SUMMARY

In the introduction a brief historical review is given of the importance of histamine as the agent responsible for the development of the signs and symptoms of anaphylactic shock in several animal species. Various aspects of histamine metabolism are considered, including the origin, the intracellular distribution and the rate of detoxication of this substance by histamine in normal and sensitive individuals, and the histamine metabolism of histamine and histamine on the other. This scheme is correlated with the ability of intracellular particles to synthesise high energy phosphate bonds.

In the experimental section it is shown that histamine-liberating drugs can release histamine from tissue homogenates, but that this action cannot be associated with changes in the activity of the enzymes. Evidence is given that the addition of ADP to ATP at extremely low concentrations, and that intravenous ATP can modify, or even abolish the action of these drugs in the intact animal. A mechanism of histamine release depending on the metabolic activity of the mitochondria is suggested in the discussion. This attempt to explain the explosive nature of histamine release during anaphylaxis and during the action of the histamine drugs. It also offers a possible explanation for the appearance of smooth muscle stimulating poly-peptide.

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In the introduction a brief historical review is given of the importance of histamine as the agent responsible for the development of the signs and symptoms of anaphylactic shock in several animal species. Various aspects of histamine metabolism are considered, including the origin, the intracellular distribution and the rate of detoxication of this substance by histaminase in normal and sensitive individuals, and the *kinetics* of its release during shock. A possible connection is suggested between the metabolic function of mitochondria and protein metabolism on the one hand and the metabolism of histamine on the other. This scheme is correlated with the ability of intracellular particles to synthesize high energy phosphate bonds.

In the experimental section it is shown that histamine-liberating drugs can release histamine from tissue homogenates, but that this action cannot be associated with changes in the activity of intracellular proteolytic enzymes. Evidence is given that three powerful histamine liberators react with adenosine triphosphate and several other phosphates at extremely low concentrations, and that intravenous ATP can modify, or even annul the action of these drugs in the intact animal.

A mechanism of histamine release depending on the metabolic activity of the mitochondria is suggested in the discussion. This attempts to explain the explosive nature of histamine release both during anaphylaxis and during the action of the liberating drugs. It also offers a possible explanation for the appearance of smooth muscle stimulating polypeptides.
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1. EARLY THEORIES OF THE MECHANISM OF ANAPHYLAXIS AND THE ORIGIN OF HISTAMINE.

The condition known as anaphylaxis was first described by Richet during investigations on poisonous proteins from sea-anemones (Richet, 1902). In dogs, small intravenous doses of extracts containing these substances initially produced very mild toxic effects which soon disappeared. When the injections were repeated three weeks later, the same animals developed such violent symptoms of poisoning that death occurred within a few minutes. This unusual response was not due simply to a cumulative effect of the poison, as a succession of injections given every 3-5 days produced only very mild toxic effects. During an interval of three weeks however, the animals apparently became so hypersensitive to the extract that another injection of it now provoked the fatal response which Richet called "anaphylactic" shock.

It was found that a comparable condition could be developed by using a wide variety of non-toxic proteins as antigens. Its successful appearance depended on two experimental conditions. Firstly, sufficient time had to elapse between the two injections of the antigen, and secondly, it was essential to use the same protein in both of them. The specificity of the reaction was in fact found to be remarkably high. After the administration of the initial sensitizing dose, a slight chemical change in the antigen molecule made it completely incapable, when injected, of eliciting a state of shock. (See Landsteiner, 1945.)
The symptoms of anaphylaxis varied considerably from species to species, a fact which was taken to indicate that different mechanisms and organs were involved. In the guinea-pig for instance, the respiratory system was principally affected, while in the rabbit and dog, circulatory and cardiac complications were prominent. In any one species however, anaphylaxis was always accompanied by the same symptoms whatever antigen was used. All the evidence eventually showed that the hypersensitivity first observed by Richet was not in any way connected with the toxicity of the antigens he happened to employ, nor was it a response peculiar to the dog. Furthermore, anaphylaxis was found to possess several features in common with the immune reactions of bacterial infections. For instance:

(i) the techniques used for developing artificial immunity and hypersensitivity both involve the use of protein antigens;
(ii) the immune reaction and the anaphylactic response are so highly specific that both are extremely sensitive to even slight changes in the antigen molecule;
(iii) if a normal animal is injected with the serum of a recently sensitized one, then it will become "passively" anaphylactic. This condition, like passive immunity is transferred only when the donor's serum already contains an appreciable concentration of the antibodies.
These similarities were justifiably regarded as evidence that in an anaphylactic shock an antigen-antibody reaction occurs, just as in an immune response to an infectious microorganism. It is, therefore, hardly surprising that the early theories of the mechanism involved in anaphylaxis were based on the assumption that the primary reaction occurs in the blood.

The first theory of the mechanism of anaphylaxis was developed by Biedl and Kraus in 1909. They noticed that in the dog the symptoms of anaphylactic shock were practically identical with those of peptone poisoning (Schmidt-Mulheim, 1880). Both conditions showed the same picture of circulatory and respiratory collapse accompanied by a lengthening of the clotting time of the blood. Anaphylaxis came to be regarded therefore as a special form of peptone poisoning.

An alternative theory, however, was developed by Dale and Laidlaw following the isolation of histamine from ergot and their discovery that its principal action in the body was to increase the tone of smooth muscle (Dale and Laidlaw, 1910). They found that in rodents the organs most sensitive to histamine were the uterus, large blood vessels and bronchioles, spasm of the latter frequently causing death by asphyxia. In carnivora, as in other species, histamine constricted the arterioles, but this circulatory response was outweighed by a general capillary vasodilation which led to a sharp fall in systemic blood pressure.
Dale and Laidlaw noticed that some of the respiratory and circulatory complications produced by histamine were very similar to those appearing in peptone and anaphylactic shock. For this reason they suggested that histamine might be involved in these two conditions. The isolation of considerable amounts of this substance from peptone by Hanke and Koersler in 1920 initially supported this view, but other evidence soon made it appear to be untenable. It was possible, by using aseptic conditions, to prepare histamine-free peptone which was still capable of producing a shock (Clark, 1924). Furthermore, a comparison of the symptoms of histamine poisoning with those of anaphylactic and peptone shock revealed so many discrepancies that it seemed rather unlikely that histamine could possibly be an important common factor in the two latter conditions as well. In the sensitized dog for instance, hepatectomy completely prevents the development of anaphylaxis. The intensity of peptone shock however is reduced to only a slight extent, while the severity of histamine poisoning remains practically unaltered (Manwaring et al, 1922). In addition, histamine does not change the clotting time in this species, while in the other two conditions it is considerably prolonged. Anaphylaxis in the rabbit causes a drop in body temperature, but this effect is absent in peptone and histamine shock (Bally, 1929). The isolated sensitized uterus of the rat contracts in the presence of the antigen but relaxes with histamine (Kellaway, 1930).
Finally, in the guinea-pig, quinine intensifies the symptoms of anaphylaxis, yet is without effect on those of histamine poisoning (Smith, 1920).

As a result of all this evidence, the suggestion that histamine was responsible for the principal symptoms of anaphylaxis seemed to be untenable. A widely accepted alternative theory, which had the added advantage of supporting the ideas of Biedl and Kraus (1909), was developed by Vaughan in 1913. He showed that the alkaline hydrolysis of proteins yields extremely toxic end-products which he collectively called "anaphylatoxins" (Best and McHenry, 1931).

These, he suggested, were normal constituents of all proteins, their presence being revealed when the macro-molecule was degraded under suitably mild conditions. The initial sensitization of an animal produced, according to Vaughan, a specific proteolytic enzyme in the blood, which hydrolysed the antigen when it was injected for the second time. The subsequent release of anaphylatoxins into the circulation then led to the development of shock. Alkaline hydrolysis of the protein in vitro would, of course, enable a single injection of the end-products to have a similar effect. Unfortunately, the nature of "anaphylatoxin" was never definitely determined. A comparison of its physiological actions with those of histamine made it probable that the two were identical. However, differences in their stability at various pH's eventually eliminated this possibility (Best and McHenry, 1931).
Vaughan's theory thus implied that the enzymic hydrolysis of the antigen molecule liberated anaphylatoxin. As this reaction was supposed to occur in the blood, the enzyme most likely to be involved seemed to be serum trypsin (Friedberger, 1909). However, attempts to show that this was activated during anaphylaxis failed. Similarly, measurements which were expected to show a rise in circulating non-protein nitrogen during shock were also unsuccessful (Auer and Van Slyke, 1913; Barger and Dale, 1914). An added complication to the acceptance of this enzymic mechanism was the fact that the symptoms of shock appeared almost immediately after the injection of the antigen (Wells, 1908). For this to happen, the speed of hydrolysis would have to be unusually high for an enzymic process. In addition, it was most unlikely that the extremely minute amount of antigen needed to precipitate a shock response could possibly supply sufficient anaphylatoxin to develop severe poisoning (Wells, 1908). This objection was partially countered by the suggestion that the enzyme digests not only the antigen molecule but the plasma proteins as well (Jobling and Petersen, 1914). The problem of the speed of the reaction however still remained and was largely responsible for the eventual rejection of Vaughan's "enzymatic" theory and its replacement by a "colloidal" mechanism of toxin formation.
This theory originated with the discovery that anaphylatoxin-like substances are produced when blood serum is brought into contact for a very short time with various gels such as agar, starch and inulin (Kopaczewski, 1919). During the reaction a flocculation appears in the serum and physical changes occur such as a drop in surface tension and a rise in viscosity of the blood. Hence Kopaczewski's suggestion that colloidal changes in the plasma were responsible for both this effect and the appearance of anaphylatoxin was very widely accepted. It had a further advantage over Vaughan's enzymatic theory in that it successfully overcame the complications connected with the speed of the reaction, originally pointed out by Wells (1908).

The similarity of the symptoms in "colloidal" anaphylatoxin poisoning and those of anaphylaxis were remarkably close and served as further support for Kopaczewski's theory. In the guinea-pig for instance, death was due to asphyxia, while in the dog it was caused by hypotension and liver oedema. Karsner and Hanzlik (1919) discovered, however, that very small quantities of the gels used to produce the toxin were themselves capable of causing thrombosis when injected into the circulation. The resulting occlusion of the capillaries in the lungs, heart and liver was shown to be responsible for the appearance of anaphylactic-like symptoms in these animals. In isolated organs they found that thrombosis was so extensive that the circulation
of the perfusion fluid was completely arrested, a condition which is never encountered in sensitized organs when they are perfused with their specific antigen. Furthermore, thrombosis is a complication which is entirely absent in both anaphylaxis and peptone poisoning in intact animals. For these reasons, Karsner and Hanzlik suggested that the toxic effects which Kopaczewski obtained were due to contamination of the serum with minute amounts of the colloid with which it was incubated. The fact that the resulting symptoms were similar or even identical with those of anaphylaxis was simply a coincidence and not an indication, as generally supposed, that similar mechanisms were involved.

The greatest objection to the colloidal theory was, however, the unphysiological treatment to which serum had to be subjected before the toxin appeared. Assuming Kopaczewski's theory to be correct, then the easiest way of making anaphylatoxin ought to have been the incubation of blood from a sensitized animal with its specific antigen. Experiments based on this conclusion invariably gave negative results (Dale, 1920), and yet the toxin readily appeared when unphysiological and unrelated materials such as agar, kaolin and starch were substituted for the specific antigen. Anaphylatoxin formation thus appeared to be a general type of toxic reaction which had very little in common with the highly specific mechanisms occurring in anaphylaxis.
The enzymatic and colloidal theories of anaphylatoxin formation both assumed that the antigen-antibody reaction occurred in the blood. Evidence gradually accumulated to show that this view was incorrect. Manwaring (1910) exsanguinated a sensitized animal and then restored its circulation with blood from a normal animal. When the specific antigen was now injected, an anaphylactic shock developed. Under these circumstances, an antigen-antibody reaction in the blood was rather unlikely but not entirely impossible. Undrained capillaries might have emptied their antibodies into the normal blood, and although considerably diluted they would still have been able to react with the antigen in the circulation. Hence, Manwaring's experiment, although making it rather unlikely, did not definitely exclude the circulation as the site of the primary shock reaction.

This was achieved by Schultz and Dale using isolated organ preparations (Schultz, 1912; Dale, 1912). The tissues of sensitized guinea-pigs were perfused with Locke solution for a considerable time so that all traces of blood were removed. In the presence of the antigen, the smooth muscles of the organs responded in the same way as in anaphylaxis. Furthermore, repeated doses of the antigen eventually produced a refractory state in the tissues, just as several inoculations of the antigen abolished hypersensitivity in the intact animal. These effects, in the absence of blood, were explained by assuming that the
antibodies were absorbed into the tissue cells where the primary anaphylactic reaction took place. An excess of antigen would eventually deplete the tissues of the antibody. No further reactions would occur and consequently a refractory state would then exist.

This cellular mechanism of anaphylaxis came to be supported by a considerable amount of further evidence. For instance, Weil showed that the presence of free antibodies in the circulation hinders the development of an anaphylactic shock in the intact animal (see Dale and Kellaway, 1921). This was confirmed by Dale and Kellaway (1921) using isolated organs. A guinea-pig was passively sensitized to egg albumen by injecting it with the specific antibodies previously developed in a rabbit. The uterus was washed free of blood and then bisected. One horn responded to 1/10,000,000 of the antigen and after a few exposures became desensitized. The other horn was then placed in a bath containing antibody, i.e. the immune serum of the rabbit. The addition of the antigen in amounts similar to those used on the other part of the uterus failed to elicit an anaphylactic response. This was developed, however, when the preparation was initially washed free of excess antibody.

Hence, in isolated organs, as in the intact animal, the appearance of a state of shock was hindered by the presence of an excessive amount of free antibody. It seemed unlikely therefore that the primary antigen-antibody reaction occurred
simply in the blood. The most likely site of this reaction now appeared to be the actual tissues themselves.

A further advantage of the cellular theory was its ability to account for the refractory period of passive anaphylaxis (Weil, 1913; Lewis, 1932). An injection of the antigen soon after an inoculation with antibody invariably fails to provoke a shock. In fact, several hours must intervene before the animal becomes hypersensitive. During this non-reactive phase, antibody is, of course, readily detected in the plasma. By the fourth hour after its administration however, its concentration noticeably drops when, simultaneously, the state of passive hypersensitivity appears. This refractory period was originally regarded as being due simply to the protective action which circulating antibodies are known to exert. As already shown, these are capable of protecting both isolated organs and the intact animal from the action of the antigen (Dale and Kellaway, 1921). This simple explanation of the refractory state was, however, shown by Dale to be inadequate for he found that before the shock reaction can occur, the tissues must first absorb antibody from the plasma. Passive sensitivity to horse serum was developed in guinea-pigs by injecting them with the corresponding antibodies formed in rabbits. The isolated perfused uterus, washed free of blood, initially contracted when small doses of the antigen were placed in the bath. After several responses however, the muscles became desensitized.
It was now soaked for at least two hours in the serum of guinea-pigs which had recently received injections of the same antigen. As a result of this treatment the uterine muscle regained its sensitivity, and after several anaphylactic responses to the antigen, again became desensitized. This experiment not only demonstrated that the anaphylactic reaction took place within the cells, but it also indicated that its development depended on the prior absorption of antibody into the tissues. The refractory period observed in passive anaphylaxis was, therefore, presumed to be due simply to the slowness of this process. After a few hours contact with the serum, sufficient antibody would be absorbed to enable the intracellular antigen–antibody reaction to provoke an anaphylactic shock.

The mechanism by which a cellular antigen–antibody reaction produces an anaphylactic shock remained obscure for a considerable time. When the blood was regarded as being the site of the reaction, the symptoms of shock were thought to be due to the formation of a toxin. Under physiological conditions, all attempts to produce this substance in the blood of sensitized animals failed completely (Dale, 1920). With the realization that the primary reaction occurred in the tissues, the search for toxic end-products centred on perfusion studies of sensitized organs. In the dog it was shown that a histamine-like substance appeared in the liver during anaphylaxis (Manwaring et al, 1925).
A similar substance was detected when the lungs of sensitized guinea-pigs were perfused with the antigen (Bartosch et al, 1932). In this species, histamine diffused from several other tissues as well, for example the aorta, heart and skin (Schild, 1939). In rabbits a shock was accompanied by a sharp rise in the concentration of circulating histamine, most of which originated in the leucocytes (Dragstedt et al, 1940).

The basis of Dale and Laidlaw's suggestion (1910) that histamine is an important factor in anaphylaxis and peptone shock was the fact that this substance is able to mimic many of the symptoms of these two conditions. It has already been mentioned that discrepancies were revealed when certain features of anaphylactic and peptone shock were compared with those of histamine poisoning. On this evidence it appeared that Dale's theory was disproved; its eventual wide acceptance was the result of two lines of evidence. Firstly, the frequent detection of histamine in the effluent after the antigen had been injected into the perfusate of a sensitized organ. Secondly, the realization that the acute symptoms of shock were due to spasm in the smooth muscle of certain strictly localised regions. These muscles were all found to be extremely sensitive to histamine, even in normal animals. Furthermore, their distribution varied from species to species and accounted for the characteristic symptoms which appeared during shock in any particular species. In the dog the hepatic veins are
constricted, in the rabbit the pulmonary artery and its branches are occluded, while obstruction of the bronchioles occurs in the guinea-pig. These species-specific responses are, in every case, the result of intense spasm of the smooth muscles in certain organs which are, for this reason, known as the "shock" organs of the particular species.

Since it was originally supposed that the primary anaphylactic reaction responsible for the production of histamine occurred in the blood, it was to be expected that the effects of an intravenous injection of histamine would closely resemble those of shock. As already mentioned however, so many differences were encountered between these two conditions that it seemed highly unlikely that histamine was the principal agent responsible for the anaphylactic condition. These discrepancies can now be accounted for by the following facts. After an injection of an antigen into a sensitized animal, a considerable amount of histamine suddenly appears within the shock organ. The smooth muscles in this tissue consequently develop very intense and prolonged spasm which precipitates the acute symptoms typical of shock in the particular species used. This very localized pocket of highly concentrated histamine within the shock organ now diffuses into the blood and so exerts its secondary effects by reaching all parts of the body. This is the phase of the reaction which most closely resembles the
effects of an intravenous injection of histamine. The highest level which this substance reaches in the blood will not, of course, be a true measure of its original concentration within the shock organ. It is therefore not surprising that an injection of histamine is usually unable to mimic anaphylaxis, even when its level in the blood is identical with the values encountered in shock.

In the rabbit, anaphylaxis causes distensions and failure of the right side of the heart and a subsequent sharp fall in arterial blood pressure. Intravenous histamine, on the other hand, usually produces hypertension due to peripheral vasoconstriction. During shock however, pressor effects are largely masked by heart failure, this condition being due to the development of intense spasm in the pulmonary artery and its branches which form, in fact, the shock organs of this particular species. In the dog, the clotting time is increased during anaphylaxis whereas histamine poisoning leaves it unaltered. This discrepancy can now be explained by the close association of heparin with histamine in this species. The isolated sensitized rat's uterus relaxes with histamine, yet contracts in the presence of the antigen. This difference in response was thought by Kellaway to disprove the histamine theory of anaphylaxis in this animal. However, by recording uterine movements in situ, Suden (1934) has shown that it responds to both stimuli with an increase in tone. A considerable amount of
work has now shown that the symptoms due to intravenous histamine are practically identical with those encountered in the later stages of a non-fatal anaphylactic shock. The symptoms which appear in the initial phases of this condition arise from the intense spasm of the smooth muscles within the characteristic shock organ of each species. This phase of the reaction can only rarely be mimicked by exogenously administered histamine.

Histamine release may occur quite apart from an antigen-antibody reaction within a specific tissue or shock organ. It has already been mentioned that the injection of a mixture of polypeptides will precipitate an anaphylactic-like condition known as peptone-shock. A very similar response frequently appears after the administration of many drugs, especially basic ones such as alkaloids (Feldberg and Paton, 1951). This particular reaction was at one time extremely difficult to explain since the hypersensitivity appeared immediately after the first injection of the drug. It was, of course, well known that the prolonged use of some chemicals, for example organic arsenicals, frequently produced a state of "drug-allergy" in man and animals. Landsteiner showed that under these circumstances, the drug reacts with the plasma-proteins to form a complex which behaves as an antigen. The body then responds by making a specific antibody which eventually reacts with its antigen within the tissues and so precipitates a state of hypersensitivity (Landsteiner, 1945).
This type of mechanism is, of course, quite unable to account for the immediate response which follows a single dose of an alkaloid. A new explanation has however recently been made by McIntosh and Paton (1949), who found that several organic bases possess properties very similar to those of the alkaloids when they are injected into the circulation of the dog or cat. These substances, now known as "histamine-liberators" have no apparent effect for twenty to thirty seconds after administration; then, quite suddenly, the blood pressure falls abruptly in the manner characteristic of an intravenous injection of histamine. This "delayed-depressor" effect is a typical feature of "histamine liberators" and is due to the fact that they must first be carried in the blood to a sensitive organ before they can release its histamine into the circulation. These organs include the skin in cats, dogs, rats and guinea-pigs, the liver in the dog, the bronchioles in the guinea-pig and the leucocytes of the rabbit.

The release of histamine by these liberators follows the same time course as in anaphylaxis; an explosive release, which is followed by diffusion at an exponentially declining rate (Feldberg and Paton, 1951). Furthermore, as in anaphylaxis, other biologically active substances besides histamine appear in the circulation, such as heparin and "slow reacting substance" (Paton, 1951). No biological evidence for cell damage is found and hence the overall effects of these liberators closely
resemble those of an anaphylactic shock. For this reason it is quite possible that in both cases similar mechanisms are involved in the release of histamine. In the case of the liberators, the suggestion has been made that basic compounds, similar to histamine, can displace the latter from its points of attachment on polypeptide chains (McIntosh and Paton, 1949). Other workers, for instance Ungar (1952), have provided evidence which suggests that proteolytic enzymes, particularly fibrinolysin, are activated by these liberators. One could then explain histamine release as being the result of protein hydrolysis within specific tissues.

The importance of proteolytic enzymes in anaphylaxis has also been emphasized by Rocha e Silva (1940), who showed that crystalline trypsin in very high dilution (1 in 30,000,000) caused contraction of smooth muscle fibres. The strength of the response varied in magnitude with the species and the tissue, and declined in the following order: guinea-pig uterus, guinea-pig ileum and then the small intestines of the cat, rabbit and rat. A similar sequence was found in their sensitivity towards histamine and hence it was feasible to suppose that the trypsin was acting by the release of histamine within these organs.

Silva has also shown that histamine release under certain conditions depends on the disintegration of "white" microthrombi in the bloodstream (Silva, 1952). In both the anaphylactic
shock in the dog and the Schwartzmann phenomenon in the rabbit, enormous clumps of white cells and platelets are formed. The appearance of histamine under these conditions is always associated with the disintegration of these thrombi, a reaction which Silva suggests must be due to the activation of a proteolytic enzyme system in the plasma.

For a considerable time the origin of the histamine appearing during anaphylaxis remained uncertain. The appearance of toxic symptoms immediately after the injection of the antigen ruled out an enzymatic mechanism for its formation. In addition, the histamine content of many antigens was far too low to be the source of the large amount appearing in shock. The possibility that histamine was derived from the histidyl radicles of the antigen molecule was also shown to be most unlikely (Schild, 1936). The work of Best and Dale (Best et al., 1927) definitely established the fact that histamine is a constituent of all mammalian tissues and not a contaminant due to bacterial action. Under normal conditions it is physiologically inert, but during anaphylaxis and peptone shock, or as a result of injury, it is liberated from the tissues and then becomes biologically active. The nature of the link between tissue protein and histamine is unknown, but it is probable that several types of chemical bond are involved. This is suggested by the fact that there is a considerable variation in the ease with which histamine is liberated from any particular tissue. For instance, quite a
large fraction passes into solution when the cells are ground up with sand and water. Similarly, digesting them with trypsin or disrupting their membranes with lysolecithin liberates a considerable amount of histamine. With all these techniques however, it is frequently found that microscopic particles of protein still possess histamine, which may be liberated into solution only after the protein is denatured with heat or hydrolysed with a strong mineral acid (Feldberg, 1941).

The distribution of histamine inside the cell is not really known. A considerable amount of it is released when the tissue is homogenized and it is quite likely that some of it is subsequently reabsorbed into cellular elements. Hence, the eventual distribution of histamine in an homogenate may be completely different from that in the intact cell. Most experiments on intracellular localization have been performed on the tissues of the dog and it has been found that histamine is highly concentrated in the mitochondria of the liver cells (Copenhaver, 1953; Hagen, 1954). This fraction of the homogenate is, however, also very heavily contaminated with granules from mast cells (Mota, 1954) which are known to contain an enormous amount of histamine (Riley and West, 1953). The intracellular distribution of this substance is still therefore largely uncertain; its possible association with mitochondria is, however, particularly interesting, as these structures contain many of the enzymes involved in the Krebs cycle
(Lehninger, 1951). This process is responsible for over 80% of
the aerobic oxidation of the cells of the body and the closely
associated process of high energy phosphate bond formation.
These facts and their possible implications will be discussed
later.
2. HISTAMINASE AND HISTAMINE METABOLISM.

Mammalian tissues possess an enzyme, histaminase, which decomposes and detoxicates histamine (Best, 1929). During the reaction, ammonia and hydrogen peroxide are formed and oxygen is absorbed. The measurement of any of these factors provides a chemical determination of histaminase activity. Alternatively, changes in the concentration of the substrate form the basis of the biological assay of enzymic activity, which is usually expressed in terms of the amount of histamine decomposed in 24 hours. The rate of this reaction, of course, depends on the concentration of the substrate as indicated by the Michaelis-Menten equation. This factor alone is found to vary considerably from the results of one worker to another. For instance, in the experiments of Best and McHenry (1930), considerable amounts of histamine, up to 40 mg/ml., were added as substrate to make sure that the enzyme was acting at its maximal rate during most of the 12 hour incubation period. Other workers however, give results which show that under their conditions the enzyme was acting far from its greatest velocity (Kapeller-Adler, 1951). Thus, the conditions under which activity is measured vary so much that it is virtually impossible to compare the results obtained by different techniques. For this reason, the conclusions of only a few recent publications will be considered.
As histamine is highly active even in low concentrations, histaminase, if it is to be of any protective value, ought to be remarkably efficient in detoxicating dilute solutions of its substrate. In most tissues however, histaminase activity is so feeble that an incubation period of about 24 hours is required to produce a measurable reaction (Cotzias and Dale, 1952; Kapeller-Adler, 1952). The assay is further complicated by a delay in the onset of enzymic action when histamine is added to a fresh tissue homogenate (Best and McHenry, 1930). This latent period may last for as long as five hours, but it can be considerably reduced, or completely eliminated, by dialysis of the enzyme (Cotzias and Dale, 1950). The nature of the inhibitor is completely unknown.

The low activity of the enzyme could, of course, be due to the destruction of intracellular organization during homogenization. Cotzias and Dale (1952) showed that in rabbit liver, the enzymic activity of the mitochondria is three times that of the rest of the homogenate. The generally low activity shown by most tissues may, therefore, arise from the medium in which homogenates are usually dispersed. Dilute saline solutions, or even distilled water, both of which are known to cause osmotic disintegration of mitochondria are frequently used. Isosmotic solutions of sucrose (0.25 M) are known to preserve the intracellular elements and consequently histaminase activity might well be considerably enhanced.
The long latency already mentioned is probably not due solely to destruction of mitochondria. There is evidence that the enzyme is a flavo-protein (Zeller et al, 1940; Kapeller-Adler, 1949), and that pyridoxal-phosphate may possibly be a coenzyme for it (Sinclair, 1952). The techniques used in preparing the enzyme extract may lead to the rupture of a chemical bond between the coenzyme and prosthetic group, and its resynthesis would probably need a considerable time under the conditions existing in a homogenate. Alternatively, the hydrolytic action of phosphatases on pyridoxal-phosphate and its subsequent gradual resynthesis by a coupled oxidation-phosphorylation process may well account for the latent period.

At the present time, it is virtually impossible to decide whether or not histaminase is an important factor in antagonizing the effects of its substrate when this is released endogenously within tissue cells. In the dog, the enzyme is in highest concentration in the intestine and kidneys (Best and McHenry, 1930). This suggests that its principal function, in this species at least, is to detoxicate the large amounts of histamine ingested in a carnivorous diet. By comparison, the enzyme is noticeably low in the corresponding organs of herbivores, the highest values in the rabbit being found in the lungs (Cotzias and Dale, 1952).
For two reasons histaminase is probably of only minor importance in antagonizing an anaphylactic shock. Firstly, it is largely absent from the shock organs of certain animals, and secondly, its overall tissue concentration bears no relation to the susceptibility of a species either to histamine poisoning or to the ease with which it develops hypersensitivity. The concentration of histaminase in a shock organ is rarely high. The one exception is the rabbit in which the lung has as much as 21 units/g. The guinea-pig, having the same shock organ, possesses an activity of only 0.6 units/g. The liver, the shock organ of the dog, is practically devoid of histaminase, whereas other structures such as the kidney are copiously supplied with the enzyme (Cotzias and Dale, 1952).

Rats and mice can tolerate large intravenous injections of histamine and are able to undergo anaphylaxis with the development of only very mild symptoms of shock (Feldberg, 1941). This can hardly be due to the levels of histaminase in their tissues, for these happen to be very similar to those of the guinea-pig (Cotzias and Dale, 1952), a species which is extremely sensitive to both endogenous and exogenous histamine. Conditions which are fatal to the guinea-pig hardly affect the other two species at all. Comparison of histaminase activity therefore is of little value in deciding the relative susceptibility of animals to histamine poisoning. Similarly, the wide variations in the severity of anaphylaxis in different animals also appears to be
independent of the level of this enzyme in their tissues. It seems most likely therefore that histaminase is largely concerned with the detoxication of histamine originating in the food, rather than with the control of its level within the cells of the animal.

Very little is known of the metabolism of histamine within the tissues, but eventually some of it is excreted in the urine, either as free histamine or in a conjugated form as acetyl histamine. According to Gaddum (1951) these two fractions have different origins since the concentration of one may vary independently of the other. For instance, the oral administration of histamine increases the amount of the conjugated form in the urine, whereas the free histamine fraction is changed only by an intravenous injection of the base. It is therefore unlikely that the free form represents histamine which has simply escaped acetylation; it is far more likely that the free base in the blood is excreted unaltered, whereas that in the intestine is either acetylated and then excreted or else decomposed by histaminase.

Although the urinary concentration of acetyl histamine varies considerably and depends on the nature of the diet, the other fraction remains remarkably constant at about 20 \( \mu g \) base per 24 hours (Gaddum, 1951). This is regarded as evidence that its origin is associated with a very steady process within the tissues, the most likely one being that of protein degradation.
So far, results have been confined to individuals in a good state of nutrition and general health. The effect of gross disturbances in protein metabolism, such as occur in malnutrition and muscular dystrophy, are unknown, but might provide further evidence for assessing the correctness of the theory.

When histamine is injected intravenously, only 1% of the dose is recovered in the urine in the free form. Should the same proportionally hold for histamine released within the tissues, then the daily loss of 20 μg. of the free base implies the release of 2 mg. of histamine from the tissue protein every 24 hours, of which 99% is detoxicated presumably by histaminase. This argument implies that injected histamine is exposed to the same conditions of decomposition as that released endogenously, an assumption which is unlikely for two reasons. Firstly, histaminase activity varies between different tissues and between tissues in general and the blood. Secondly, most of the injected histamine will promptly pass through the kidneys, which, in most species, are exceptionally rich in histaminase. There is the added complication that the enzyme would show very little activity towards its substrate if it appeared at such a slow rate as 2 mg. per 24 hours. For this reason, it is possible that the daily loss of intracellular histamine might be considerably greater than 2 mg. and might reach a level at which histamine could show a high degree of activity. On the other hand, such a concentration of histamine would inevitably produce
toxic complications such as contraction of smooth muscle, increased glandular secretion, hypotension and oedema. The lack of these typical symptoms implies therefore either that histaminase is exceptionally active within the intact tissues, or that other mechanisms are involved in keeping histamine in a biologically inert form. In the following section Gaddum's theory of the origin of free urinary histamine will be examined in the light of recent work on protein metabolism and an alternative mechanism of detoxication suggested.
3. PROTEIN METABOLISM AND ITS RELATION TO THE PHYSIOLOGICAL ACTIVITY OF INTRACELLULAR HISTAMINE

Ideas on the mechanism by which proteins are metabolized have changed considerably over the last twenty years. The first comprehensive theory of this process was developed by Folin in 1905, who examined the effect of the diet on the distribution of nitrogen between the various constituents of the urine (Folin, 1905). He found that the daily output of urea depended on the amount of protein ingested by a subject. In contrast to this, changes in the diet hardly affected the daily loss of creatinine, which remained remarkably constant over a very wide range of protein intake. From these results Folin concluded that protein degradation occurs by two independent but simultaneous processes, the particular mechanisms employed on any specific protein molecule being determined by its origin.

Urea was regarded as the end product of dietary (exogenous) protein metabolism, while the metabolism of the tissue (endogenous) proteins gave rise to urinary creatinine as its final end product. This mechanism represented the effect of "wear and tear" on the body proteins, which, once they had been formed, were supposed to be inert molecules as far as the metabolism of their constituent amino acids were concerned. As a result of their participation in metabolic activities however, some tissue-protein molecules gradually became "worn out", and were then completely degraded by the mechanism which Folin called the "endogenous" aspect of protein metabolism.
Folin's concept that tissue proteins are structurally inert substances held the field for a considerable time. An alternative theory gradually developed however, which implied that the apparently stable tissues and organs of the body were constantly being degraded and resynthesised throughout the life of the organism. This idea of a "continuous protein metabolism" was first suggested by Borsook and Keighley (1935) as a result of metabolic studies on human beings.

Direct and convincing evidence in support of this process was supplied by Schoenheimer and Rittenberg (Schoenheimer, 1942). Isotopically labelled amino acids, when injected into the circulation of adult animals in a state of nitrogenous equilibrium, are very rapidly incorporated into certain tissue proteins. After only a short interval of time however, they are to be found either in a different location or in the general metabolic pool. The use of N amino acids has shown for instance that the half-life of rat liver protein is about seven days, and that in this species, labelled glycine and other amino acids are incorporated into the tissues and then lost from them at a remarkably high rate (Shemin and Rittenberg, 1944).

This complex process of co-ordinated protein synthesis and degradation is probably performed by intracellular proteases, the cathepsins. A peculiar feature of these enzymes is the unphysiological pH at which their activity is greatest for the pH optima of most of them lie between the values of 3.5 and 4.5.
At first sight this presents a difficulty since it would mean that these enzymes are unable to function to any great extent at the normal intracellular pH of 7.4. However, since it is impossible to provide them in vitro with their natural substrates, the low pH optima are probably an indication that hydrolysis is unable to occur until the artificial substrate has been denatured into a suitable configuration which will enable it to combine with the enzyme.

Preparations of these intracellular enzymes are able to synthesize simple peptides under certain limited conditions in vitro. Papain, a plant cathepsin, forms hippurylanilide when it is incubated with hippurlamide and aniline (Bergmann, 1937), the maximal yield being obtained at pH 4.5 to 5.0:

\[
\begin{align*}
&\text{CO NH CH}_2\text{ CO NH}_2 + \text{NH}_2\text{CO} \\
\Leftrightarrow &\text{CO NH CH}_2\text{ CO NH} + \text{NH}_3.
\end{align*}
\]

Two special features of this reaction explain the ease with which this peptide is formed. Firstly, the substrates are unable to form zwitterions. This is particularly important as the production of a peptide bond between un-ionized molecules needs far less energy than that between strongly ionized structures. Secondly, the hippurylanilide is insoluble in water. The equilibrium of the reaction is therefore continually displaced to the right, and, as a result of mass action, an appreciable yield is obtained.

The synthesis of proteins by proteases has not yet been achieved in vitro, largely because this process is not simply
a reversal of enzymatic hydrolysis. The absence of synthesis is an indication that the amino acids presented to the enzymes are in an unsuitable energy state for their incorporation into a polypeptide complex. The formation of one gram equivalent of peptide bond requires about 3000 calories (Lipmann, 1949), but before this can occur, additional energy is needed to convert the zwitterion of the amino acids into the non-ionized form (Haurowitz, 1950). The synthesis of peptide bonds depends therefore on a readily available supply of energy, the importance of which has been revealed in studies on the bio-synthesis of very simple peptides.

The mechanisms which have been most fully examined are those involved in the synthesis of hippuric acid (Borsook and Dubnoff, 1947), and glutathione (Block, 1949, 1951) and the acetylation of sulphonilamide (Lipmann, 1945). These peptides are formed under aerobic conditions only when the cell extracts are able to esterify inorganic phosphorus so that AMP is converted either into ADP or ATP. Under anaerobic conditions, the velocity of the reaction gradually diminishes and eventually stops, but it is resumed when ATP is added to the system. Although the enzymes responsible for the formation of these three types of peptide bond have not yet been isolated, the intracellular cathepsins provide the only known mechanism which could be responsible for their formation. Moreover, the large amount of energy needed to form a peptide bond suggests that
these enzymes are coupled to the energy yielding mechanisms associated with the synthesis of ATP in the tricarboxylic acid cycle. This would explain the high synthetic activity of the cathepsins in media whose pH values are between 7 and 8.

Schoenheimer's work firmly established the dynamic state of the tissue proteins, in that one protein molecule exists as an intact entity for only a very short period of time. Within a day, some of its component amino acids will have been replaced by other like molecules from the metabolic pool; this process of amino acid exchange applies also to structural proteins as well as to those functioning as enzymes and hormones. The resulting state of flux occurs in varying degrees throughout the tissues and is so rapid that the average half-life of human protein is only 30 days, the half-life of liver and serum proteins is approximately 10 days, while that of the carcase as a whole is 158 days (see Lancet, 1953).

The fact that histamine is a normal constituent of all mammalian tissues was established at a time when Folin's theory of protein metabolism was in vogue. On the basis of this theory, it seemed reasonable to suppose that tissue histamine was kept in a physiologically inactive state simply by its union with the stable structural proteins of the cell. The development of a new dynamic theory of protein metabolism seems to have had no effect on this concept of the histamine-protein complex. As far as histamine metabolism is concerned, cell proteins are still regarded in the same way as Folin pictured them. Gaddum's
explanation of the origin of the stable fraction of urinary histamine is apparently derived from Folin's concept of endogenous protein catabolism. In one case it is used to explain the excretion of a constant amount of creatinine; in the other, it is responsible for the unvarying quota of histamine lost from the body.

If Schoenheimer's theory is correct, proteolysis must be a continuously occurring reaction within the tissue cells. The in vitro hydrolysis of proteins by acids is frequently accompanied by histamine release, it is however extremely unlikely that the intracellular degradation of protein involves the liberation of physiologically active histamine. The body contains so much of this substance, and the half-life of many proteins is so relatively short, that the amount of histamine liberated would probably be sufficient to overwhelm the histaminase system. The result would be an accumulation of histamine in the body fluids, with all its attendant disastrous effects. The concentration, and so presumably the activity of histaminase is however extremely variable. In some organs in fact, it can hardly be detected at all. It is in these particular structures, at least, that some other mechanism must be present for inactivating or masking histamine when it is liberated during the normal course of protein metabolism.

It seems highly probable that during protein hydrolysis histamine is chemically masked and then promptly recombined with
fresh polypeptide chains which are about to be incorporated into new protein molecules. Histamine loses its biological activity when its $\alpha$-amino group is condensed with the terminal carboxyl radicle of a synthetic peptide chain (Silva, 1943). Neither these artificial histamine-peptides nor natural protein-histamine complexes possess any vaso-depressor activity when injected intravenously into a cat; after acid hydrolysis however, the presence of free histamine is promptly revealed in both types of molecule. Silva's work shows how readily histamine may be masked by a relatively simple chemical process, i.e. peptide bond formation. These facts do not, of course, provide any evidence for the nature of the link between natural proteins and histamine. The peptide bond may be only one mode of combination, for the fact that histamine can be explosively liberated in a tissue without the appearance of histological changes suggests that its union can, in some cases, involve a link which is far weaker than the relatively strong and stable peptide bond.
4. EXERGONIC REACTIONS AND THE INTRACELLULAR STABILIZATION OF HISTAMINE

All these aspects of histamine metabolism which have been discussed tend to suggest therefore that normal cells possess an extremely active mechanism which keeps the potentially labile histamine in a physiologically inert state.

The distribution of histamine within a cell is such that a steep concentration gradient exists between different intracellular compartments such as the mitochondria and the intracellular fluid. The problem now arises as to how this difference is maintained, especially as the histamine protein link is highly labile. By comparison with other processes which involve the development of concentration gradients, it seems very likely that an active transfer mechanism is involved, analogous with the mechanism for glucose absorption. We know that no matter how low the concentration of glucose may be in the gut, it is always completely absorbed against the relatively high glucose content of the blood (Verzar and McDougall, 1936). Energy for this "active" process is provided by ATP. Similarly, the ionic gradients developed across mitochondrial membranes persist only as long as ATP is made available by the Krebs cycle (Bartley and Davies, 1954). Hence it is possible that the intracellular histamine gradient is maintained by a similar "active" process which depends on the utilization of the high energy phosphate bonds provided by ATP.
It has previously been mentioned that the granules of mast cells and the mitochondria of many tissue cells apparently contain a considerable amount of histamine. Furthermore, mitochondria are known to possess a distinct outer membrane (Glimstedt et al., 1954), across which they are able to maintain an ionic gradient (Bartley, Davies and Krebs, 1954). A similar set of conditions probably exist for mitochondrial histamine with respect to its concentration in the intracellular fluid. This is all the more likely when it is remembered that these particles contain enzymic mechanisms responsible for at least 80% of the high energy phosphate production of the cells. The fact that this energy exchange process is so closely connected with the mitochondria would explain the ability of these structures to maintain concentration gradients with respect to the intracellular fluid.

Tissue histamine however, is also present in the non-particulate protein fraction of the cell, for example the cell membrane (Feldberg, 1941). Sensitized tissues, such as smooth muscles, immediately respond when they are brought in contact with their specific antigen. The promptness of the response and the size of most antigen molecules make it fairly certain that, under these particular conditions, the liberating reaction occurs on the cell surface, which is presumably also the source of the histamine responsible for the muscular contraction. Although the cell membrane is not separated from the intracellular
fluid by a distinct barrier, it would still be possible for a histamine gradient to be developed between these two cellular components. In this case, a delicate energy-yielding mechanism would again be involved, but this time, instead of acting as a "pump", it would chemically activate histamine molecules and bring them into a suitable energy state for their combination with the polypeptides of the cellular membrane. The ease with which histamine is liberated might then be an indication either of the weakness of the histamine-protein link in this particular protein complex, or of the sensitivity of the exergonic reaction which enables histamine to be combined with the components of the cell membrane. In this way an energy-yielding process could provide at least two mechanisms for preserving histamine in a non-toxic state, whether it is combined with the proteins of the cell membrane or concentrated within the mitochondria in the intracellular fluid.

Histamine release in an anaphylactic reaction occurs explosively (Schild, 1939); a large amount of histamine is liberated in an extremely short time. When small pieces of sensitized guinea-pig lung are suspended in saline at 37°C, there is practically no release of histamine over a prolonged period. The addition of the specific antigen to the saline causes an immediate liberation of histamine, whose rate of diffusion from the tissue decreases exponentially. These results indicate that (i) within the tissues an initially high concentration of free
histamine suddenly appears which promptly diffuses into the adjacent tissues, and (ii) after this initial phase of release very little, if any further histamine is liberated even at a slow rate.

If the mechanism of release is dependent on proteolysis, it is difficult to explain (i) how the explosive release can possibly occur without an unprecedented increase in proteolytic activity, (ii) what factor could be responsible for the equally prompt arrest of this activation so that a slow and prolonged phase of histamine liberation does not occur, and (iii) how such a process could take place without the slightest sign of tissue damage, which would be bound to appear with extensive proteolysis.

These complications are overcome if one assumes that the antigen-antibody reaction inhibits an energy-transferring system. Interference with ATP synthesis in the wall of the gut (Verzar and McDougall, 1936), in isolated mitochondria (Bartley and Davies, 1954), or in the renal tubules (Taggart et al., 1950) promptly abolishes the ability of these tissues to perform "active" processes such as absorption against a gradient, the maintenance of ionic concentration differences across a membrane, or of renal transport. The eventual exhaustion of high energy phosphate bonds finally allows diffusion processes to abolish the concentration gradients in a relatively short time. Similarly, if an energy-transferring system is involved in the stabilization of histamine, its disruption would immediately lead to a
considerable quantity of this substance suddenly spilling over from its region of high concentration, in the mitochondria, into the intracellular fluid. Simultaneously, histamine–protein complexes, whose formation depends on a ready supply of energy, would also break down and histamine would promptly be liberated from the cell membrane. Should the mechanism of anaphylaxis involve a considerable disorganization of the coupling of ATP utilization with peptide metabolism, then protein synthesis would also be disturbed. Many intermediates involved in this process, such as peptones and polypeptides of various molecular weights, would accumulate within the cell. Their presence, together with that of histamine, would then account for the closer resemblance of anaphylaxis to peptone shock than to pure histamine poisoning. Furthermore, the intracellular accumulation of peptides during anaphylaxis could also explain the appearance during this condition of large biologically active molecules, such as the "slow-reacting substance" of Kellaway and Trethewie (1940).

It is frequently found that a physiological process operates far more efficiently in a structurally organized system than in a preparation in which this has been destroyed. For example, it is known that the respiratory rate of the cyclophorase system is far higher when the mitochondria are intact than when they are disrupted by a high-speed blender (Green, 1951). This difference could, of course, be due solely to the dilution or destruction of essential coenzymes during homogenization or the subsequent
incubation of the tissues. However, it persists even after the non-protein components of the mechanism have been reinforced to such an extent that their final concentration in the homogenate is identical with or exceeds that in the intact cell. The fact that this difference in respiratory rate still persists under these conditions might, of course, be due to the lack of an unidentified metabolic factor. The possibility of this being true is extremely doubtful however, as both preparations are able to metabolize the substrates of the Krebs cycle to CO₂ and H₂O without the accumulation of any intermediates. The difference between the two respiratory rates is in all probability an indication that the high activity of the organized system depends on the spatial arrangement of its components with respect to each other. This intracellular orientation of functionally related enzymes would have two important effects on the rate of metabolism. Firstly, the likelihood of a fruitful collision taking place between an enzyme-coenzyme complex and its substrate is far greater than the chance meeting of these three individual components in a random mixture. Secondly, the orientation of functionally allied enzymes on a mitochondrion enables their substrates to be passed along the metabolic chain with a minimum of delay.

Structural orientation within the cell is of considerable importance in other metabolic processes such as the coupling of oxidation with phosphorylation, the synthesis of high energy
phosphate bonds and the formation of urea. Aerobic oxidation involves at least ten enzyme systems, all of which are concentrated in the mitochondria (Lehninger, 1951). Disruption of these particles by osmotic effects promptly results in a drop in respiration and the complete arrest of phosphate esterification. Thus, any treatment which destroys cellular organization leads to the random positioning of functionally related enzymes. The resulting delay in the rate of enzyme catalysed reactions may then have profound effects on the supply of energy that both the formation of metabolic intermediates and the co-ordinated activity of inter-related processes are completely arrested.

Similarly, in the hypothesis put forward, the process which may keep histamine stabilized on tissue protein, or concentrated within the mitochondria, probably depends on the integrity of intracellular organization for its greatest efficiency. This process, like many others, would therefore be extremely sensitive to disintegration of the cell, and even slight mechanical or proteolytic trauma would lead to the release of considerable amounts of histamine into the intracellular fluid.
EXPERIMENTAL SECTION

1. HISTAMINE RELEASE IN HOMOGENATES.

As a result of the conclusions mentioned in the Introduction, the action of histamine-liberators was tested on intracellular enzyme systems. Three particular groups were examined: firstly the catherinins, secondly the endo- and thirdly the enzymes associated with aerobic respiration and the formation of high-energy phosphate bonds. The isolation of these enzymes involved the homogenization of the tissues, a treatment which not only released a considerable amount of bound histamine, but also involved the risk of inactivating some, if not all, of the components of the histamine-liberating mechanism. This possibility led to a further study which these histamine-liberators happened to have on the remaining enzyme systems might well be completely unconnected with the histamine-releasing action which they displayed on normal intact tissues.

At this time (early in 1953) no reports had appeared in the literature of the effect of histamine-liberators on homogenates. An attempt was therefore made to see whether or not they could release histamine in this type of preparation. If they could, then it would have been justifiable to assume that the components of the releasing mechanism were still intact. Furthermore, a comparison could then be made between the amount of histamine actually liberated and the effect of the drugs on the particular enzyme systems under consideration.
1. Histamine release in Homogenates.

As a result of the conclusions mentioned in the Introduction the action of histamine-liberators was tested on intracellular enzyme-systems. Three particular groups were examined: firstly the cathepsins, secondly adenosine triphosphatase and thirdly the enzymes associated with aerobic respiration and the formation of high-energy phosphate bonds. The isolation of these enzymes involved the homogenization of the tissues, a treatment which not only released a considerable amount of bound histamine, but also involved the risk of inactivating some, if not all, of the components of the histamine liberating mechanism. This possibility led to a further complication in that any effects which these histamine liberators happened to have on the remaining enzyme systems might well be completely unconnected with the histamine-releasing action which they displayed on normal intact tissues.

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It has already been mentioned that histamine metabolism may be connected with the energy processes linked to aerobic respiration. These initial experiments on the effect of liberators on tissue homogenates thus needed a preparation in which the aerobic processes were maintained at a level of activity similar to that of intact tissues. For this reason, homogenization was performed in 0.25M sucrose in order to preserve the mitochondria. At the same time, 0.02M sodium citrate, 0.02M sodium succinate and horse-heart cytochrome-C were included to provide essential substrates and co-factors for the enzymes of the tricarboxylic cycle. These happen to be so sensitive that irreversible inactivation occurs when they are deprived of their substrates even for a short period of time. This will, of course, occur when the cellular contents are diluted by a homogenization fluid devoid of these particular substrates. Under these circumstances, enzymic activity is retained only when all manipulations are performed at zero or below. As these conditions could not be attained, the citrate and succinate were incorporated in the sucrose solution from the very beginning, so that some degree of protection would be afforded when the temperature rose during subsequent operations. The pH of the homogenizing fluid was adjusted so that it had a value of pH 7.3 at 37°C. Before use, both it and the homogenizers were thoroughly cooled in ice.
Preparation of tissues. A guinea-pig was killed by a blow on the back of the head and the blood drained out by a cut across the neck. The lungs, heart and diaphragm were then removed and separately cut into thin slices which were immersed in ice cold 0.25M sucrose solution. The slices were blotted, weighed on a torsion balance and samples of 400 mg. homogenized with 1.5 ml. of sucrose solution for two minutes. When all the tissue had been so treated, the homogenates were spun at 2500 r.p.m. for five minutes to remove pieces of connective tissue. Unfortunately, this procedure did not spin down intact cells; to do so would have required a much higher speed. Iced sucrose solution was added to the lung homogenate, so that the final preparation contained 5 g. of tissue dispersed in 30 ml. (tube 1). Due to the small amount of diaphragm muscle (2 g.), its homogenate was added to that of the heart muscle (3 g.) and the combined preparation then brought up to 30 ml. with more sucrose solution (tube 2).

Experimental procedure. Each suspension was equally divided between two flasks which were oscillated in a bath at 37°C. A stream of oxygen containing 5% CO₂ was passed through them and after a ten minute equilibration period, 7 ml. samples were removed for the determination of the initial histamine concentration. The rest of the procedure is shown in Table 1.
**TABLE 1.**

Sequence of incubation experiment with fresh tissues.

*(P.I. = propamidine isethionate)*

<table>
<thead>
<tr>
<th>Initial Specimen for histamine content</th>
<th>Addition of P.I.</th>
<th>Final Specimen for histamine content</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flask 1</strong></td>
<td>Specimen S1</td>
<td>NONE</td>
</tr>
<tr>
<td><strong>Tube 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Flask 3</strong></td>
<td>S3</td>
<td>8 mg.</td>
</tr>
<tr>
<td><strong>Flask 2</strong></td>
<td>S2</td>
<td>NONE</td>
</tr>
<tr>
<td><strong>Tube 2</strong></td>
<td>S4</td>
<td>8 mg.</td>
</tr>
<tr>
<td><strong>Flask 4</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Assay of histamine. Before any incubation experiments were started, trials were made on two techniques for assaying histamine; these were Schild's four point assay method (Schild, 1942) and Feldberg's method. The proper performance of the former technique needs an automatically controlled gut bath, but this complication is avoided in Feldberg's procedure which was eventually used for all the assays in this work. The lower part of the ileum of a guinea-pig (fasted for 18 hours) was removed and washed out with Tyrode solution. A piece, 4 inches long, was suspended in an aerated 15 ml. gut bath so that intestinal secretions could easily drain away into the surrounding fluid. The lever was adjusted to enable the tissue to remain slightly flabby and not held taut. At frequent intervals the bath fluid was changed and 0.2 ml. of 1:500,000 atropine sulphate added to the fresh saline. This "rest" period lasted two hours and was found to be essential for developing a preparation having a reasonable degree of sensitivity and a steady baseline. At the end of this period the gut was stimulated at regular intervals with 0.10 μg. histamine base. Once the responses had become constant, which was usually after 10 to 20 stimulations, the assay was commenced.

A constant volume of the unknown solution was alternated with graded doses of the standard so that the latter produced responses which initially just matched the unknown, then were slightly in excess and then slightly smaller than the matching dose. In some cases the volume of "unknown" was adjusted as well, but this
procedure was not often used for besides tending to alter the sensitivity of the gut, it also varied the concentration of gut-stimulating substances other than histamine which might have been present in the extract. The use of a constant volume of "unknown" throughout the assay enabled a measurement to be made of the extent to which other substances had affected the gut. This was achieved by repeating the "unknown" dose in the presence of mepyramine maleate (1:25,000,000), a highly specific anti-histamine. In this way the presence of other gut-contracting substances was revealed and their activity relative to histamine determined.

The following sequence was used in all assays, the standard or unknown solution acted on the gut for twenty seconds. The lever was then clamped and the bath washed out twice. After the third refilling the clamp was removed, the gut relaxed and 0.2 ml. of (1:500,000) atropine sulphate added. The preparation was stimulated every two minutes and clamping was used to prevent the gut from being suddenly stretched when the bath fluid was emptied out.

Results. Part of the assay is shown in Fig. 1, the solution S1 being the first specimen removed from Flask 1. The standards, all labelled S, consisted of 0.1 µg. of histamine. In "A" the effect of 0.5 ml. of undiluted S1 is shown; the large contraction it caused altered the sensitivity of the preparation to such an extent that normal responses to the standard were not obtained for a considerable time. Eventually, matching occurred between 0.50
ml. of SI diluted 1/10 (Fig. 1 D) and the standard; this dose was repeated five times to make sure that the matching and gut-sensitivity were reasonably constant. At F, 0.55 ml. of diluted SI exceeded the standards, while 0.45 ml. (G) gave a smaller response. From this assay it was calculated that one ml. of undiluted SI contained 20.8 µg. of histamine. At the end of the assay the gut was treated with mepyramine maleate (Fig. 2). This abolished the fast concentrations in both the standards and incubation liquids, but in two of the latter a small amount of a slow contracting substance was present. Its effects were however negligible compared with those of histamine and so it was ignored when the activities of the different incubation liquids were assessed.

Results. The following table shows the results:

**TABLE 2.**

(P.I. = propamidine isethionate)

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>10 mins. incubation</th>
<th>Histamine-releasing drug added</th>
<th>30 mins. incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) lung</td>
<td>20.8</td>
<td>NONE</td>
<td>25</td>
</tr>
<tr>
<td>3)</td>
<td>20.0</td>
<td>8 mg. P.I.</td>
<td>25</td>
</tr>
<tr>
<td>2) heart muscle</td>
<td>0.50</td>
<td>NONE</td>
<td>0.4</td>
</tr>
<tr>
<td>4) diaphragm</td>
<td>0.40</td>
<td>8 mg. P.I.</td>
<td>0.5</td>
</tr>
</tbody>
</table>
FIG. 1.
Incubation of fresh tissue homogenates (Table 1). Assay of solution Sl.

S - dose of 0.1μg. of histamine

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>effect of 0.5 ml. of undiluted Sl</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.2 ml. of Sl diluted 1 in 10</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.3 ml. of Sl diluted 1 in 10</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.5 ml. of Sl diluted 1 in 10</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.5 ml. of Sl diluted 1 in 10</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.55 ml. of Sl diluted 1 in 10</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.45 ml. of Sl diluted 1 in 10</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>0.40 ml. of Sl diluted 1 in 10</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 2.
Incubation of fresh tissue homogenates (Table 1). Effect of fluids on guinea-pig ileum after treatment with mepyramine maleate (1:25,000,000).

S - 0.1μg. histamine; the mepyramine was then added to the bath half a minute before the incubation liquids.

2S - 0.2μg. histamine after mepyramine.

1 = specimen Sl1
2 = specimen Sl2
3 = specimen Sl3
4 = specimen Sl4
In flasks the inhibitory sensitivity was reduced and the base line made so irregular that it was impossible to obtain consistent gradations between two flasks differed by as much as 0.1 mg. histamine. This change in sensitivity and the alteration in base line are shown in Fig. 1.

The experiment was therefore repeated using a far lower concentration of inhibitor in the incubation fluid together with considerably more drug and by the method put forward in a previous paper. A solution containing 0.1 mg. of succinylcholine was prepared and by a ten minute incubation the initial samples were removed from both flasks. Preparations of succinylcholine was added to one, giving a concentration of 0.1 mg. per ml. Samples for assay were removed from both flasks after a further ten minute incubation. The results were as follows:
It can be seen that no histamine was apparently liberated in flasks 2 and 4. This may well have been due to the fact that the incubation solutions contained so much propamidine that the sensitivity of the gut was considerably reduced. The histamine standards were also prepared with an equivalent amount of the drug and, under these conditions, the responses of the gut were so reduced and the base line made so irregular that it was impossible to obtain consistent gradations between doses which differed by as much as 0.1 μg. histamine. This change in sensitivity and the alteration in base line are shown in Fig. 3.

The experiment was therefore repeated using a far lower concentration of liberator in the incubation fluid together with considerably more tissue to magnify any release that might occur. By the method previously described an homogenate was prepared containing 8 g. of heart muscle and 3 g. of diaphragm muscle in 30 ml. of sucrose solution. After equilibration for ten minutes, the initial sample was withdrawn for assay and the remainder of the suspension divided equally between two flasks. Propamidine isethionate was added to one, giving a concentration of 0.1 mg/ml. Samples for assay were removed from both flasks after a further ten minutes' incubation. The results were as follows:—
### TABLE 3.

<table>
<thead>
<tr>
<th>Treatment during incubation</th>
<th>Solution No.</th>
<th>Histamine concentration in μg/ml. of incubation fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Removed after 10 min. equilibration period</td>
<td>S17</td>
<td>0.03</td>
</tr>
<tr>
<td>Incubate divided into two equal portions</td>
<td></td>
<td>Specimen lost</td>
</tr>
<tr>
<td>Removed after 10 min. incubation</td>
<td>S19</td>
<td>0.06</td>
</tr>
<tr>
<td>Removed after 10 min. incubation with propamidine isethionate</td>
<td>S20</td>
<td>0.12</td>
</tr>
</tbody>
</table>

In this experiment histamine was released in the homogenate by the addition of propamidine isethionate. Part of the assay is shown in Fig. 4.

Although the last experiment showed that propamidine isethionate can act in a homogenate, the amount of histamine liberated was considerably less than that released by its action on intact cells. One reason for this could have been the effect of dilution on a necessary component of the releasing mechanism. An attempt to overcome this difficulty was made in two ways; firstly, by isolating mitochondria so that they could be resuspended in a smaller final volume, and secondly, by desiccating the tissues, then grinding and dispersing them so that a
FIG. 3.

Incubation of fresh tissue homogenates (Table 1). Effect of propamidine isethionate on guinea-pig ileum during histamine assay.

S - 0.1 μg. histamine.
Sp - 0.1 μg. histamine in a solution containing propamidine isethionate 1 mg/ml.

FIG. 4.

Incubation of fresh tissue homogenates (Table 3). Part of the assay of solutions S19 and S20, the volume of the incubation fluid being recorded in ml.

$\frac{S}{2} - 0.05 \, \mu g. \, histamine.$
The attempt experiments were particularly rich in large mitochondria. The usual procedure is to homogenize in 0.25M sucrose, remove connective tissue and intact cells by spinning at 600 g and then to sediment the mitochondria in considerably stronger gravitational fields (Bogeboom, Schneider and Fälade, 1948). The highest speed of the centrifuge available at the time was insufficient to produce the necessary gravitational force. However, it is possible to deposit these particles in a strong solution by a procedure known as ultracentrifugation. Instead, the apparatus was chilled in an ice-salt mixture at between -10 and -20°C. As soon as the tissues were removed from the animal, they were immersed in cold 0.25M sucrose and cut into
preparation more concentrated than a homogenate was obtained. It
was also hoped that the isolation of mitochondria would show
whether or not the liberators acted on this cellular component
alone, or whether other constituents of the cell or tissue extract
were needed for the reaction to occur.

The attempt to isolate mitochondria was unsuccessful. Trial
experiments were made on homogenates from rat liver as this tissue
is particularly rich in large mitochondria. The usual procedure
is to homogenize in 0.25M sucrose, remove connective tissue and
intact cells by spinning at 600 g and then to sediment the mito-
chondria in considerably stronger gravitational fields (Hogeboom,
Schneider and Palade, 1948). The highest speed of the centrifuge
available at the time was insufficient to produce the necessary
gravitational force. However, it is possible to deposit these
particles at a lower speed if they are first agglutinated with a
strong solution of potassium chloride (Lehninger, 1951). This
procedure did not give satisfactory yields however, and, in
addition, the tissue preparations became very warm during centri-
fugation. As mitochondria could not be isolated from rat liver,
no attempt was made to apply the technique to guinea-pig tissues.
Instead, the second method was adopted, even though it has obvious
disadvantages.

All the apparatus was chilled in an ice-salt mixture at
between -10 and -20°C. As soon as the tissues were removed from
the animal, they were immersed in cold 0.25M sucrose and cut into
thin slices; after blotting these were spread on cold Petri dishes, which were quickly placed in a cooled vacuum-desiccator. Phosphorus pentoxide was introduced, the vessel sealed and then evacuated to a pressure of less than 0.5 mm. Hg. with a Speedivac pump. This low pressure was maintained for two hours while the vessel stood in the ice-salt mixture. It was then disconnected from the pump and placed in the refrigerator for 24 hours. As a result of this treatment the tissue was found to be thoroughly dried and brittle and easily powdered. The ground-up tissue was kept ice-cold, spread out on Petri dishes and exposed to fresh phosphorus pentoxide at 0.5 mm. Hg. pressure. The final product was stored in the refrigerator over the same dehydrating agent. When suspended in Tyrode it was further disrupted by a homogenizer for two minutes.

Two sets of four tubes, each containing either 80 mg. of lung powder or 70 mg. of heart muscle powder in 10 ml. Tyrode, were incubated at 37°C. The sequence followed in the experiment was as follows:
TABLE 4.

Incubation of desiccated lung and heart tissue.

(P.I. = propamidine isethionate)

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Treatment</th>
<th>Specimen No.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>lung</td>
<td>heart</td>
</tr>
<tr>
<td>1</td>
<td>Incubated for 10 mins.</td>
<td>S1</td>
<td>S11</td>
</tr>
<tr>
<td>2</td>
<td>Incubated for 20 mins.</td>
<td>S2</td>
<td>lost</td>
</tr>
<tr>
<td>3</td>
<td>Incubated for 30 mins.</td>
<td>S3</td>
<td>S12</td>
</tr>
<tr>
<td>4</td>
<td>Incubated for actual period of 30 mins. and 0.1 mg. P.I. added after 10 mins.</td>
<td>S4</td>
<td>S13</td>
</tr>
</tbody>
</table>

At the end of the appropriate period, each tube was centrifuged at 2,500 r.p.m. for three minutes, the supernatant collected and then kept in ice until assayed. The histamine assay was performed on the same day as the incubation. The result of the experiment is given in Fig. 5, which shows that histamine was released in the tube containing propamidine isethionate. Parts of the actual assay are shown in Figs. 6-10.

These results were confirmed with another preparation of dried lung tissue. The same procedure was used as before except that the incubations were continued for an extra ten minutes to see whether longer contact with propamidine would alter the amount
FIG. 5.

Histamine release from desiccated lung and heart muscle powder in the presence of the histamine-liberator propamidine isethionate (solutions S4 and S13). For sequence see Table 4.

Open columns - control tubes
Black columns - tubes containing propamidine isethionate.

FIG. 6.

Incubation of desiccated lung and heart tissue (Table 4). Assay of solution S1.

H - histamine standard \((10^{-7})\); volume in ml. recorded below.
V - effect of 0.3 ml. of S1, diluted 1 in 10.

FIG. 7.

Incubation of desiccated lung and heart tissue (Table 4). Assay of solution S3.

H - \(10^{-7}\) histamine.
\(u - 0.5\) ml. of S3, diluted 1 in 20.
FIG. 8.
Incubation of desiccated lung and heart tissue (Table 4). Part of the assay of solutions S4 (desiccated lung) and S11 (desiccated heart) after propamidine isethionate.

H - histamine solution (10^{-7}); volume in ml. recorded below.
x - effect of 0.5 ml. of solution S4 diluted 1 in 20.
y - effect of 0.5 ml. of solution S11 diluted 1 in 5.

FIG. 9.
Incubation of desiccated lung and heart tissue (Table 4).

H - histamine solution (10^{-7}); volume in ml. recorded below.
W - effect of 0.5 ml. of solution S13 diluted 1 in 5.
(Desiccated heart after addition of propamidine isethionate.)
FIG. 10.

Incubation of desiccated lung and heart tissue (Table 4). Effect of incubation fluids (see Table 3) on guinea-pig ileum before and after treatment with 1:25,000,000 mepyramine maleate.
The results are shown in Fig. 10 and Table 1. In this experiment, the presence of the H liberated was confirmed by the addition of trypsin to the incubation mixture. The results are shown in Fig. 10.

![Fig. 10.](image)

**Incorporation of Histamine Liberated**

<table>
<thead>
<tr>
<th>Time</th>
<th>Sample</th>
<th>B.I. concentration ppm in 300 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero + 4 mins.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero + 8 mins.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero + 12 mins.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero + 15 mins.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero + 18 mins.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sample S3 removed Sample S4 removed Sample S5 removed
of histamine liberated. The results are shown in Fig. 11 and parts of the assay in Figs. 12-14. In this experiment the presence of the H liberator increased the concentration of histamine by 68%, while in the previous test the increase was 45%.

The amount of propamidine used was sufficient to release all the drug-labile histamine in the preparations and this was confirmed by another experiment (see Fig. 15) in which two additions of the drug were made. Dried lung powder (250 mg.) was incubated in 50 ml. of Tyrode solution with the following sequence:

**TABLE 5.**

Incubation of dried lung powder in Tyrode solution.
(P.I. = propamidine isethionate)

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero + 4 mins.</td>
<td>Sample S1 removed.</td>
</tr>
<tr>
<td>Zero + 8 mins.</td>
<td>Sample S2 removed. P.I. added to give final concentration of 100 μg/ml.</td>
</tr>
<tr>
<td>Zero + 12 mins.</td>
<td>Sample S3 removed. P.I. concentration raised to 200 μg/ml.</td>
</tr>
<tr>
<td>Zero + 15 mins.</td>
<td>Sample S4 removed.</td>
</tr>
<tr>
<td>Zero + 18 mins.</td>
<td>Sample S5 removed.</td>
</tr>
</tbody>
</table>
FIG. 11.

Release of histamine from dried lung tissue. Propamidine isethionate added after 10 mins. and remained in contact with the incubate for 30 mins.

Open columns - control tubes.
Black column - tube containing propamidine isethionate.

FIG. 12.

Incubation of dried lung tissue with propamidine isethionate. Assay of incubation fluids.

H - histamine (10^{-7}); volume in ml. indicated.
a - 0.2 ml. of solution S1, diluted 1 in 10.
b - effect of 0.25 ml. of same solution.

(See Fig. 11 for final result.)
**FIG. 13.**
Incubation of dried lung tissue with propamidine isethionate. Assay of solution S2.

H - histamine \((10^{-7})\); volume in ml. indicated.

C - effect of 0.3 ml. of solution S2 diluted 1 in 10.

**FIG. 14.**
Incubation of dried lung tissue with propamidine isethionate. Assay of solutions.

H - histamine \((10^{-7})\); volume in ml. indicated.

d - effect of 0.2 ml. of solution S3 diluted 1 in 10.

e - effect of 0.3 ml. of solution S5 diluted 1 in 20.

(See Fig. 11 for final result.)
FIG. 15.

Incubation of dried guinea-pig lung. Sequence shown in Table 5, at A and B propamidine isethionate was added, its initial concentration being 100 \( \mu g/ml \) and the final one 200 \( \mu g/ml \).

Open columns - control specimens.
Black columns - specimens containing propamidine isethionate.
In another flask an identical amount of lung tissue was incubated as a control from which two samples were removed: S6 after four minutes equilibration and S7 at the end of zero + 18 mins. As can be seen from Fig. 15, the second addition of propamidine did not alter the amount of free histamine already present in the incubation fluid.

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**Fig. 15.**

- pg. histamine per ml.
- Mins.
2. EXPERIMENTS ON PROTEOLYTIC ENZYMES.
2. EXPERIMENTS ON PROTEOLYTIC ENZYMES.

The experiments in which fresh or dried tissue homogenates were incubated with propamidine isethionate showed that this drug was able to liberate histamine in these preparations just as it does in whole organs. The components of the reacting system were therefore still intact and it now seemed justifiable to see whether or not specific enzyme systems were involved in the releasing process. At about this time, the results of the effect of liberators on the skeletal muscles and skin of the cat, rat and guinea-pig were published (Feldberg and Talesnik, 1952), showing that these drugs were particularly active on the skin of the rat, which, for this reason, was used as the source of intracellular enzymes in the initial experiments. The cathepsins were first studied because many authorities regard proteolysis as an essential step in the release of bound histamine (Feldberg, 1941; Silva, 1952; Ungar, 1952).

The enzyme extracts were made as follows: the skin was quickly removed from a stunned rat and cooled in iced water; after cutting into narrow strips it was blotted and passed through a mincer, the macerated residue being collected in cold carbon tetrachloride. The subcutaneous fat was extracted with two separate volumes of this solvent, which was itself removed with several washings of diethyl ether. The tissue was then suspended in an equal volume of 2% KCl in acetate buffer at pH 6 (Snoke,
1950) and allowed to extract in the cold for two hours; enzyme assays were performed on the filtrate, proteolytic activity being measured by Anson's method (Anson, 1939). The substrate, crystalline horse haemoglobin, was prepared by a modification of Keilin's method given in Sumners and Somers (1944).

Preliminary tests carried out at pH 3 to 8 completely failed to show any proteolytic activity between pH 3 and 5. However, as histamine release occurred in the homogenates at pH 7.3, it seemed rather unlikely at first sight that these cathepsins could be involved in this particular reaction. Nevertheless, experiments were continued for two reasons. Firstly, because it was considered that the discrepancy between pH optima for proteolytic activity and histamine release might have been due to the use of an abnormal substrate; secondly, because the effect of the liberators on cathepsins had not been examined previously.

A few additional experiments were performed in the absence of foreign protein substrates to see whether or not the cathepsins would act on the proteins extracted from the cells. In these tests however, no measurable change in tyrosine concentration occurred, either in the controls or in the presence of up to 100 µg/ml. of propamidine isethionate.

Initial experiments showed that more tyrosine was liberated from urea-denatured haemoglobin than from the substrate in the native state. Twenty-five grams of the protein were dissolved in a solution whose final composition was 36% urea and 0.08 N sodium
hydroxide. The mixture was incubated at 37°C for four hours, dialysed against tap water for four days and its volume then adjusted to give a 20% solution of the protein.

In the determination of the pH optima of the skin cathepsins, samples of the solution of denatured haemoglobin were adjusted to give the following range of pH values at 37°C: 3.5, 4.0, 4.5 and 5.0. Samples of enzyme solution were similarly adjusted and a series of 2M acetate buffers of corresponding pH values were also prepared. The actual incubation mixtures were made up in the following way:

- 12 ml. of enzyme extract.
- 1.5 ml. of urea-denatured haemoglobin solution (20% Hb).
- 1.5 ml. of 2M acetate buffer of appropriate pH.

All the components were warmed to 37°C before mixing, the concentrated buffer being added last. At zero time and at 45 minute intervals, 2 ml. of the incubate were placed in 2 ml. of 10% trichloracetic acid and the precipitate spun down. 2 ml. of the supernatant was then used for tyrosine determination using the Folin-Ciocalteu colour reaction. The optical density of the solution was read on the absorptiometer using a spectrum red filter (660 mμ). The result of the experiment is shown in Fig. 16, which indicates that the enzyme showed optimum activity at pH 4.5 approximately.
Other specimens of rat skin cathepsin were incubated in the presence of propamidine isethionate (100 μg/ml.) at the optimum pH. The results of a typical experiment are shown in Fig. 17. Enzyme activity in both flasks was low, and over a period of fifteen minutes the drug had no obvious effects.

The persistent low activity of many preparations suggested that natural activators were missing. Schales and Hill (1949) showed that cathepsins are stimulated by cysteine (20 milli-moles/litre) and the ferrous ion of ferrous ammonium sulphate (0.02M). In these skin preparations both substances, even in low concentration, reacted so strongly with Folin and Ciocalteu's reagent that no satisfactory readings could be made. The development of this intense colour could largely be prevented by adding formaldehyde to the incubate after it was placed in trichloracetic acid; this modification nevertheless failed to show any difference between the preparations with and without activators, whether propamidine was present or not.

An attempt to increase enzymic activity was made by concentrating the extracts from several skins; the solutions were treated with ten times their volume of ice-cold acetone, the protein precipitate spun down and resuspended in 0.2N sodium acetate-acetic acid buffer at pH 4.5. The protein concentration was therefore theoretically increased ten times but its enzyme activity was however extremely low, even in the presence of the two activators. Saturated ammonium sulphate was also used, the
precipitated proteins again being resuspended in acetate buffer which was then dialysed in the cold for 48 hours. The final volume was adjusted to give a ten-fold concentration. Enzymic activity was almost identical with that of the starting material. Hence, although the enzymes were present, manipulations apparently inactivated them to a considerable extent.

Other tissues such as the kidney, liver, spleen, heart and lungs, were now examined as a source of a reasonably active, liberator-sensitive cathepsin. This work had the obvious disadvantage that the pharmacological effect of these drugs was unknown on most of these organs, but as proteolytic activity was so low in the skin it was felt that other tissues should be tested. The kidney, spleen and liver of the rat and guinea-pig turned out to be convenient sources of proteolytic enzymes. The lungs of the latter species were also examined but found to be devoid of activity. Fig. 18 shows the results obtained when the tissues were treated in the following manner: 5 g. of tissue were homogenized in 25 ml. of 0.2N of acetic acid-sodium acetate buffer and extracted in the cold for two hours; connective tissue was spun down and the enzyme containing supernatant (12.5 ml.) was then incubated with 3 ml. of 20% urea-denatured haemoglobin. The cathepsins from all three organs possessed the same pH optimum as that from the skin and similarly showed no change in activity in the presence of up to 100 μg/ml. of propamidine isethionate.
FIG. 16.
Determination of the pH optimum of rat skin cathepsin using urea-denatured haemoglobin as substrate.

FIG. 17.
The activity of rat skin cathepsin with and without histamine liberator.

- enzyme alone.
- enzyme and propamidine isethionate (100 μg.ml.). The arrow shows the time at which the drug was added.

FIG. 18.
Cathepsin activity in rat tissues. Optimum activity at pH 4.5.

1 - kidney
2 - spleen
3 - liver

(See text for reference to A, B and C.)
FIG. 16.

Optical density

2.0
1.0
0.0

1.5
4.0
5.0

20 40 60 80 100 120
Mins.

FIG. 17.

Optical density

0.1
0.05

15 30 45 60
Mins.

FIG. 18

Optical density

-0.6
-0.4
-0.2
-0.0
0.0

20 40 60 80 100 120 140
Mins.
Several months after the previous experiments had been completed, it was found that a proteolytic enzyme having a pH optimum at 7.4 had been isolated from the skin of the rat (Beloff and Peters, 1946–7). For two reasons this seemed to be a particularly useful system on which to test the histamine liberators. Firstly, the incubations would be at normal intracellular pH, and secondly, the source of the enzyme would be a tissue known to be particularly sensitive to histamine-liberators in the intact animal (Feldberg and Talesnik, 1952).

The techniques used in the isolation of the enzyme were practically identical with those in the original paper. The skin on the abdomen and chest of young rats was shaved, quickly dissected off and placed in iced water; after fat and muscle tissue had been removed, the material was weighed and then frozen with gaseous carbon dioxide on the platform of a microtome; sections 40 µ thick were cut and suspended for an hour each in three separate volumes of ice-cold acetone. The tissue was finally dried over phosphorus pentoxide in a vacuum desiccator kept in the refrigerator.

The enzyme was extracted from the dried tissue by suspending it for half an hour at 37°C in 5% potassium chloride containing 0.21% sodium bicarbonate, while 5% carbon dioxide in oxygen was bubbled through the mixture to keep the pH at 7.4. In their original method, Beloff and Peters controlled the pH with a M/20 phosphate buffer. These, however, were unsuitable for this work.
as it has been found that at normal pH values, several powerful histamine liberators react with a variety of phosphates, even when both are in low concentration. Further details of this complication will be given later, but as far as these experiments were concerned, CO₂-bicarbonate buffers were used in preference to barbiturate or borate systems. All solutions contained 0.21% sodium bicarbonate (as in Krebs-Ringer-bicarbonate) and before use they were equilibrated with 5% CO₂ for half an hour at 37°C. The substrate was a solution of casein, prepared as in Beloff and Peters' paper, while proteolytic activity was measured by Peters and Van Slyke's (1932) manometric method for amino nitrogen.

In each determination, 5 ml. of buffered casein was incubated with 5 ml. of the enzyme extract for 1 hour. Five percent carbon dioxide was bubbled through all the time and the reaction was stopped by the addition of 10 ml. of 0.3N trichloracetic acid. The drugs were added to the active enzyme at zero time, and as they contained a high percentage of amino nitrogen, an identical amount was added to the blanks when the initial amino nitrogen values were being determined. Precipitated proteins were removed by centrifugation and 2 ml. of the supernatant used for the manometric estimations which were performed in duplicate.

The results are shown in the following Table:-
TABLE 6.

Incubation of rat skin protease with casein in the presence of histamine liberators.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Increase in amino nitrogen expressed as mg. amino nitrogen/gm.fresh skin/hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Control Expt.1.</td>
<td></td>
</tr>
<tr>
<td>(b) with 100 μg/ml. propamidine isethionate</td>
<td>0.99</td>
</tr>
<tr>
<td>(a) Control Expt.2. (b) 500 μg/ml. Compound 48/80</td>
<td>1.42</td>
</tr>
<tr>
<td>(c) 100 μg/ml. antrycide</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Although only a small number of measurements were made, they show that enzyme activation, if it occurred at all, could not be detected by this technique. The obvious disadvantage was the length of the incubation time, for in the course of an hour, an initially high rate of proteolysis, caused by the drug, might easily decline to normal values; the long incubation period would therefore tend to mask the original high enzymic activity.

The really important factor to be measured is the rate of proteolysis immediately after the enzyme has come in contact with
the drug. After all, it is the explosive nature of histamine release which suggests that this opening phase is of outstanding importance in the release mechanism compared with the long term or chronic effects which the drugs may then exert on other enzyme systems. Shortening the incubation period would, of course, have required the use of highly concentrated enzyme solutions if measurable changes in the amino nitrogen were to be obtained. However, the preparation of such a solution could not be attempted at this time and for this reason further experiments were not continued.
3. EXPERIMENTS WITH ADENOSINE TRIPHOSPHATASE.
3. EXPERIMENTS WITH ADENOSINE TRIPHOSPHATASE.

In the Introduction two possible mechanisms for histamine release were mentioned; firstly, the activation of proteolytic activity, and secondly, an interference with the production and utilisation of molecules containing high energy phosphate bonds. The inconclusive results with the cathepsins resulted in experiments being started to test the truth of the second possibility.

At the moment there is an overwhelming amount of evidence to show that the principal source of energy in all the activities of the body is provided by high energy phosphate bonds. When muscular and secretory work or chemical synthesis is performed, the necessary energy is supplied by the rupture of these high energy phosphate bonds. This cleavage is an essential step in the transfer of their energy from the donor molecule (usually ATP) to the energy-deficient receptor system, and is catalysed by the enzyme adenosine triphosphatase. This enzyme thus occupies an extremely important position in energy metabolism. In fact, in one of the most active mammalian tissues, skeletal muscle, this enzyme is so intimately connected with its energy-consuming activity that it is virtually impossible to separate it from myosin, the contractile protein of the muscles (Engelhardt and Lyubimova, 1939). Now, should histamine release involve an interference with energy utilisation, it seemed
possible that one point of attack might be this enzyme. The effect of propamidine isethionate was therefore tested on this system, whose activity was measured by the method of Potter and Dubois (1943).

Adenosine triphosphate was isolated from rabbit skeletal muscle by Needham's procedure (1942) and stored as the barium salt; when a solution of sodium ATP was required, the barium salt was treated with sodium sulphate by McIlwain's method (1951). In later experiments adenosine triphosphoric acid purchased from Light & Co. was used; sodium bicarbonate (0.2M) was added dropwise to a solution of the nucleotide until its pH, measured with a glass electrode, reached 7.5. Guinea-pig organs such as the lungs, kidney or liver were homogenized in ice-cold distilled water to extract the ATPase, connective tissue was removed and the pH of the suspension adjusted to 7.5. In a typical experiment the following mixture was incubated at 37°C and slowly rocked in the Warburg bath:—

0.02M sodium-ATP 1 ml.
enzyme extract 1 ml.
0.1M Barbiturate buffer pH 7.5 1 ml.
0.02M Calcium chloride 0.5 ml.
water or solution of drug 0.1 ml.

(final concentration of sodium ATP approximately 0.012M)
Three mixtures were used in each determination; the first contained the enzyme, substrate and drug; the second, the enzyme and substrate alone; the third, boiled enzyme, substrate and drug. At regular intervals, usually 2½ to 5 minutes, 1 ml. of the incubate was placed in 5 ml. of ice-cold 8% trichloracetic acid, the protein precipitate removed and the supernatant used for the estimation of the inorganic phosphorus by King's method (1932). The result of a typical experiment is shown in Fig. 19A. From the very start of the incubation one of the flasks contained 80 µg/ml. of propamidine isethionate. As can be seen, there is very little difference between the amount of phosphorus liberated in the presence and absence of the drug. In Fig. 19B the drug was added ten minutes after the incubation commenced, giving a concentration of 5 mg/ml. The incubation mixture was prepared in double volume and then divided before the drug was added, but as can be seen, the concentration of inorganic phosphate in tube 2 (enzyme alone) slightly exceeded that in tube 1 (enzyme + drug). The difference between the two curves in both Figs. 19 A & B is so slight that it is evident that the drug is having hardly any effect.

The mechanism of histamine-release suggested in the Introduction implied that the activity of ATPase would be diminished rather abruptly by liberator drugs. The two experiments just described did not, of course, give any support to this idea and
at first sight appeared to dispose of this hypothesis completely. However, it must be remembered that in these preparations the drug is working under very adverse conditions, even though its concentration is far higher than that needed to cause histamine release in intact tissues. One possible reason for its lack of effect is the presence of an enormous excess of the substrate. Assuming that the drugs act by combining with the active sites of the enzyme, then the chance of such a molecule reaching one of them would be considerably reduced by an excess of ATP. Even in the most active tissues the concentration of adenine nucleotides is extremely low, a state of affairs which probably helps to control the activity of ATP-ase quite apart from the inhibitory effect which ADP has on this enzyme. Hence, tissues in which phosphate esterification occurs at a very low rate would not only have a low concentration of the adenine nucleotides but also a relatively small proportion of them in the form of ATP. This state of affairs would be expected to occur in organs particularly rich in connective tissue stroma such as the lungs, skin and muscles. In these organs it might therefore be possible for a very small amount of the drug to compete successfully with the natural substrate and so completely disrupt enzymic activity.

With this possibility in mind, further tests were performed in which the concentration of sodium-ATP was considerably reduced. For example, 3 g. of guinea-pig lung, dispersed in 150 ml. were used in the following preparation.
The mixture was divided between two flasks and samples removed every five minutes; the drug was added to one of the flasks after withdrawing the ten-minute sample, its final concentration being 700 mg/ml. The absence of any effect even under these conditions is shown in Fig. 20A.

Although these experiments gave negative results, another series of tests were made using massive doses of the drug. An extract of rat skin was first tried but it showed no activity at all, probably because an insufficient number of cells had been ruptured during mincing. Guinea-pig lung was again used as the source of the enzyme and the following fluids prepared:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>prop. isethionate mg/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>all</td>
</tr>
<tr>
<td>2</td>
<td>5 tubes</td>
</tr>
<tr>
<td>3</td>
<td>9 contain</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
</tr>
</tbody>
</table>

Na ATP - 5 ml. 0.117M barbiturate buffer - 1 ml. 0.0217M calcium chloride - 0.5 ml.

The final concentration of Na ATP being 0.008M. The mixtures were warmed for ten minutes, 1.5 ml. of enzyme preparation added and
samples removed at $2\frac{1}{2}$ minute intervals. The results, which show a considerable reduction in enzyme activity, are given in Fig. 20B.

During these experiments with propamidine isethionate and ATP-ase, it was frequently noticed that a faint, white precipitate appeared when the drug was added to the incubation mixture. Although, on shaking, the precipitate often dissolved, it was obviously important to identify the substances responsible for this effect since an unknown proportion of the drug was probably being removed from the solution. Alteration of the pH on mixing could not be the cause of the precipitation since the propamidine solutions were always adjusted before being mixed with the enzyme. Tests were therefore performed on combinations of the various components in the incubation fluid. No reaction occurred between solutions of the drug and those of calcium chloride and sodium barbiturate, but when added to Na-ATP at pH 7.4, a copious, stable, white precipitate appeared. This result might have been due to contaminants in the nucleotide such as other organic and inorganic phosphates. Their activity, and that of pure ATP, was therefore examined as well. The results are shown in Table 7; the weight of the tested substance, which was suspended in 2 ml. of 0.1M barbiturate buffer at pH 7.4, is shown: 1 mg. of propamidine isethionate, also in the buffer, was added and the effect noted.
FIG. 19A.
Effect of propamidine isethionate (30 μg.ml.) on adenosine triphosphatase from guinea-pig kidney. Concentration of Na ATP - 0.012 M.

1 - enzyme + drug.
2 - enzyme alone.
3 - boiled enzyme + drug.

FIG. 19B.
Adenosine triphosphatase activity in guinea-pig lung homogenates. Addition of propamidine isethionate (5 mg.ml.) at arrow. Curve numbering as in A.

FIG. 20A.
Guinea-pig lung adenosine triphosphatase; diluted enzyme. Concentration of Na ATP - 0.008 M. Propamidine isethionate added at the arrow to give 700 μg.ml.

1 - enzyme alone.
2 - enzyme + drug (0.0012 M)

FIG. 20B.
Guinea-pig lung adenosine triphosphatase; enzyme diluted. Na ATP - 0.008 M with the following drug concentrations.

1 enzyme alone.
2 5 mg.ml. propamidine isethionate (0.008 M)
3 9 " " (0.016 M)
4 18 " " (0.032 M)
TABLE 7.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Weight</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium pyrophosphate</td>
<td>100 mg.</td>
<td>precipitate which persists</td>
</tr>
<tr>
<td>disodium hydrogen phosphate</td>
<td>100</td>
<td>precipitate appears; then dissolves</td>
</tr>
<tr>
<td>potassium dihydrogen phosphate</td>
<td>100</td>
<td>no precipitate</td>
</tr>
<tr>
<td>sodium glycerophosphate</td>
<td>100</td>
<td>precipitate which persists</td>
</tr>
<tr>
<td>glucose - 1 - phosphate</td>
<td>75</td>
<td>&quot;    &quot;    &quot;    &quot;    &quot;</td>
</tr>
<tr>
<td>fructose 1-6 diphosphate</td>
<td>40</td>
<td>&quot;    &quot;    &quot;    &quot;    &quot;</td>
</tr>
</tbody>
</table>

This precipitating effect could, of course, have been due to the isethionate part of the drug rather than the histamine liberating propamidine portion, but this possibility was excluded in two ways:

(i) the precipitates were collected, carefully washed and fused with sodium; no sulphur could be detected in the melt.

(ii) other histamine-liberators not possessing an isethionate radicle were examined. Compound 48/30 and antrycide, both, like propamidine, very powerful releasers, also reacted with ATP and the other phosphates. A relatively weak liberator, the alkaloid thebaïne, did not however.

The fact that propamidine isethionate reacts with sodium pyrophosphate was shown also by a pH titration curve. Sodium
pyrophosphate (50 mg.) was dissolved in a final volume of 20 ml. to give an 0.005M solution. This was added, 0.1 mls. at a time, to 10 ml. of distilled water and the pH measured after mixing. The result is shown in Fig. 21, curve A. An identical volume of 0.007M propamidine isethionate was similarly treated (curve B) which shows that a "buffering" action took place during the initial phase of the titration. Eventually the two curves became parallel, showing that this action had ceased. A distinct precipitate appeared by the time 0.4 ml. of pyrophosphate had been added, but the shape of curve B shows that before this became visible, a reaction had occurred between the two substances.

One of the most convenient methods of detecting a chemical reaction is to examine the ultra-violet absorption spectra of the supposed reactants before and after they are brought together. This technique is of greatest value when the regions of absorption maxima in the spectra of the components are widely different; unfortunately those of propamidine isethionate and ATP are practically identical (see Fig. 22). In spite of this complication, the spectra of these two substances were examined before and after they were mixed. All the solutions were centrifuged before and after mixing at 5000 r.p.m. for half an hour to remove any fine precipitates. No indication of a reaction was obtained however, for the optical density of the mixture equalled the sum of the individual components at the same concentration. Increasing their concentrations to such an extent
FIG. 21.

Reaction between propamidine isethionate and sodium pyrophosphate.

A - 10 ml. water which was titrated with 0.005 M sodium pyrophosphate.
B - 10 ml. of 0.0066 M propamidine isethionate which was similarly titrated.

The arrow shows the point at which a distinct precipitate was first seen during the second titration.

FIG. 22.

Ultra-violet absorption spectra of 0.000033 M propamidine isethionate (curve 1) and of 14 μg/ml. of Na ATP (curve 2) to show that their absorption maxima and minima correspond for all practical purposes.
FIG. 21.

\[
\begin{align*}
\text{pH} & \quad 8 \\
7 & \quad 6 \\
6 & \quad 6 \\
5 & \quad 2 \\
4 & \quad 0 \\
\end{align*}
\]

Mls. of Na pyrophosphate.

FIG. 22.

Opt. dens.

200

200
that a precipitate was formed gave solutions of such high optical density that a considerable dilution had to be made before any measurements were possible.

For convenience, the experiments were continued with sodium triphosphate instead of ATP. This substance was chosen as it virtually lacks an absorption spectrum in the ultra-violet yet is so similar to ATP from a physiological point of view that it competitively inhibits ATP-ase in very low concentration (Engelhardt, 1946). By using this reagent it was hoped that the minimum concentration at which a triphosphate would react with a histamine liberator could be determined in vitro. It would then be possible to decide whether or not such a reaction might conceivably occur in vivo when a minute dose of the liberator is administered to an intact animal.

The spectra in Figs. 23A & B and 24A show the effect of increasing the concentration of sodium triphosphate in the presence of a constant amount of propamidine isethionate ($1.6 \times 10^{-5}$M). The gradual removal of the drug from the solution could have been due to two processes: (i) the formation of an insoluble complex with triphosphate, followed by (ii) the adsorption of the soluble drug on to the precipitate once it had been formed. To see whether or not this occurred, the propamidine concentration was increased to $1 \times 10^{-4}$M so that the turbidity of the mixtures came within the range of the Spekker absorptiometer. After making this measurement, each preparation was centrifuged at
Evidence for a reaction between propamidine isethionate and sodium triphosphate. Note the gradual reaction in absorption with an increase in the concentration of the triphosphate.

<table>
<thead>
<tr>
<th>Curve No.</th>
<th>Conc. of propamidine isethionate</th>
<th>Conc. of sodium triphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.000016 M</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>0.0001 M</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>0.0002 M</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>0.0003 M</td>
</tr>
</tbody>
</table>

FIG. 23B.

Shows an enlarged portion of Fig. 23A. By means of a calibration curve (Fig. 24B) it can be shown that 0.0001 M sodium triphosphate reduces the concentration of 0.000016 M propamidine to 0.000013 M.

Addition of sodium triphosphate to propamidine isethionate.

<table>
<thead>
<tr>
<th>Curve No.</th>
<th>Conc. of propamidine isethionate</th>
<th>Conc. of sodium triphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0001 M</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>0.0006 M</td>
</tr>
<tr>
<td>17</td>
<td>&quot;</td>
<td>0.003 M</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0.0025 M. alone.</td>
</tr>
</tbody>
</table>

Calibration of propamidine isethionate at 260 mμ.

<table>
<thead>
<tr>
<th>vol. prop. iseth.</th>
<th>conc. of drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>.5</td>
<td>0.000033 M</td>
</tr>
<tr>
<td>1.0</td>
<td>0.000066 M</td>
</tr>
<tr>
<td>1.5</td>
<td>0.0001 M</td>
</tr>
</tbody>
</table>
5000 r.p.m. and the ultra-violet density of the clear supernatant fluids was determined. The results are shown in Figs. 24C & D.

The similarity of the shape of the two curves indicates that the reduction in ultra-violet density (Fig. 24C) is paralleled by an increase in turbidity (Fig. 24D) of the suspension. Hence more of the insoluble complex is being formed between triphosphate and the drug, yet the latter is not adsorbed to any great extent on to the precipitate. Fig. 25 shows a further point of interest which was not revealed by the turbidimetric results, namely that above a certain critical concentration of the triphosphate, the ultra-violet absorption reading starts to rise, showing that dissociation of the complex is taking place.

The dissociation of the precipitate could not be due to pH changes arising from the use of large amounts of sodium triphosphate. This was assured by keeping the pH of all solutions to the range 7.5 ± 0.2 and checking with a glass electrode. Every preparation contained 0.02M borate buffer (pH 7.6) so as to facilitate subsequent pH adjustments with dilute hydrochloric acid or sodium hydroxide. The removal of the precipitate by centrifugation at 5000 r.p.m. for half an hour noticeably warmed the solutions which were therefore cooled to 20°C before spectrum readings were made.

The reaction just described for propamidine isethionate and sodium triphosphate was also obtained with two other powerful histamine-liberators, Compound 48/80 and antrycide.
Precipitation of propamidine isethionate by sodium tripolyphosphate. An 0.0001 M solution of the drug was treated with the following volumes of sodium tripolyphosphate.

<table>
<thead>
<tr>
<th>Vol. of Na tripolyphosphate</th>
<th>Molarity of Na tripolyphosphate</th>
<th>% of propamidine removed from solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ml.</td>
<td>0.0001 M</td>
<td>13</td>
</tr>
<tr>
<td>0.3</td>
<td>0.0003</td>
<td>37</td>
</tr>
<tr>
<td>0.6</td>
<td>0.0006</td>
<td>69</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0010</td>
<td>75</td>
</tr>
<tr>
<td>1.6</td>
<td>0.0016</td>
<td>83</td>
</tr>
<tr>
<td>3.0</td>
<td>0.0030</td>
<td>79</td>
</tr>
</tbody>
</table>

Turbidimetric measurements on Spekker using propamidine isethionate (0.0001 M) and sodium tripolyphosphate.

<table>
<thead>
<tr>
<th>Vol. of Na tripolyphosphate</th>
<th>final conc. of Na tripolyphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 ml.</td>
<td>0.00003 M</td>
</tr>
<tr>
<td>0.6</td>
<td>0.0006</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0010</td>
</tr>
<tr>
<td>3.0</td>
<td>0.0030</td>
</tr>
</tbody>
</table>

The reaction between propamidine isethionate and sodium tripolyphosphate. Evidence for the disruption of the precipitate when the concentration of the tripolyphosphate rises.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Conc. of propamidine</th>
<th>Conc. of sodium tripolyphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.000015 M</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>0.00025 M</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>0.00033 M</td>
</tr>
</tbody>
</table>

Initially, the addition of sodium tripolyphosphate gradually reduced the absorption readings, but beyond a certain critical concentration, a rise occurred. The rise in curve 7 between 220 mμ and 280 mμ cannot be explained.
Typical results with ultra-violet absorption measurements are shown in Figs. 26 and 27. A weak histamine liberator, thebaine, apparently did not participate in a reaction with either inorganic triphosphate or ATP under these experimental conditions.

The use of ultra-violet absorption measurements showed that, even in low concentration, three powerful histamine-liberators reacted with sodium triphosphate. Evidence was also obtained that these drugs combined with biologically important materials such as adenosine triphosphate. It was decided to see whether this substance could annul or decrease the effect of the liberators if it was mixed with them before they were injected into the circulation of a cat. In addition, there was a possibility that an excess of ATP might modify the responses of the preparation to the remainder of the drug which failed to combine in the initial reaction. Finally, there was a chance that a previous injection of organic or inorganic phosphates might annul the effect of a dose of the liberators. The chlororolosed cat was used for these experiments and the following sequence was followed:

(i) several intravenous injections of histamine were made to ensure that the animal was sufficiently sensitive and gave graded responses of lowered blood pressure with different doses. These were followed by

(ii) an injection of the histamine liberator to show a typical delayed depressor effect; this was followed by
Effect of adding sodium triphosphate to a solution of Compound 48/30 (0.000085 M).

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Ml. of sodium triphosphate</th>
<th>Molarity of sodium triphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.00005</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>0.00015</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>0.0003</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>0.0005</td>
</tr>
<tr>
<td>6</td>
<td>2.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Proportion of Compound 48/30 removed by the addition of sodium triphosphate.

<table>
<thead>
<tr>
<th>Molarity of sodium triphosphate</th>
<th>% of Compound 48/30 removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00005</td>
<td>3</td>
</tr>
<tr>
<td>0.00015</td>
<td>15</td>
</tr>
<tr>
<td>0.0003</td>
<td>25</td>
</tr>
<tr>
<td>0.0005</td>
<td>28</td>
</tr>
<tr>
<td>0.001</td>
<td>30</td>
</tr>
</tbody>
</table>
**FIG. 27A.**
Effect of sodium tripolyphosphate on a solution of antrycide (0.0026 M).

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Ml. of sodium tripolyphosphate</th>
<th>Molarity of sodium tripolyphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.00005</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>0.00015</td>
</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

**FIG. 27B.**
Calibration curve for antrycide.

**FIG. 27C.**
Proportion of antrycide removed from solution by sodium tripolyphosphate.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Ml. of sodium tripolyphosphate</th>
<th>Molarity of sodium tripolyphosphate</th>
<th>% of antrycide removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.00005</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>0.0001</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>0.00015</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>0.4</td>
<td>0.0002</td>
<td>66</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>0.00025</td>
<td>58</td>
</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>0.0004</td>
<td>36</td>
</tr>
<tr>
<td>8</td>
<td>1.2</td>
<td>0.0006</td>
<td>26</td>
</tr>
</tbody>
</table>
(iii) an identical dose of the liberator plus ATP; and then by

(iv) another injection of the liberator to make sure that releasable histamine was still available and that the sensitivity of the preparation had not declined; then

(v) an injection of ATP alone.

In most cases a standard dose of histamine was given between each of these injections as it was found that after some drugs a refractory state developed which lasted for as long as half an hour. Quite frequently, a delay of more than 30 minutes was necessary between (ii) and (iii) as a prolonged state of hypotension made the preparation unresponsive to further injections of depressor drugs.

A typical result obtained with propamidine isethionate is shown in Fig. 28. A stock solution of the liberator drug was adjusted to pH 7.5 with sodium borate and hydrochloric acid on the glass electrode and the final concentration brought to 4 mg/ml. A dose of 0.5 mg/kg. of propamidine was given (Fig. 28, No. 3) and a typical delayed-depressor response obtained. Two more solutions were prepared; one contained 20 mg. of propamidine and the other 14 mg. of ATP, the pH of both being 7.5. After they were mixed, a heavy white precipitate appeared which was removed by centrifugation. The supernatant fluid was collected, the precipitate washed twice with normal saline and the rinsings added to the supernatant whose volume was then adjusted to 5 ml. after its pH had been corrected. Hence, if no
reaction had taken place between the drug and ATP, this solution would have contained 4 mg/ml. of propamidine isethionate and 2.8 mg/ml. of ATP.

This mixture was now injected into the animal so that a theoretical dose of 0.5 mg/kg. of propamidine isethionate was given. The result is shown in Fig. 28, No. 5. There is a complete absence of the delayed depressor effect which usually appears within 20–30 seconds after the intravenous injection of a liberator. In this particular experiment the recording was made for 90 seconds without any response developing, whereas in Fig. 28, No. 3 a depressor effect was obtained within 25 seconds. The records labelled 4 and 6 show that the sensitivity of the preparation towards histamine had not been altered to any great extent during this part of the experiment.

If no reaction had occurred between the liberator and ATP, the cat would have received a dose of 0.35 mg/kg. of ATP. The effect of this quantity of ATP injected alone is shown in Fig. 28, No. 7; the depressor effect was immediate and disappeared rapidly. A second injection of the liberator was given (Fig. 28, No. 9) and a delayed depressor response obtained. This time however, the recovery in blood pressure was much quicker. Whether or not this difference was due to the effect of previous injections of phosphate is not at present clear. The response does show however that releasable histamine was still available and that the results obtained in No. 5 were due to a reaction between the two depressor substances in the mixture.
FIG. 28.
Cat chloralosed; recording from the carotid artery; injections into the femoral vein; time trace 5 seconds.

1  0.3 μg.kg. of histamine
2  0.2 μg.kg. " (≡ H)
3  0.5 mg.kg. propamidine isethionate
4  H
5  propamidine isethionate + ATP
0.5 mg.kg. of propamidine and
0.35 mg.kg. of Na ATP
6  H
7  0.35 mg.kg. Na ATP alone
8  H
9  0.5 mg.kg. propamidine isethionate
10 H
These results show that both propanidide and the same effect is injected into the heart. A dose of ATP will abolish the latter interval to sixty seconds as shown in Fig. 10. An interval of five seconds apparently enables the phosphate to develop the effects (Fig. 11).

There is an indication of two ways. Firstly, so to the heart, and therefore to cause death, its might well have a chronic toxic effect and be responsible for slight lowering of the blood pressure for a short hour.

The reaction occurs to both a drug remaining in the heart. ATP would influence the potentiation of the phosphatase liberator unless...
These results thus indicate that the depressor effect of both propamidine and ATP are abolished when the two are mixed outside the body. Experiments were now performed to see whether the same effect could be obtained when the drugs were separately injected into the circulation. Fig. 29 shows that a 'protective' dose of ATP followed four minutes later by the liberator does not abolish the latter's delayed depressor response. Reducing the interval to sixty-five seconds gave the rather unusual response shown in Fig. 30. An interval of five seconds apparently enabled the phosphate to combine with the drug and annul most of its effects (Fig. 31). However, even by the end of one minute there is an indication of slight depression which probably developed in two ways. Firstly by the traumatic effect of a fine precipitate on the heart, and secondly by the fact that, as previously shown, the reaction between the liberator and phosphate does not go to completion. A very small amount of propamidine would therefore remain free, and although unable to release sufficient histamine to cause acute depression, it might well exert a chronic toxic effect and be responsible for a slight lowering of the blood pressure for a considerable time.

The reaction between Compound 48/80 and sodium triphosphate occurs to such a limited extent that a considerable amount of the drug remains in solution. It thus seemed rather unlikely that ATP would influence the depressor effect of this very powerful liberator unless the doses used were, at the most, only 30%
FIG. 29.
Chloralosed cat; carotid blood pressure.
1 - 1.4 mg.kg. ATP (i.v.) followed by
2 - 0.5 mg.kg. propamidine isethionate
4 minutes later.

FIG. 30.
Chloralosed cat; carotid blood pressure.
1 - 1.4 mg.kg. ATP (i.v.) followed by
2 - 0.5 mg.kg. propamidine isethionate
65 seconds later.

FIG. 31.
Chloralosed cat; carotid blood pressure.
1 - 0.5 mg.kg. propamidine isethionate (i.v.)
followed by 1.4 mg.kg. ATP 5 seconds
later. The depressor effect was
immediate and the blood pressure
recovered within 30 seconds of the
first injection.
2 - Effect of 0.5 mg.kg. propamidine
isethionate alone to show (i) the
delayed-depressor effect which persists
beyond the response obtained in 1 above,
and (ii) that releasable histamine was
still present in the preparation.
Fig. 29.

![Graphs showing ATP and Propamidine effects](chart1.png)

Fig. 30.

![Graphs showing ATP and Propamidine effects](chart2.png)

Fig. 31.

![Graphs showing ATP and Propamidine effects](chart3.png)

It should be noted that the results shown in the graphs were obtained using another preparation, extracts from adult nerve tissue, and that the effects were not as pronounced as in the original experiment. The use of esterified nerve tissue allowed for a more accurate representation of the effects of the different compounds.
greater than the threshold value (which is about 5 mg/kg., see Paton, 1949). Doses above this amount, e.g. 10 mg/kg., gave the results shown in Fig. 32; in (3) a depressor effect with slowing of the heart occurred and the systemic blood pressure remained low for a considerable time; in (5), using more ATP, the immediate depressor effect of this substance was followed by one of delayed depression due to Compound 48/80. However, the drop was considerably smaller than those in (2) and (6) and lasted for a much shorter time. Furthermore, the prolonged depression which these liberators invariably produce was completely absent with this particular solution. Similar results were obtained in other experiments as shown in Fig. 33. Hence, it does seem justifiable to conclude that ATP will annul the histamine-liberator effects of 48/80 to a limited extent, though not so completely as it does those of propamidine isethionate.

Results similar to those with propamidine were obtained by using another powerful liberator, antrycide (4-amino-6-(2'-amino-6'-methylpyrimidyl-4'-amino) quinaldine-1:1'-dimethyl). As in the other experiments the drug and the ATP were mixed and allowed to react outside the body, and the effect of the centrifuged mixture was tested on the cat's blood pressure. The results are shown in Fig. 34.

It should be noticed that in (5) the injection of the antrycide-ATP mixture caused an immediate drop in blood pressure (due to an excess of nucleotide), which was followed by a sharp
FIG. 32.

Chloralosed cat; carotid blood pressure.

Effect of ATP on Compound 48/80; the two substances were mixed in vitro, allowed to react for 30 minutes at 20°C, centrifuged and the clear supernatant fluid injected into the femoral vein.

1 - 0.2 μg.kg. histamine.
2 - 10 μg.kg. Compound 48/80.
3 - 10 μg.kg. Compound 48/80 + 0.1 mg.kg. ATP.
4 - 0.2 μg.kg. histamine.
5 - 10 μg.kg. Compound 48/80 + 0.14 mg.kg. ATP.
6 - 10 μg.kg. Compound 48/80 alone.
Fig. 32.
FIG. 33.

Chloralosed cat; carotid blood pressure.

1 - 0.2 μg/kg. histamine.
2 - 10 μg/kg. Compound 48/80 + 0.05 mg/kg. ATP (Solution L).
3 - 0.2 μg/kg. histamine.
4 - 0.05 mg/kg. ATP alone.
5 - 10 μg/kg. Compound 48/80 alone.
FIG. 34.

Reaction between antrycide and ATP. Chloralosed cat; carotid blood pressure; time trace 10 seconds.

The liberator reacted with ATP in vitro and the centrifuged supernatant was injected into the femoral vein.

1 - 0.2 μg.kg. histamine (≡ H).
2 - 1 mg.kg. antrycide.
3, 4 - H.
5 - antrycide + ATP, 1 mg.kg. antrycide and 0.7 mg.kg. ATP.
6, 7 - H.
8 - 0.7 mg.kg. ATP.
9, 10 - H.
11 - 1 mg.kg. antrycide.
12 - H.
rise in pressure of 0.2 mg/kg. of hycanthine in a normal animal gave the same dose effects that were observed in the case of the self-poisoned animals in which the liberator was given a dose of 0.5 mg/kg. in each instance, the response obtained in this manner was marked by an examination of drugs in the presence of the presamid physiological reaction leaves and, as was to be less diminished these substances in vivo, their physiological actions are considerably reduced.
rise in pressure and a prompt return to normal. A dose of 0.2 mg/kg of histamine seventy seconds afterwards gave a normal type of response (6). This should be compared with (3) in which a similar dose of histamine failed to produce a response five minutes after the antrycide (2), which caused a prolonged state of hypotension.

However, the effects shown in (5) were not the only type of response obtained with the antrycide-ATP mixture. Using the same dose, solutions and animal, subsequent injections gave the results shown in Fig. 35, which can be explained as being due to the self-potententiating effect of several sub-threshold doses of the liberator (McIntosh and Paton, 1949; Paton, 1949). In both cases the initial sharp fall in blood pressure was followed by a short period of hypotension of approximately the same extent, during which completely different responses to histamine were obtained.

These results thus confirm the conclusion reached by an examination of the ultra-violet absorption spectra of these drugs in the presence of sodium triphosphate. A high proportion of the propamidine and antrycide is precipitated and rendered physiologically inert. In the case of Compound 48/80 the reaction leaves a considerable amount of the drug in solution, and, as was to be expected, depressor effects in the cat were less diminished with this material. Hence, when ATP reacts with these substances in vitro, their physiological actions are considerably reduced.
Subsequent responses of the chloralosed cat to the mixture of antrycide and ATP used in Fig. 34. Compare with the effects in (5) of Fig. 34.
Fig. 35.
EXPERIMENTAL SECTION

4. THE CHEMICAL ANALYSIS OF THE COMPLEX FORMED BETWEEN
   PROPAMIDINE AND ADENOSINE TRIPHOSPHATE.
4. THE CHEMICAL ANALYSIS OF THE COMPLEX FORMED BETWEEN PROPAMIDINE AND ADENOSINE TRIPHOSPHATE.

Chemical analysis of precipitate.

The precipitate formed between propamidine isethionate and Na-ATP was collected by centrifugation, washed several times with small volumes of distilled water, then acetone, and finally dried over calcium chloride in a vacuum desiccator. Sodium fusion tests showed that the precipitate contained nitrogen and phosphorus. Sulphur could not be detected however, an indication that the isethionate radical (β-hydroxyethane sulphonate) was apparently not involved in the reaction. The precipitate was therefore formed solely from the histamine-liberating part of the molecule, a point which was uncertain for this particular drug until this analysis was made.

The reactants and the precipitate were quantitatively analysed for nitrogen, phosphorus and ribose. Samples of propamidine isethionate, ATP and the precipitate were decomposed by Kjeldahl's method using Chibnall's catalyst. Nitrogen and phosphorus were then estimated in the residue after it had been suitably diluted. The Kjeldahl nitrogen results were checked by a more reliable gasometric method which was performed by the Microanalytical Laboratory at the Imperial College of Science. Ribose was estimated by Mejbaum's method (Mejbaum, 1939) after control experiments had shown that propamidine did not interfere with the colour reaction.
### TABLE 1.

**Theoretical composition of Reactants**

<table>
<thead>
<tr>
<th></th>
<th>M.W.</th>
<th>%N</th>
<th>%P</th>
<th>%ribosyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propamidine isethionate</td>
<td>564</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Propamidine</td>
<td>312</td>
<td>17.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
<td>507</td>
<td>13.7</td>
<td>18.3</td>
<td>26.4</td>
</tr>
</tbody>
</table>

In fact the following analytical results were obtained as shown in Table 2 for propamidine and Table 3 for ATP.

### TABLE 2.

<table>
<thead>
<tr>
<th>Mg. of drug</th>
<th>Mg. N found</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.5</td>
<td>1.66</td>
<td>10.5</td>
</tr>
<tr>
<td>13.55</td>
<td>1.22</td>
<td>9.0</td>
</tr>
<tr>
<td>9.50</td>
<td>1.12</td>
<td>11.8</td>
</tr>
<tr>
<td>20.9</td>
<td>2.08</td>
<td>9.95</td>
</tr>
<tr>
<td>16.85</td>
<td>1.97</td>
<td>11.6</td>
</tr>
<tr>
<td>21.40</td>
<td>1.99</td>
<td>9.3</td>
</tr>
<tr>
<td>42.90</td>
<td>4.14</td>
<td>9.7</td>
</tr>
</tbody>
</table>

Average % of nitrogen is 10.26.

The gasometric analysis from Imperial College gave 9.7%.
TABLE 3.

Analysis of adenosine triphosphate.

<table>
<thead>
<tr>
<th>Mass of specimen in mg.</th>
<th>mg. N</th>
<th>% N</th>
<th>mg. P</th>
<th>% P</th>
<th>mg. ribose</th>
<th>% ribose</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.6</td>
<td>2.36</td>
<td>13.4</td>
<td>2.90</td>
<td>16.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.2</td>
<td>2.16</td>
<td>11.25</td>
<td>2.80</td>
<td>14.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.15</td>
<td>2.74</td>
<td>10.09</td>
<td>3.30</td>
<td>13.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29.95</td>
<td>3.42</td>
<td>11.4</td>
<td>4.35</td>
<td>14.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.35</td>
<td>2.30</td>
<td>11.9</td>
<td>2.90</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.95</td>
<td>1.87</td>
<td>11</td>
<td>2.20</td>
<td>12.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.50</td>
<td>2.52</td>
<td>13.6</td>
<td>2.80</td>
<td>15.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.55</td>
<td>2.22</td>
<td>13.4</td>
<td>2.70</td>
<td>16.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.72</td>
<td>31.3</td>
</tr>
<tr>
<td>10.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.10</td>
<td>30.1</td>
</tr>
</tbody>
</table>

Average results from above gave:

- N: 12%
- P: 14.8%
- Ribose: 30.7%

Gasometric analysis gave 12.25% nitrogen.

It is worth noting that the gasometric nitrogen result agrees very closely with the Kjeldahl determinations on both propamidine and ATP.

Using the same methods the precipitate was now analysed, the results being tabulated in Table 4.
Table 4.

Analysis of complex between propamidine and ATP.

<table>
<thead>
<tr>
<th>Mass of precipitate in mg.</th>
<th>mg. N</th>
<th>% N</th>
<th>mg. P</th>
<th>% P</th>
<th>µg. ribose</th>
<th>% ribose</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.05</td>
<td>1.09</td>
<td>13.6</td>
<td>0.64</td>
<td>7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60.10</td>
<td>8.73</td>
<td>14.5</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.05</td>
<td>1.86</td>
<td>15.4</td>
<td>0.86</td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.55</td>
<td>1.63</td>
<td>14.1</td>
<td>1.04</td>
<td>9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.55</td>
<td>1.15</td>
<td>12.1</td>
<td>0.50</td>
<td>5.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.55</td>
<td>1.25</td>
<td>11.8</td>
<td>0.61</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.2</td>
<td>-</td>
<td>-</td>
<td>0.82</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.2</td>
<td>1.28</td>
<td>10.5</td>
<td>0.61</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36.3</td>
<td>5.2</td>
<td>14.2</td>
<td>2.60</td>
<td>7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31.3</td>
<td>4.5</td>
<td>14.2</td>
<td>2.30</td>
<td>7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>560</td>
<td>10</td>
</tr>
<tr>
<td>21.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2000</td>
<td>9.5</td>
</tr>
<tr>
<td>31.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3035</td>
<td>9.7</td>
</tr>
</tbody>
</table>

The final average result from the above table gave 13.4% N, 6.7% P, and 9.7% ribose.

The gasometric analysis gave 14.3% nitrogen.

The molecular composition of the precipitate was determined by comparing this analysis with the theoretical values obtained by calculating the percentage composition of various molecular complexes of propamidine and ATP. Table 5 shows the values obtained for these different complexes.
TABLE 5.

Composition of theoretical complexes between the histamine liberator and ATP.

P = 1 molecule of propamidine.

PI = 1 molecule of propamidine isethioninate.

ATP = 1 molecule of adenosine triphosphate.

<table>
<thead>
<tr>
<th>Molecular proportion in complex</th>
<th>M.W.</th>
<th>% N</th>
<th>% P</th>
<th>% ribosyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P + 1ATP</td>
<td>819</td>
<td>15.4</td>
<td>11.4</td>
<td>16.4</td>
</tr>
<tr>
<td>1P + 2ATP</td>
<td>1326</td>
<td>14.8</td>
<td>14</td>
<td>20.2</td>
</tr>
</tbody>
</table>

Hence increasing the number of ATP molecules simply elevates the P and ribose values while those for N drop.

| 2P + 1ATP                     | 1131 | 16.1 | 8.2  | 11.9      |
| 3P + 1ATP                     | 1443 | 16.5 | 6.4  | 9.3       |
| 4P + 1ATP                     | 1755 | 16.8 | 5.3  | 7.6       |

More propamidine molecules would obviously lower the P and ribose values and elevate those of nitrogen. Using propamidine isethionate gave no better values:

| 1PI + 1ATP                    | 1071 | 11.8 | 8.7  | 12.5      |
| 2PI + 1ATP                    | 1635 | 11.1 | 5.7  | 8.2       |
| 3PI + 1ATP                    | 2199 | 10.3 | 4.2  | 6.1       |

There was the possibility that the reaction between ATP and the liberator decomposed the nucleotide to ADP or AMP and that these substances reacted with the drug to form a precipitate.

Table 6 shows the analytical results which would be obtained under these circumstances.
TABLE 6.
Composition of theoretical complexes between histamine liberators and ADP and AMP.

<table>
<thead>
<tr>
<th>Molecular proportion in complex</th>
<th>M.W.</th>
<th>% N</th>
<th>% P</th>
<th>% ribosyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P + 1ADP</td>
<td>739</td>
<td>17</td>
<td>8.4</td>
<td>18.3</td>
</tr>
<tr>
<td>2P + 1ADP</td>
<td>1051</td>
<td>17.3</td>
<td>5.9</td>
<td>12.8</td>
</tr>
</tbody>
</table>

and so obviously no use to continue the series.

| 1P1 + 1ADP                     | 991  | 12.7 | 6.3  | 13.5      |
| 2P1 + 1ADP                     | 1555 | 11.7 | 4.0  | 8.6       |
| 1P + 1AMP                      | 659  | 19.1 | 4.7  | 20.4      |
| 2P + 1AMP                      | 971  | 18.8 | 3.2  | 13.8      |
| 1P1 + 1AMP                     | 911  | 13.9 | 3.4  | 14.7      |
| 2P1 + 1AMP                     | 1475 | 12.4 | 2.1  | 9.1       |

The values which are fairly close to those of the analysis are underlined. It has been previously mentioned that no sulphur could be detected in the precipitate. Hence, the molecular ratios involving propamidine isethionate may be neglected, leaving the only alternative structure for the precipitate as a compound consisting of 3 molecules of propamidine linked to one of ATP. The average phosphorus and ribose values are in very close agreement but the nitrogen values by both the Kjeldahl and gasometric methods are low. It is of course possible that this discrepancy is due to the partial decomposition of the amidine radicals of
the drug when it reacts with ATP. This would lower the % of nitrogen in the final complex without altering the relative values of the other constituents to any great extent. The results suggest that the propamidine molecules are united to ATP via the latter's phosphate group. This supposition could be checked by an analysis of the complexes formed between this drug and ADP and AMP.
DISCUSSION
DISCUSSION.

The finding that three powerful histamine liberators react with organic phosphates when all the reactants are in very low concentrations suggests a new explanation to account for the physiological action of these liberators and for the processes occurring in anaphylaxis.

The mechanisms of histamine release which have been proposed up to now may be divided into two groups: (a) the displacement of histamine by structurally related bases; (b) the activation of intracellular or plasma proteolytic enzyme systems. In the "displacement" theory the union between histamine and the polypeptide chain is regarded as being so weak and unspecific that the histamine molecule can be replaced by another type of basic molecule. In a mechanism of this sort one would expect to find that one molecule of the liberator would eject only one of histamine. In fact however, some powerful liberators, such as Compound 48/80 and propanididine, are capable of displacing more than 100 molecules of histamine (McIntosh and Paton, 1949). In addition, if the basic nature of both histamine and the liberator is such an essential feature for the release, it is difficult to see how such a property could also be responsible for liberating acidic molecules like heparin or amphoteric substances such as the polypeptides, which also appear after the administration of the liberators. This displacement mechanism is therefore too
non-specific to account adequately for the action of these drugs, which are effective in such low concentrations and must act, presumably, in a very precise manner. The complications involved in the second mechanism, proteolytic activation, have been discussed already in the Introduction (p. 39).

An alternative view of histamine release, which has also been presented (p. 39), is that it is associated with a disruption in energy metabolism within the sensitive tissue and that this disruption involves an intracellular mechanism which will be called the "histamine pump". It is now well established that the "large granules" (mitochondria) of many cells contain a considerable amount of histamine. The localization of this substance on these particles is particularly interesting when it is remembered that 80-90% of the aerobic respiration of the cell occurs in the mitochondria together with the associated process of oxidative phosphorylation. The presence of 60-70% of the tissue histamine on structural units, which also contain these highly active metabolic centres, can hardly be fortuitous and it does suggest that the accumulation of histamine, its maintenance in a non-toxic state and its intracellular function must all be connected with oxidative phosphorylation or some other aspect of mitochondrial metabolism.

Mitochondrial histamine does not appear to be combined in any way for it can be released by suspending the particles in a hypotonic solution (Hagen, 1954). Hence, within these

† Direct evidence for the existence of the "pump" is incomplete; it is based largely on the observations mentioned in the Introduction.
cytoplasmic granules, an extremely high local concentration of uncombined histamine is maintained in a physiologically non-toxic form by processes which are at the moment unknown. It seems rather unlikely that mitochondrial membranes are structurally impermeable to a substance like histamine, which readily diffuses through most tissues and is able to pass many structural barriers in the process. Furthermore, if the mitochondrial membrane were not permeable, then histamine release could occur only after the granule had undergone lysis. Such a change could be revealed histologically, whereas, in fact, the only cells which do show intracellular alterations during histamine release are the mast cells whose granules, probably modified mitochondria, undergo disruption (Riley and West, 1953). All other tissues containing histamine in excess of that of their mast cell population do not show any ordinary histological change after treatment with liberators or after an anaphylactic shock.

The maintenance of the steep concentration gradient between the intra-mitochondrial histamine and the intracellular fluid would depend on the activity of the "histamine pump". This mechanism would be analogous to other active transfer enzyme systems which maintain a concentration gradient across membranes. All these processes depend on the availability of energy-rich phosphate bonds supplied by ATP, which in turn are largely provided by the aerobic metabolism of intermediates in the tricarboxylic acid cycle.
FIG. 36.

SCHEME FOR THE HISTAMINE PUMP.

<table>
<thead>
<tr>
<th>INTRACELLULAR FLUID</th>
<th>MITOCHONDRIAL MEMBRANE</th>
<th>INTRA-MITOCHONDRIAL COMPARTMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CELL MEMBRANE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HISTIDINE</td>
<td>HISTAMINE</td>
<td>HISTAMINE</td>
</tr>
<tr>
<td>DECARBOXYLASE (intracellular)</td>
<td>ENZYME A</td>
<td>ENZYMES B, C</td>
</tr>
<tr>
<td>HISTIDINE</td>
<td>ENZYME D</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>TRICARB CYCLE</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DIFFUSION EFFECT</td>
<td></td>
</tr>
</tbody>
</table>
An advantage of this concept of a "histamine pump" mechanism is that one can thereby account for the following features of histamine release: (i) the explosive nature of the release, (ii) the action of the histamine liberators which react with phosphates, (iii) the histamine-liberating properties of some antihistamines (Arunlakshana, 1953), and (iv) the appearance of polypeptides after the injection of histamine liberators or after the development of anaphylactic shock.

A general scheme for the working of the pump is shown in Fig. 36 opposite, in which A, B and C represent individual enzymes present in the mitochondrial membrane and required for the pump mechanism. D is an enzyme or carrier which transfers the energy of the phosphate bond of ATP to the pump. Histamine within the mitochondrial membrane is presumed to exist as a phosphate ester; this is meant to imply that in this state histamine is physiologically non-toxic but chemically very highly reactive. In this form it will pass either to enzymes B and C to be secreted into the particle, or else diffuse into the intracellular fluid to reach and combine with other cell proteins. It is of course quite possible that other types of complex, e.g. a thio-ester, is responsible for the chemical activation of histamine. Whatever its form however, this substance would have to be capable of reacting with the proteins and phosphatides of the cell membrane to account for the presence of bound histamine in extra-mitochondrial regions.
There is a possibility that histamine may react with acetyl-coenzyme A. Stadtman and White (1953) have found that in some bacteria imidazole reacts with acetyl-CoA to yield N-acetyl imidazole. This substance is remarkable in having an exceptionally high heat of hydrolysis (−16,000 to −18,000 cal.s.) which implies that it could function as an extremely potent acetylating agent. Functioning in this role, it would be possible for the imidazole radical to be linked to a polypeptide once it had lost its acetyl group. Similarly, a chemically reactive form of histamine would also be able to link itself onto the structural proteins and phosphatides of the cell membrane and thus account for its distribution between two distinct cellular localities, i.e. the cell membrane and the mitochondria.

The "histamine pump" mechanism will now be used to explain the phenomena listed above.

(a) The explosive nature of histamine release; this has already been discussed in the Introduction (p.38-9) and depends on the abrupt inhibition of a secretory process which normally maintains a concentration gradient between intra-mitochondrial and intra-cellular histamine.

(b) The action of the histamine liberators: this may be accounted for by the "histamine pump" as follows. The pump consists of several enzymes, some of which introduce ATP into the mechanism to provide the necessary energy while other enzymes handle the histamine. On account of their basic properties, drugs such as
propamidine, Compound 48/80 and antrycide will be taken into the histamine pump which they will then disorganise by preventing ATP from transferring its energy. The very low concentration at which these liberators cause histamine release shows that their mode of action is highly specific. In vitro, their reaction with ATP occurs when the latter is in solution. Now, although the tissues contain a considerable amount of both ATP and ADP, there is evidence that these nucleotides are bound to the proteins and not dispersed throughout the intracellular fluid (Perry, 1954). Hence, when the liberators are injected, they will not be exposed to a large pool of organic phosphates which would tend to cushion their effects; instead, they will accumulate in highly specific localities within the cell where they will then be brought into contact with localized high concentration sites of the nucleotides.

It is most unlikely that all the mitochondria within a cell are concerned with storing histamine; it is far more probable that this function is performed by only a relatively few particles. Recent studies on the centrifugation of mitochondria show that they vary in size and possess different densities (Kuff and Schneider, 1954). Under intense gravitational fields, these particles will therefore be deposited in distinct layers whose sequence will depend on the sedimentation constants of the different granules. In this way it is possible to isolate different types of mitochondria and to examine their biochemical activity. The results show that some enzymes are more highly
concentrated on some particles than on others and so we must conclude that the mitochondrial fraction of granules is biochemically heterogenous. Similarly, we may conclude that when the histamine liberators have entered the cells of the sensitive tissues, they will be active on only a fraction of all the available mitochondria present.

The experimental results show that the reaction between the histamine-liberators and phosphates can occur at a very low concentration. In the case of propamidine, the end-product is an insoluble complex having three molecules of the drug linked to one of ATP. It is most likely that the reaction proceeds in several stages in which first one molecule and then two molecules of the liberator combine with the nucleotide. Should these two intermediate complexes be water soluble, then their formation would not be revealed by the methods used. The combination of only one molecule of the drug with ATP might suffice to render the nucleotide quite inactive as far as the enzymic splitting and utilisation of its high-energy phosphate bond is concerned. Thus, these drugs would be physiologically active at much lower concentrations than the ultra-violet measurements imply, simply because the depression of the U/V spectra depends on the appearance of a water insoluble complex containing three molecules of the drug combined to one of the phosphate.

Some histamine liberators have been found to have a slight stimulating effect on tissue respiration when this is measured
in a phosphate-free buffer. In these initial experiments a rather
toxic buffer system (sodium borate) was used, but it is intended
to repeat the work in a bicarbonate-CO$_2$ system. This respiratory
effect is similar to that of "uncoupling agents" which dissociate
oxidation in the Krebs cycle from the esterification of inorganic
phosphate and the formation of high energy phosphate bonds. This
disruption has two principal results; firstly the concentration
of ATP diminishes, and secondly the respiratory rate rises. It
appears that the esterification of inorganic phosphate acts as a
brake on aerobic oxidation in the mitochondria. When the link
between the two processes is broken, the oxidative mechanism then
tries to function at its maximal rate.

Hence, there may be an indication here that some histamine
liberators are "uncoupling agents" of a very special kind.
Dinitrophenol and gramicidin, two powerful uncoupling compounds,
do not release histamine. However, the action of the liberators
may consist of blocking the utilization of organic phosphates in
the "histamine pump" rather than in preventing the esterification
of phosphate molecules.

(c) The histamine-liberating properties of some antihistamines
in high concentration: the action of an antihistaminic depends on
its ability to compete with histamine for the receptors on smooth
muscle fibres and other effector cells. An important property of
a successful competitor is that it must be so strongly bound to the
receptor that histamine is unable to dislodge it. This property
FIG. 37

H-C≡C-CH₂-CH₂-NH₂

H₂ C — C — H₂ 
(CH₃)₂N
Benadryl

H₂ C — C — N — N(CH₃)₂
Anthergan

H₂ C — C — N — (CH₃)₂
Phenergan

H₂ C — C — CH₃

H₂ C — C — CH₃

Mepyramine

Thymoxethyldimethylamine.
depends largely on a close similarity between the chemical structure of the antihistaminic and that of histamine. Powerful antihistamines such as thymoxethyldimethylamine, Antergan, Phenergan, Benadryl and Mepyramine are all structurally related to histamine (Fig. 37) (Wooley, 1952). At relatively low concentrations, these drugs compete against histamine for its receptors on the cell surface. In high concentrations however, these same substances will liberate histamine from the cell.

In terms of the "histamine pump", this action can be explained as follows. The pump will consist of several enzymes, some for utilising ATP and others for handling histamine. The latter will possess active-sites having a configuration best suited for the formation of an enzyme-histamine reversible link. When however a high concentration of the antihistamines are given, these drugs, instead of keeping to the surface of the cell, will penetrate into the intracellular fluid and then come in contact with the enzymes of the "histamine pump". The antihistamines will preferentially adhere to the histamine-enzyme sites in an irreversible manner, just as they do on the extracellular receptors. As a result, the action of the pump will be arrested and the intra-mitochondrial histamine will spill out into the cells and extracellular fluid.

Thus, the action of the pump may be abolished in one of two ways: either by interference with the energy supply to the system (block between enzymes A and D), or else by inactivation of the
histamine-carrying group of enzymes (block between enzymes A and B or B and C).

(d) The release of polypeptides by histamine liberators and their appearance in anaphylaxis: So far it has been assumed that the only histamine in a tissue is that contained within the mitochondria. There is evidence however that other structural units such as the cell membrane also possess some histamine. The reason for this view is that histamine release may occur when a sensitized organ is perfused with its antigen. The response of a sensitized uterus to its specific antigen is almost immediate and the release of histamine occurs within a fraction of a second, even when the antigen belongs to the globulin class of proteins whose molecular weights may be as high as 2,000,000. Taking into account the size of the antigen molecule and the promptness of the response, it is most unlikely that such a molecule could reach the inside of the cell within a fraction of a second. It is remarkable enough that it can even penetrate the vascular bed and reach the cell surface in such a short time. For these reasons it must be assumed that large antigen molecules interfere with a metabolic process on the cell membrane. The union of the antigen and antibody could cause a considerable alteration in the spatial configuration of the antibody, a change which could mask enzymic sites on the protein and so reduce or abolish its normal metabolic activity or prevent it from interacting with other enzyme systems. Should the stability of the histamine-protein link depend on
metabolic processes involving the use of high-energy phosphate bonds, it can be seen that after the antigen-antibody reaction this part of the cell surface would release histamine which would stimulate the smooth muscle in the tissue.

Hence, in anaphylaxis, the metabolic processes taking place at the cell surface will be disrupted whereas the histamine-liberators will disorganise the metabolic processes of the mitochondria. It is frequently stated that the chemical reactions within a cell are all held in a delicate state of equilibrium so that marked changes in the activity of one group of enzymes will have repercussions on related chemical processes. The appearance of various polypeptides during anaphylaxis or following the effect of liberators suggests that protein synthesis and the formation of peptide bonds is altered under these conditions.

Up to now it has not been possible to synthesize a protein in tissue extracts in vitro, but the formation of peptide bonds in substances like glutathione has been shown to depend on energy from donors such as ATP (see Introduction p.31). Many authorities therefore conclude that protein and polypeptide synthesis is vivo is also ATP-dependent. It is suggested that the disruption in metabolism which leads to the release of histamine will affect other processes within the cell, including that of peptide bond formation and protein synthesis. As a result, polypeptides will tend to be dissociated from their sites of formation and will then pass into the extracellular fluid.
The scheme for the "histamine pump" shows that once the histamine is concentrated within the cytoplasmic granules, it tends to diffuse out again and so has to be 're-secreted'. At the moment, the normal functions of histamine are largely unknown. It is an important factor in the protective mechanisms of inflammation but the only physiological function which has been ascribed to histamine is its stimulation of gastric acid secretion. Code, in fact regards it as the gastric secretory hormone (Code, 1955).

The principle results of the effects of histamine are (i) stimulation of the contraction of smooth muscle fibres, (ii) the secretion of an acid gastric juice, (iii) stimulation of the secretion of mucous, and (iv) an increase in cell permeability. All these effects can be obtained with very low concentrations of histamine. It seems reasonable to suppose therefore that these results may be regarded as the normal, non-toxic actions of this substance. It is only when histamine appears in relatively excessive amounts that a pathological condition develops.

All the four actions of histamine make the effector cells perform work, either mechanical (muscle shortening) or chemical (separation and concentration of ions, mucous secretion or changes in permeability). The rise in the oxygen consumption of histamine stimulated tissues shows that these processes are closely connected with the oxidative cycles of metabolism. The fact (a) that histamine in very low concentrations can stimulate these active processes, and (b) that it is concentrated at metabolically
active sites, i.e. the mitochondria and cell membranes, implies that histamine is intimately connected either directly with the aerobic process or indirectly with the distribution of energy from the tricarboxylic acid cycle to different metabolic processes. The precise function is of course unknown but one possibility is that histamine influences the overall aerobic process by alteration of the permeability of the mitochondria themselves. This would control the entry of substrates into these granules and therefore regulate the rate at which energy donors (ATP) could leave. Its diffusion from the granules would depend on the ratio of the concentration of histamine within the mitochondria to that in the intracellular fluid. The value of this ratio would in turn depend on the availability of ATP and the proportions used for essential but different metabolic processes.

The constant turnover of histamine may seem a wasteful process but it is analogous to the way in which the tissue cells deal with other substances including water. It is usually assumed that tissue cells are in osmotic equilibrium with the extracellular fluid. This condition appears to be exceptional however for it has been found that many cells behave as though their osmotic pressures are two to three times that of the plasma (Bartley, Davies and Krebs, 1954). The use of isotopes has shown that tissue slices are constantly pumping out water by active processes and in this way maintain a concentration of electrolytes which give an osmotic pressure far greater than that of the extra-
cellular fluid. This constant "secretion" of water depends on the availability of the substrates of the Krebs cycle. Thus the way in which cells deal with water is analogous to the mechanism suggested for the mitochondrial metabolism of histamine.

The work of Schayer (1952) provides some data both for and against this idea of a "histamine pump". Subcutaneous injections of $^{14}C$ labelled histamine ($0.05 \mu g/g.$) were given to guinea-pigs whose tissues were then examined four hours later; after this interval, no radioactive histamine could be detected in the blood, lungs, kidney or intestines, whereas practically all the dose was recovered in the urine. These results were then compared with the effect of subcutaneous injections of $^{14}C$ labelled histidine ($2 \mu g/g.$). This time, radioactive histamine was detected in the tissues for as long as 15 days and only a relatively small proportion appeared in the urine. Schayer concludes that the histamine bound to the tissues originates from the decarboxylation of histidine and not from circulating exogenous histamine. Some tissues, e.g. the lungs, kidney and intestine, apparently contain a binding mechanism which enables them to retain endogenous histamine for a considerable period of time.

These results support the "pump" theory to the extent that the existence of a "histamine-binding" mechanism is suggested, but they oppose it by showing that exogenous histamine is not taken up by the pump. As histamine is so diffusible, the labelled material must certainly have reached the inside of the tissue
cells but the failure to retain it may be accounted for by the possibility that the mitochondria were already carrying their full load of histamine and could not hold any more. This situation is rather unlikely however, since the histamine derived from histidine was kept in the tissues for a considerable time.

There are other possible explanations for these facts however. One is that tissue histamine originates from precursors such as histidine and that the enzyme, histidine decarboxylase, is so closely associated with the mitochondrial "pump" system that before histamine can be secreted it must first be transferred from the decarboxylase enzyme, which thus acts as a highly specific donor to the pump. Another possibility is that the rate at which histamine diffuses from the mitochondria and the rate at which it is recaptured and pumped back is so high that exogenous histamine cannot enter the mechanism. A third suggestion is that before being secreted, the histamine molecule must either be fixed to a suitable "donor" as suggested above or else that it must be chemically activated in some way. This process would appear to be unavailable to exogenous histamine.

Schayer's results show that the turnover of histamine is very slow and that in the body the half-life of bound histamine is approximately 50 days. Feldberg and Talesnik (1953) also found that depleted tissues, for example the skin of dogs or of rats, regained histamine at a very slow rate, which approximates to Schayer's values.
FIG. 38. POSSIBLE PRECURSORS of TISSUE HISTAMINE.

- HEXOSE MONOPHOSPHATE
- OXIDATIVE PROCESS
- RIBOSE
  - C fragment of
  - IMIDAZOLE GLYCERYL PHOSPHATE
  - HISTIDINE
  - HISTIDINE DECARBOXYLASE
  - ENZYME CARRIER of HISTAMINE
- NUCLEIC ACIDS
- URIC ACID
  - URICASE
  - IMIDAZOLIDONE DERIVS.
- MITOCHONDRIAL HISTAMINE PUMP
The origin of histamine, apart from its production from the amino acid histidine, is largely unknown. Other substances may well be precursors - for instance, Canellakis and Cohen (1955) have shown that uricase forms imidazole derivatives (5-ureido-2-imidazolidone-4,5 diol, 4 carboxylic acid) from uric acid, while Ames and Mitchell (1955) have found that imidazole-glyceryl phosphate is a precursor of histidine in Neurospora and that in turn the carbon atoms of the imidazole-glyceryl base arise from sugars such as ribose. The origin of histamine may well depend therefore on several metabolic reactions which transfer their end products to the histamine pump via enzymic carriers. Under these circumstances, the free exogenous histamine within the cell will neither be secreted into the mitochondria nor bound on to the tissue protein (Fig. 38).

Zeller (1951) has suggested that the shock which follows an injection of various snake venoms may be due to exhaustion of the animal's usual sources of energy. Many venoms contain ophio-adenosine-triphosphatase, together with other enzymes, which either destroy intermediates of energy metabolism such as hexose phosphate, or else attack essential coenzyme phosphates such as CoE 1 and 2. The formation of high-energy phosphate bonds is therefore blocked at several stages of the metabolic chain and so their concentration rapidly diminishes.

Further evidence for a connection between histamine and energy metabolism is provided by measurements of the concentration
which histamine reaches in the skin of animals with certain diseases. In rats with over-active thyroids, skin histamine concentration rises from 11 to 22 \( \mu g/g \) (Goltz and Dragstedt, 1940), while in humans with advanced cancer of the breast, subnormal skin histamine values are obtained (Feldberg and Loesser, 1954). The results from hyperthyroid animals are of interest when it is remembered that energy is increased in this condition and that its utilisation is highly inefficient. One effect of this abundance of energy might be that a considerable proportion of the available energy-donors are diverted into the secretion of histamine into the mitochondria. In the case of cancer, energy is liberated largely via an anaerobic mechanism which is considerably slower and less efficient in forming high-energy phosphate bonds when compared with the Krebs cycle. A restriction in the availability of ATP or a change in the direction in which it is utilized would thus reduce the action of the "pump" considerably.

Recently, it has been shown that various organic phosphates (cetylphosphate and cetyl methyl phosphate) form stable complexes with a variety of amines at water-chloroform interfaces (Hirt and Berchtold, 1954). Complex formation is particularly marked with histamine for it undergoes this reaction at very low concentrations \( (5 \times 10^{-6} \text{ M. histamine } \text{2HCl}) \). A further remarkable point is that the combination between histamine and these organic phosphates can be blocked by Neo-antergan in a concen-
tration of $10^{-5}$ M. These facts suggest that (a) Neo antergen is capable of blocking the "pump" and hence cause histamine release by antagonising the reaction between histamine and the enzymes "A" and "D", and (b) that the receptors on smooth muscles and other cells which respond to histamine involve a phosphate grouping.

The last suggestion is reinforced by the finding that histamine is taken up by phosphatides such as lecithin and cephalin (Lindahl, 1955). It is not certain whether a true histamine-phosphatide molecular compound is formed for there is a possibility that the phenomenon may be due to adsorption. However, as phosphatides are important constituents of all tissues, these results may be an indication of how histamine is held on some tissue components.

**Further experimental work.**

It has already been mentioned that some initial experiments showed that histamine-liberators slightly increase the oxygen consumption of guinea-pig tissues. This work was performed in borate buffers to avoid inactivation of the drugs which would have occurred if a phosphate buffer had been employed. As borate is rather a toxic substance, it is intended to repeat these experiments in a bicarbonate-$\text{CO}_2$ system, using normal and sensitized tissue with liberators and specific antigens. Should a change in oxygen consumption be detected again, it will then
be necessary to (a) eliminate the respiratory effect due to the
spasm of smooth muscle fibres following the release of histamine,
(b) to find the point at which the respiratory process is
affected, and (c) to see whether or not there is a change in the
rate at which oxidative phosphorylation occurs.

Other experiments, which might show whether or not histamine
is actively transferred to the mitochondria, would involve the
following sequence:— (a) organs, such as guinea-pig lungs,
would be perfused and part of their histamine released with
liberators or antigens; (b) the mitochondria of these tissues
would then be isolated and attempts made to see whether histamine
secretion could occur under various metabolic conditions. If
the presence of a pump was revealed, then the effects of drugs
(librators and antihistamines) could be determined and the
changes occurring in anaphylaxis would be open to examination.

Another aspect of histamine metabolism which would be worth
examining is the mechanism by which it is bound on proteins
during its transference through the pump by Enzymes A, B and C.
As already mentioned, it has been shown (a) that histamine is
readily attached to lecithin and cephalin either by a chemical
bond or by adsorption, (b) that histamine reacts with long-chain
phosphates at chloroform-water interfaces, and (c) that N—acetil
imidazole is a potent acetylated agent. A recent paper by
Burnett and Kennedy (1955) shows that rat liver mitochondria
contain an enzyme which transfers the phosphate of ATP to the
Fig. 39
Suggested mechanism by which histamine combines with cell receptors and with enzymes in the "pump".

Enzyme or receptor protein

\[
\begin{align*}
&\text{CO—CH—NH—CO—CH—NH} \\
&\text{CH}_2 \\
&\text{O} \\
&\text{CO} \\
&\text{CH}_3
\end{align*}
\]

+ \[
\begin{align*}
&\text{CH}—\text{C—CH}_2—\text{CH}_2—\text{NH}_2 \\
&\text{CH}_3\text{CO—N—CH}
\end{align*}
\]

\[
\begin{align*}
&\text{CO—CH—NH—CO—CH—NH} \\
&\text{CH}_2 \\
&\text{O} \\
&\text{CO} \\
&\text{CH}_3
\end{align*}
\]

+ inorganic Phosphate.
serine residues of casein. There is, admittedly, some doubt regarding the exact structure of the phospho-serine complex for it may be a simple or a di-ester. Nevertheless, these results do suggest the possibility that phosphate groups exist on some protein molecules, a fact which may be of some significance in the mechanism by which histamine combines with Enzymes A, B and C and with its receptors on effector cells, as shown in Fig. 39 in which the di-ester formed by two serine residues act as the acetyl-receptor and histamine-binding groups.
APPENDIX

ESTIMATION OF RIBOSE BY MEJBAUM'S METHOD.
ESTIMATION OF RIBOSE BY MEJBAUM'S METHOD (MEJBAUM, 1939).

The reagent consisted of 1% orcinol with 0.1% ferric chloride dissolved in concentrated hydrochloric acid. Three ml. of this solution and an equal volume of the unknown were heated in a boiling water-bath for twenty minutes. After cooling and making the volume up to 10 ml. with water, the optical density was read in the Spekker absorptiometer using 1 cm. cells and red filters (Chance No. 0R2). Standards were prepared with arabinose.

As propamidine isethionate is very stable, even in acid solution, the effect of this drug on the colour reaction was tested before any analyses were started. The following table shows that pentose estimations were completely unaffected by its presence.

TABLE
Estimation of ribose (in terms of arabinose) in the presence of propamidine isethionate (P.I.).

<table>
<thead>
<tr>
<th>Conc. of arabinose in ψ per ml.</th>
<th>Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standards alone</td>
</tr>
<tr>
<td>5</td>
<td>0.036</td>
</tr>
<tr>
<td>10</td>
<td>0.062</td>
</tr>
<tr>
<td>15</td>
<td>0.103</td>
</tr>
<tr>
<td>20</td>
<td>0.140</td>
</tr>
<tr>
<td>25</td>
<td>0.175</td>
</tr>
</tbody>
</table>
The procedure described above was slightly modified for the analysis of the ATP-propamidine complex. About 5 mg. of the complex was first hydrolysed in concentrated hydrochloric acid and aliquots were then assayed for ribose. Carefully weighed samples of arabinose were similarly treated and used as standards in preparing the calibration curve.
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