appears to be able to bring about the depletion of noradrenaline, and since at the same time reserpine impairs the synthesis of noradrenaline by decreasing the rate of penetration of its precursor dopamine, such an investigation might be difficult.

The hypothesis that the uptake of ^{14}C-adrenaline by the chromaffin granules from carbachol, insulin or reserpine treated animals represents the uptake by newly formed granules is strengthened by the findings that the granules from reserpinized guinea pigs behave in the same manner as those from the two other groups of treated animals. It is well established that reserpine inhibits the storage of catecholamines in the chromaffin granules (136,137). Since there is no inhibition of the uptake during the repletion experiments, it implies that the measured uptake is taking place in newly synthesized granules which have not been subjected to the effect of reserpine. The hypothesis that emptied granules are reutilized is thus indirectly eliminated by this experimental finding.

SUMMARY AND CONCLUSION

The effect of ethionine, an ATP depleting agent was studied in the adrenal glands, brain and liver of the female or male rats. Except for a marked decrease of the liver ATP, ethionine does not deplete the other organs studied of their ATP.

The rate of ATP and catecholamine repletion in the granular fraction of the guinea pig adrenal glands was studied following the depletion of catecholamine and ATP in the glands by carbachol, insulin or reserpine treatment of the animals. The effect of carbachol is marked and of long duration. It is specific since adrenaline only is depleted. The depletion of adrenaline is accompanied by a similar depletion of ATP. The repletion of the adrenal glands has a duration of about eight days. Both ATP and adrenaline rates of appearance are closely related, while the rate of noradrenaline disappearance is not directly comparable to that of adrenaline appearance. It was concluded from this finding that part of the newly synthesized noradrenaline is found in the catecholamine containing granules while some of it might be present in the cytoplasmic sap.

The effect of insulin on the depletion of catecholamines and of ATP is also specific. Only ATP and adrenaline concentrations are lowered after a single injection of insulin to the animals. The effect of insulin appears to be more rapid and of less duration than

that of carbachol. The repletion of catecholamine and of ATP is comparable to that of carbachol treated group. However, after the insulin treatment, it starts earlier. The normal level of total catecholamines was reached within eight days after treatment.

Reserpine is not specific since noradrenaline as well as adrenaline were depleted in the chromaffin granules of reserpinised guinea pigs. The level of ATP was also markedly decreased. The repletion of the granule content was not complete eight days after reserpine treatment. The level of noradrenaline, during the repletion period was never very high. It was concluded that reserpine is a long acting agent which causes a delay in the repletion of the content of the granules. The delay is probably due to two main factors. First the long lasting action of reserpine might mask an early repletion; secondly, an impairment in the penetration of dopamine, precursor of noradrenaline, results in a slow rate of catecholamine synthesis.

The capability of depleted granules to pick up ^{14}C-adrenaline was investigated in the catecholamine containing granules from the adrenal glands of guinea pigs which had been pretreated with carbachol, insulin or reserpine. It has been found that the capability of the granules to pick up exogenous adrenaline was impaired by any of the treatments. There was, however, a return to normal value which was in phase with the repletion of the granule content. The rate of the increase in the uptake was almost

identical for the three treated groups. The increase in the uptake of exogenous adrenaline by the chromaffin granules was dependent upon the time following the treatment of the animals; it decreases with time. The half-life of the granules appears to be about twelve days. The uptake of adrenaline by the chromaffin granules from reserpinized guinea pigs was similar to that observed with the two other groups. On this experimental finding, it was concluded that the uptake of adrenaline is taking place in newly formed granules since reserpine is known to inhibit the storage of catecholamines within the chromaffin granules. This experimental evidence would indicate that the reutilization of depleted granules is unlikely.

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