THE PURIFICATION AND IDENTIFICATION OF PLACENTAL HISTAMINASE

by

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CONTENTS.

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Chapter 1. Introduction.

1.1 Historical introduction to histaminase and diamine oxidase.

1.2 The role of histamine in normal physiology.

1.3 Histamine metabolism in pregnancy.

1.4 Aims of the present work.

Histaminase is a soluble enzyme catalysing the oxidative deamination of histamine. For many years, the enzyme was studied only in the context of its relation to histamine metabolism, but Zeller (1938a) found that aliphatic diamines also act as substrates of the enzyme; histaminase from some mammalian tissues oxidises the diamines at a faster rate than histamine. Further, it is now known that many human tissues possess the enzyme imidazole-N-methyl transferase (Nilsson et al., 1959) which catalyses methylation of histamine in the imidazole ring, and that the serum benzylamine oxidase of certain species also oxidises histamine to some extent (Blaschko, 1962). These developments, combined with repeated failures to demonstrate an important function for histaminase in normal human physiology, have only recently begun to enliven research into the metabolism of histamine on a broader basis, unhampered by the preconception that there is a unique relationship between histamine and histaminase.

Since changes in histamine metabolism during pregnancy are accompanied by synthesis of histaminase in the placenta and appearance of the enzyme in the serum (Section 1.3), many attempts have been made to establish links between placental histaminase and histamine metabolism in pregnancy and its disorders. This introductory chapter shows, however, that even in the field of human pregnancy, the lure of a special relationship between histamine and histaminase may have proved misleading.

A brief historical outline will emphasise the classification of histaminase as a diamine oxidase. Current concepts relating histaminase to the functions of histamine will then be discussed.

1.1 Historical introduction to histaminase and the diamine oxidases.

In 1915, Eustis reported the inactivation of histamine by a heat-labile component of turkey-buzzard liver, and Koessler and Hanke (1924) concluded that histamine was inactivated in the intestine of the guinea-pig, after oral administration. Best (1929) found that the vasodilator activity of histamine could be destroyed by incubation with autolysed ox or horse lung; this property of the autolysed tissue did not survive heating at 90°, and Best suggested, by analogy with tyramine oxidase, that histamine might be destroyed by an oxidative enzyme. Best and McHenry (1930) were the first to attempt detailed characterisation of the histamine-inactivating enzyme from dog kidney and intestine. They found that the enzyme could be inactivated at 60°, had optimum activity between pH 7 and pH 8, and that it was inhibited by cyanide or lack of oxygen. On the basis of an observed decrease in the diazo reaction during the inactivation of histamine, these authors proposed that rupture of the imidazole ring took place during the enzymic reaction; this theory figured prominently in the literature on histaminase for some twenty years, when Tabor (1951) showed satisfactorily that the imidazole ring remained intact during histamine inactivation. Best and McHenry can also be credited with the first detailed review of histamine inactivation (Best and McHenry, 1931). McHenry and Gavin (1932) prepared a stable acetone powder from hog kidney, and this has remained the most popular source of crude histaminase for general use.

Zeller (1938, a, b) established that hog kidney extracts also catalysed the oxidation of many diamines. The oxidation of both histamine and the diamines was subject to inhibition by carbonyl reagents, and histamine and the diamines competed as substrates of the oxidative enzyme. Zeller concluded that a single enzyme was responsible for the oxidation of both histamine and the diamines, and proposed that Best and McHenry's term "histaminase" should be replaced by the

more general term "diamine oxidase". Zeller (1938a) was the first to identify the products of the enzymic reaction, and to define the following equation for the overall reaction of diamine oxidase on its substrates.

$$R.CH_2NH_2 + H_2O + O_2 \longrightarrow R.CHO + H_2O_2 + NH_3$$

Zeller and his associates have been most prominent in determining the structural requirements of substrates of diamine oxidase.

Danforth and Gorham (1937) demonstrated histaminase activity in the human placenta, and Marcou and his co-workers (1938) found greatly increased histaminase activity in the serum of pregnant women. Zeller, Schär and Staehlin (1939) claimed that histaminase of human pregnancy plasma oxidised aliphatic diamines as well as histamine, thus conforming with the specificity of hog kidney diamine oxidase. Zeller and Birkhäuser (1940) applied a colorimetric indigo test to the estimation of serum diamine oxidase levels in different stages of pregnancy and its disorders.

Subsequently, similar enzymes oxidising histamine and the diamines were found in many mammalian tissues, plants and micro-organisms. The latter have provided some interesting varieties of diamine oxidase. Gale (1942) claimed that Ps. pyocyanea possessed a constitutive enzyme for the oxidative deamination of aliphatic diamines, but that histamine and tyramine were oxidised only after a period of adaptation by the organism. Satake, Ando and Fujita (1953) also observed that Achromobacter could form adaptive enzymes specific for the oxidation of histamine, and of putrescine. These are exceptional instances of enzymes attacking only histamine, or only diamines. The weight of evidence supports Zeller's contention (Zeller, 1956) that there exists a very wide range of diamine oxidases, occurring in many species, varying in their affinity for

TABLE 1.1.1 Properties of Some Amine Oxidases

Common name of enzyme	Substrates oxidised (and relative rates)	Prosthetic groups	Inhibitors
Human placental histaminase	C > P > H (1)	Unknown	Aminoguanidine carbonyl reagents (2)
Hog kidney DAO	H > C > P > B (1,3)	Possibly FAD and P.Pal (4)	As for histaminase (3)
Pea seedling DAO	C > P > H > B (3,5)	Cu^{++}, probably P.Pal (5)	As for histaminase (3)
Plasma benzylamine oxidase	B > H C and P not oxidised (6)	Cu^{++}, probably P.Pal (6)	As for histaminase (3)
Mitochondrial monoamine oxidase (MAO)	Alkylamines and arylalkylamines, long-chain diamines. C and P not oxidised. (3)	Unknown	Not inhibited by carbonyl reagents or aminoguanidine. (3)

Abbreviations: H - Histamine, C - Cadaverine, P - Putrescine,
B - Benzylamine, FAD - Flavin adenine dinucleotide,
P. Pal - Pyridoxal phosphate.

References :

(1) Zeller (1956)
(2) Kapeller-Adler (1956, b)
(3) Zeller (1963)
(4) Kapeller-Adler and MacFarlane (1963)
(5) Hill and Mann (1964)
(6) Buffoni and Blaschko (1964)

histamine and the diamines, but all capable of oxidising both kinds of substrate to a measurable extent. The highly-purified hog kidney preparations of Mondovi et al. (1964) showed the same ratio of rates of oxidation of histamine and cadaverine (H/C ratio) throughout purification. McEwen (1964) has reported that the increase in serum "histaminase" throughout human pregnancy is paralleled by an increase in the capacity of serum to oxidise putrescine, but not benzylamine. Southren et al. (1964) used putrescine as substrate in their study of serum histaminase levels throughout pregnancy; the pattern of enzymic activity resembled closely the activity of pregnancy serum towards histamine as found by Ahlmark (1944).

Table 1.1.1 summarises a few of the similarities and differences between some common varieties of amine oxidases. Zeller has exhaustively described the known varieties of diamine oxidase in two reviews (Zeller, 1951 and 1963). The properties of intensively studied diamine oxidases, such as those from pea seedlings and hog kidney, will be discussed in later chapters only when comparison with the placental enzyme is especially interesting. Current confusion over diamine oxidase specificity is due largely to over-zealous analogies being drawn between the properties of enzymes prepared from different species, without adequate specifications of purity, and assayed under radically different conditions.

The author's choice of the name "histaminase" for the enzyme in the present work requires some explanation. A review of the experimental evidence suggests that the "histaminase" of human placenta, hog kidney, etc., oxidises both histamine and the aliphatic diamines, and that the general term diamine oxidase (or Zeller's contraction, DAO) is the better name, even though histamine is not, strictly speaking, a diamine. The Enzyme Commission's designation is "Diamine : O$_2$ oxidoreductase (deaminating) 1.4.3.6."(Report of the Commission

on Enzymes of I.U.B. 1961). Despite these recommendations, the placental enzyme is still almost exclusively known as histaminase. Most investigations have been directed towards finding physiological functions of the enzyme in relation to histamine, the substrate considered to have the most potent pharmacological activity. Repeated failures to demonstrate any such function in vivo do not appear to have diminished the popularity of the name histaminase; the author's choice is justified only by common usage. Kapeller-Adler, however, has repeatedly contended that histamine and the diamines are oxidised by different enzymes, and that these enzymes should be called histaminase and diamine oxidase, respectively (Kapeller-Adler, 1944, 1949, 1951, 1956(b); Kapeller-Adler and MacFarlane, 1963). The basis of these claims will be discussed later, but it must be emphasised at this point that the term "histaminase" is used in the present work only as an acceptable synonym for DAO, and does not imply support for Kapeller-Adler's nomenclature.

1.2. The role of histamine in normal physiology

It has been known for fifty years that histamine injected into mammals can exert profound pharmacological effects. According to the dose and the experimental animal, injection of histamine may result in arteriolar and capillary dilatation, flushing, itching or pain in the skin, increased gastric secretion of acid, and increased tone of smooth muscle, e.g., the uterus. Anaphylactic shock and certain human allergic conditions can also be attributed to histamine release.

The role of histamine in normal physiology, when the organism is not under overt stress, is less clear. The body possesses two distinct pools of histamine which are not normally in equilibrium, histamine bound firmly in mast cells, and histamine in tissue fluids. Mast cells, found in almost all tissues, may release histamine under stress, e.g. tissue injury, antigen-antibody reactions, or contact with certain polybasic compounds. Histamine produced and bound in mast cells is not known to be released under normal physiological conditions.

It has recently been suggested (Green, 1964) that histamine may play a part in the mediation of nervous impulses. Fuché and Kahlson (1957) have presented evidence that histamine may exercise control of the pituitary gland. These functions have been more tentatively suggested than the comprehensive theories put forward by Schayer and by Kahlson, which seek to involve histamine in the normal control of the microcirculation, and in the promotion of rapid tissue growth.

Histidine, always present in blood, can be decarboxylated to histamine by an enzyme present in all tissues. In Schayer's view (1962), this histidine decarboxylase controls the microcirculation by synthesising histamine in response to the local need for the supply of blood. He has suggested that the

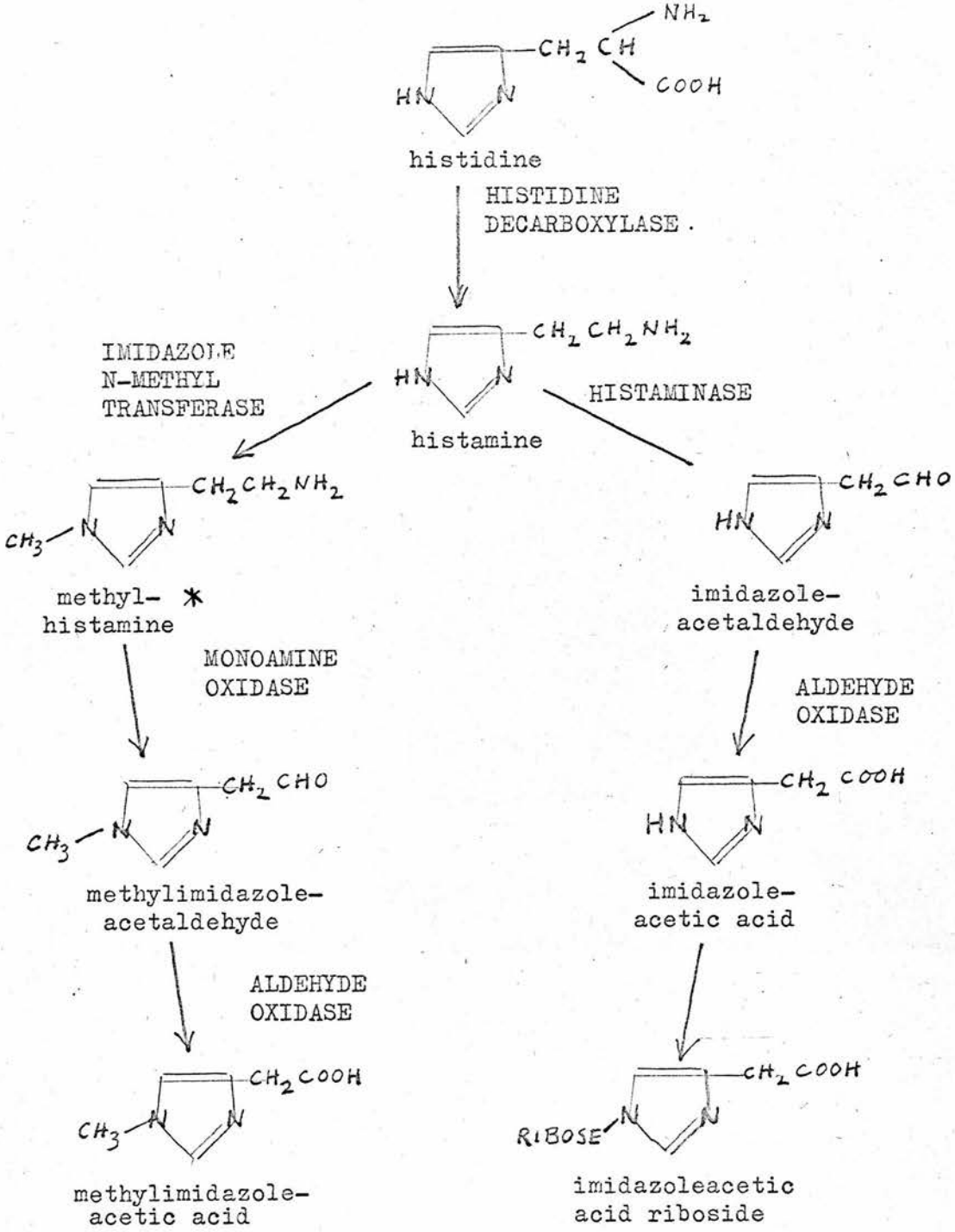


Fig. 1.2.1. Major pathways of histamine metabolism in man.

* Trivial name for 1-methyl, 4-(β-aminoethyl) imidazole.

enzyme is activated, or "induced" by chemical stimulus, and is controlled by a feedback mechanism arresting histidine decarboxylase activity when the appropriate local concentration of histamine in the tissue has been attained.

Kahlson has presented evidence that histamine formation is important in rapidly growing tissues. He has shown that the rat foetus, (Kahlson, Rosengren and Westling, 1958), ascites tumour cells (Kahlson, Rosengren and Steinhardt, 1963) and healing wound tissue (Kahlson et al., 1960), all have a very high histidine decarboxylase activity, and he has shown a correlation between activity of this enzyme and frequency of mitosis (Kahlson, Rosengren and Steinhardt, 1963). He emphasised that these growth-promoting effects seem to be linked to the formation of histamine (i.e. histidine decarboxylase activity) rather than to the total histamine content of the tissue (Kahlson, 1960).

Schayer's development of techniques for measuring the products of metabolism of ^{14}C -histidine and ^{14}C -histamine (Schayer, Kobayashi and Smiley, 1955; Schayer and Cooper, 1956), has given fresh impetus to the study of the normal metabolism of physiological amounts of these amines in several animals. He has reviewed the subject of histamine catabolism (Schayer, 1959), elucidated chiefly by the determination of urinary metabolites after injection of ^{14}C -histamine. Fig. 1.2.1 summarises the two most important pathways in mammals. Acetylation of the side-chain primary amino-group has been described, but does not appear to be quantitatively important in man (Urbach, 1949).

In man, little histamine is normally excreted in the urine. The chief urinary metabolites of histamine are imidazoleacetic acid, largely conjugated as the riboside, and 1,4 methylimidazoleacetic acid.* The pathways involving oxidative deamination and methylation are of approximately equal importance in man; however, if large doses of histamine are given, or if histaminase is blocked by injections of aminoguanidine, the methylating system

* Trivial name for 1-methyl, 4-(β-acetic acid) imidazole.

alone is capable of inactivating all the extra histamine. In the rat, methylation is normally of minor importance, most of the histamine being inactivated by histaminase in the intestine.

1.3 Histamine metabolism in pregnancy

Discovery of placental histaminase.

Danforth and Gorham (1937), were the first to observe histaminase activity in human placenta. Subsequently, Marcou et al. (1938), Werle and Effkemann (1940) and Zeller and Birkhäuser (1940), reported that, while normal blood had negligible histaminase activity, the plasma of pregnant women had high histaminase activity. These earlier workers were cautious in discussing possible functions of increased histaminase activity in pregnancy. Marcou et al. considered that the function of the enzyme was to protect the mother from the vasodilator and anticoagulant effects of histamine in case of haemorrhage. Although Danforth (1939), found that the histaminase content of placenta varied inversely with the strength of uterine contractions, he did not suggest that histamine induced labour.

Clinical significance of histidine and histamine excretion in pregnancy.

Kapeller-Adler (1941), claimed that a decrease in normal urinary histidine output was diagnostic of severe pre-eclamptic toxaemia. Kapeller-Adler and Adler (1943) estimated the urinary histamine of pregnant subjects by a gravimetric method using flavianic acid: with this relatively insensitive method, only traces of histamine were found in the urine of normal patients, and those with severe pre-eclamptic toxaemia or eclampsia. However, histamine excretion was elevated in cases of hyperemesis gravidarum or mild pre-eclamptic toxaemia. Kapeller-Adler and Adler proposed that the balance of histidine decarboxylase and histaminase was disturbed in these disorders, and that many symptoms of toxaemia might be ascribed to histamine intoxication.

Bjüro, Lindberg and Westling (1961), re-examined urinary and blood levels of histamine during human pregnancy, using a more sensitive and specific

~~biological assay method. They found a significant increase in urinary~~
biological assay method. They found a significant increase in urinary

histamine in about half of normal pregnancies, especially if prolonged. These authors agreed with Kapeller-Adler and Adler (1943), that little histamine could be found in the urine of patients with severe toxaemia, but that increased histamine was excreted by patients with mild or moderately severe toxaemia. They found no correlation, however, between urinary and plasma histamine concentrations of the mother in normal or toxaemic pregnancy, and they could not suggest any causal relationship between histamine and toxaemia.

Clinical significance of histaminase activities in pregnancy sera.

Zeller (1941), found that serum histaminase activity was lower in patients with hyperemesis gravidarum than in normal pregnancy.

In 1944, Ahlmark published an extensive study on histaminase in pregnancy plasma. He found a gradual increase in plasma histaminase throughout pregnancy, (except for a temporary lowering at about the eighth month), and rapid loss of the plasma activity in cases of toxaemia. Kapeller-Adler (1944), using an insensitive indigo test, showed that serum histaminase was decreased in mild toxaemia, and severely decreased in severe pre-eclamptic toxaemia and eclampsia. Ahlmark and Werkö (1950), after a survey of a large number of pregnancies, re-affirmed that there was no correlation between the severity of toxaemia and decrease in plasma histaminase, and suggested that any changes in enzymic activity might be secondary to toxaemic changes in the placenta, or to changes in hormone levels. Swanberg (1950), found no correlation between toxaemia and histaminase content of the placenta at term.

Kapeller-Adler (1951, 1952), repeated her earlier histaminase measurements on pregnancy sera, using a microvolumetric indigo test of improved precision, and again found a significant correlation between severity of toxaemia and reduction of histaminase in serum. It is conceivable that the discrepancies in

these reports are a result of the different assay methods used. Ahlmark (1944), and Swanberg (1950) employed a biological test for histamine recovered from the histaminase assay, whereas Kapeller-Adler's method depended on the oxidation of indigo by H_2O_2 formed by the action of histaminase on histamine. Kapeller-Adler's method is probably more specific for histaminase, but less sensitive.

There has been no more recent attempt to relate serum histaminase activities to toxaemia, and the possibility of a causal relationship cannot yet be dismissed. A recent review in British Medical Journal (Leading article, 1964), indicates that monoamine oxidase is now receiving attention as a possible influence in toxaemia, to the neglect of histaminase.

The source of pregnancy serum histaminase and its effectiveness in vivo.

Swanberg (1950), showed that the foetus played no part in the increased production of histaminase in pregnancy. Treatment of non-pregnant women with progesterone stimulated histaminase production in the uterine endometrium; pseudo-pregnancy of the rabbit resulted in increased serum histaminase, in the absence of a foetus. Dissection of human placenta revealed that the source of placental and plasma histaminase was the maternal decidua.

Wicksell (1949), found that the blood of the human foetus had no histaminase activity but had a higher concentration of histamine than maternal blood. He could detect no significant arterio-venous difference in the histamine content of the umbilical blood vessels. Wicksell concluded that foetal blood histamine was carried mainly in the cells and that most of the plasma histamine was bound in a form insusceptible to histaminase.

Wicksell also made the important observation that pregnancy did not change a woman's response to subcutaneous injections of histamine; the diameter of skin flare was not reduced at any stage of pregnancy, even when very high levels of plasma histaminase could be demonstrated. Nilsson et al. (1959),

supported Wicksell's observation. On injecting ^{14}C - histamine into pregnant and non-pregnant women, they could detect no significant difference in the distribution of urinary metabolites in the two groups of subjects. Methylation accounted for about half of the injected histamine, and no increase in the final product of histaminase activity, namely imidazoleacetic acid, was observed in the pregnant group.

In recent years, many aspects of histamine metabolism have been clarified by two independent lines of enquiry led by Kahlson and Lindberg, both employing ^{14}C-histamine techniques devised by Schayer. These two systematic studies represent the most determined attempts to elucidate the physiological roles of histamine, especially in pregnancy, and it is appropriate that their results should be given some prominence in this review.

Foetal Histamine.

Kahlson, Rosengren and Westling (1958), found that the rat excretes increased amounts of urinary histamine in the later stages of pregnancy, and that this histamine excretion subsides to a normal level at term. Surgical removal of embryos before term also reduced urinary histamine. The authors concluded that the embryo was the chief source of urinary histamine in the pregnant rat; although the rat had a high level of histaminase in the placenta, passage of blood through this organ was too brief to inactivate foetal histamine completely, and histamine was excreted in the mother's urine.

Comparison of human umbilical venous and arterial histamine content suggested that the human foetus also produces histamine (Kahlson, Rosengren and White, 1959). This interpretation was supported by the high histamine content and weak histamine-binding capacity of the human foetus (Kahlson and Rosengren, 1959).

Björo, Lindberg and Westling (1961), confirmed the observation of

Kahlson et al. (1959) of an arterio-venous difference in the histamine content of umbilical blood vessels, and agreed that the sharp fall in urinary histamine after parturition suggested that, as in the case of the rat, the human foetus was the source of histamine excreted by the mother.

Kahlson and Rosengren (1959), devised a means of reducing histidine decarboxylase activity in rats. The inhibitor semicarbazide had some effect in reducing histamine formation, but it could be shown that new molecules of the enzyme were formed adaptively during inhibition, resulting in a pronounced "rebound" in histidine decarboxylase activity when semicarbazide treatment was discontinued. However, if the rat was starved of pyridoxine, a cofactor of histidine decarboxylase, semicarbazide treatment reduced histamine formation to 10-20% of the normal rate. When pregnant rats were given semicarbazide and a pyridoxine-free diet, surgery revealed that foetal development ceased at the stage when histidine decarboxylase inhibition became effective. While semicarbazide also inhibits histaminase, this did not cause the arrest of foetal development, since in separate experiments complete inhibition of histaminase by aminoguanidine had no effect on the course of embryonic development. Comparisons of histamine formation rates in foetal and adult rat tissues confirmed that the foetal liver, in particular, produces histamine at a much faster rate than any maternal tissue.

Kahlson (1962), views these results as part of a pattern of increased histamine formation in tissue growth and repair rather than phenomena peculiar to pregnancy. Lindell and Westling (1957), have supported Kahlson's contention that human foetal tissues have a high capacity for histamine formation, especially the skin and kidney. Rosengren (1963) found that the skin of the mouse embryo showed a steadily increasing capacity for histamine formation throughout development. Since the maternal kidneys also produced much histamine

the relative importance of histamine in foetal development was less clear than in the case of the rat. Rosengren's results, however, do not conflict with Kahlson's theory that tissue histidine decarboxylase activity, or some concomitant reaction, is more relevant to tissue growth than the total concentration of histamine in the tissue.

Although it appears that histamine formation by the rat and mouse foetus is associated with development, it may still be premature to extrapolate these findings to the human foetus, whose histamine forming capacity is substantially lower. (Lindberg, Lindell and Westling, 1963b).

Consequences of *in vivo* inhibition of histaminase.

As stated in the previous section, Kahlson and Rosengren (1959) reported that the development of the rat foetus was undisturbed by administration of aminoguanidine to the mother, although histamine catabolism in the rat depends almost solely on histaminase. This is in complete disagreement with the work of Roberts (1954), who found that when pregnant rats were given injections of aminoguanidine, increasing maternal and foetal mortality were inflicted by increasing the dose of the inhibitor. Roberts could not determine whether toxicity was inherent in aminoguanidine itself, or due to toxic accumulation of histamine or diamines, but he suggested that at least the maternal mortality was the result of amine intoxication. The discrepancy between these two reports has not been explained.

Mitchell (1963), found little change in urinary histamine after injecting aminoguanidine (or isoniazid or iproniazid) into normal men, who possess the alternative methylating enzyme system for histamine catabolism. Lindell, Nilsson, Roos and Westling (1960), studied the urinary metabolites of non-pregnant women given injections of ^{14}C-histamine after treatment with histaminase inhibitors; they detected an increase in urinary methylimidazole-

acetic acid, and a decrease in imidazoleacetic acid. No ill effects of aminoguanidine administration were reported in either of these groups.

Bjüro, Lindberg and Westling, (1964), injected aminoguanidine into pregnant women and found that inhibition of histaminase had no effect on their urinary excretion of histamine. The fact that no immediate ill effects of histaminase inhibition were observed is perhaps the most striking argument yet produced against the importance of histaminase in pregnancy. However, Bjüro et al. seem to have used a dose of aminoguanidine calculated to inhibit the histaminase of non-pregnant women. No experimental check was made that serum histaminase was completely inhibited in their pregnant groups, and no attempt was made to determine whether pregnant women were protected against aminoguanidine by some detoxicating enzyme system. Since it is unlikely that repetition of these important experiments would be justified, it is particularly disappointing that they were not supported by direct proof of histaminase inhibition in the pregnant group.

In vivo experiments on histamine metabolism in pregnancy.

The possible importance of histaminase in pregnancy has appeared to recede even further with the confirmation by Lindahl (1961), that the histamine-methylating enzyme system, as well as histaminase, is present in human placenta. Lindahl pointed out that the observations of Nilsson et al. (1959), on histamine catabolism in pregnancy were consistent with the hypothesis that, in spite of the enormous increase of histaminase in placenta and plasma, the methylating pathway remained the most important histamine detoxicating system in pregnancy.

Lindberg and co-workers have since published the results of a remarkable series of experiments on the metabolism of histamine, in vivo and in vitro, in late human pregnancy. Because of their direct approach to the

problem, and the proven sensitivity of the ^{14}C-histamine analyses employed, their results command attention as the most coherent view we have, so far, of this aspect of histamine metabolism.

During surgery for abortion or hysterectomy, pregnant and non-pregnant women were given a continuous infusion of ^{14}C-histamine; after attainment of a steady histamine concentration in the arterial blood, samples were withdrawn simultaneously from the brachial artery, ante-cubital vein, uterine vein and umbilical vein (Lindberg, 1963a). It was found that, at the same infusion rate, pregnant women maintained an arterial histamine concentration 50% lower than that of non-pregnant women, indicating efficient histamine catabolism operating at lower blood histamine concentrations. In pregnant women about half, and in non-pregnant women only about one third, of the histamine was catabolised during passage through the uterus. Low histamine concentrations in the umbilical vein also suggested efficient histamine inactivation in the pregnant uterus. The distribution of metabolic products indicated that in non-pregnant women methylation is the predominant inactivation mechanism, while in pregnant women histaminase assumes greater importance; it had to be concluded that the increase in histaminase metabolites in pregnancy blood is due to plasma histaminase rather than histaminase active in the placenta.

Some of the unexpected features of these experiments (which are not in complete agreement with urinary analyses of histamine metabolites) were confirmed by <u>in vitro</u> incubation of ^{14}C-histamine with maternal tissues (Lindberg, 1963b). In the placenta and pregnant myometrium, oxidative deamination by histaminase predominated, and could largely be inhibited by aminoguanidine, whereupon efficient methylation of histamine occurred. Uterine muscle from pregnant women also possessed both enzyme systems, but non-pregnant myometrium inactivated almost all the histamine by methylation.

In another series of experiments, (Lindberg, Lindell and Westling, 1963a) ^{14}C-histamine was injected into the ante-cubital vein or umbilical artery during surgical abortion, and the metabolites in the mother's urine analysed. After injection into the umbilical artery, histamine appeared in the urine almost solely as methylated products, whereas histamine injected into the ante-cubital vein was partly methylated and partly attacked by histaminase - as shown by Nilsson et al. (1959). These findings indicated that inactivation of histamine in the pregnant uterus was chiefly performed by a methylating enzyme present in either the placenta or the foetus, or both.

It was subsequently demonstrated (Lindberg, Lindell and Westling, 1963b), that many foetal tissues possessed imidazole N-methyl transferase activity, the main product of <u>in vitro</u> activity being methylhistamine; the foetus did not appear to possess the enzymes (possibly MAO and aldehyde oxidase) oxidising this primary product to methylimidazoleacetic acid, as does the adult. Histaminase was present only in the kidney and intestine of the foetus.

These experiments suggest that in spite of its high histaminase content, the placenta itself plays little part in histamine inactivation, except by methylation. The placental histaminase becomes important only when it enters the maternal plasma; the methylating enzyme is present in most tissues but is absent from blood. When the high histamine production of the foetus is considered, it is tempting to believe that maternal plasma histaminase is a protection against foetal histamine, but in view of the ability of the methylating enzyme to take over its role if histaminase is inhibited, such protective capacity seems superfluous. Histaminase, both in placenta and in plasma, may rather be directed against aliphatic diamines, but our knowledge of the metabolism and function of these other substrates of histaminase is deficient.

1. 4. Aims of the present work.

Zeller (1963) classifies placental histaminase as a diamine oxidase. Kapeller-Adler (1956b) believes that histamine and the diamines are oxidised by discrete enzymes. McEwen and Cohen (1963) have found benzylamine oxidase activity in human serum, and Blaschko (1962) has emphasised that histamine is also a substrate of typical mammalian serum benzylamine oxidases. No classification of amine oxidases has been found to be generally acceptable (cf. Zeller, 1963 and Blaschko, 1963), and the separate identity of enzymes capable of oxidising histamine is a particularly controversial subject, partly obscured by disagreement over assay methods and the validity of classical substrate-competition experiments as criteria of specificity. The most convincing approach to this problem seemed to be to determine the specificity of highly purified placental histaminase, using an assay method applicable to all classes of substrate.

Similarly, cofactors could best be studied at a stage of purification where simple measurements of cofactor concentration in the enzyme preparation would be unambiguous - that is, when all contaminants had been removed or identified. In view of recent work on the cofactors of pea seedling DAO (Hill and Mann, 1964), hog kidney DAO (Mondovi et al., 1964), Kapeller-Adler and MacFarlane, 1963) and serum amine oxidase (Yamada and Yasunobu, 1962a) (Buffoni and Blaschko, 1964), determination of the cofactors of placental histaminase might aid classification of the enzyme.

Kapeller-Adler's (1951) microvolumetric indigo test was adopted for the assay of histaminase throughout the first year of this project. It became increasingly obvious during this period that Kapeller-Adler's method was not sufficiently reproducible for the comparison of histaminase activities towards different substrates throughout purification. Chapter 2 of this thesis

described the author's reasons for abandoning Kapeller-Adler's test, and the development of a more sensitive and reproducible indigo test applicable to all substrates, and especially suitable for the assay of limited amounts of purified enzyme. The investigation of the validity of indigometric tests for histaminase necessarily became one of the major aims of this project.

The reluctance of biochemists to exploit the placenta as a rich source of human enzymes is based on the difficulty of extracting enzymes from the tough placental tissue, and the problem of removing the blood proteins from the extract. Hagerman's recent review (1964) emphasises the difficulties of working with the placenta, and how few enzymes have been extensively purified from this source. It was hoped that determined efforts to solve some of these practical problems might result in procedures applicable to other placental enzymes.

Chapter 2. The assay of histaminase activity.

2.1 Available methods of assaying histaminase.

2.2 Kapeller-Adler's indigo test.

2.3 A new spectrophotometric indigo test.

2.4 Other assay methods examined.

2.5 Critical assessment of histaminase assays.

2.1 Available methods of assaying histaminase.

Two main considerations guided the initial selection of an assay method for histaminase. The method had to permit comparison of enzymic activities towards both histamine and the diamines at successive stages of purification, and in response to different experimental conditions, such as the addition of inhibitors. Secondly, it was essential to choose a convenient method of assaying numerous samples arising from column chromatographic separations; ideally, the same method should be sufficiently valid for detailed study of the purest enzyme preparations.

The first of these requirements was the more restrictive. The different classes of substrate, and the aldehydes formed from them by the action of histaminase, share few chemical properties which might be exploited in a common assay method. For instance, imidazoleacetaldehyde, formed from histamine, bears little resemblance to Δ^1-pyrroline, which is formed from the oxidation product of putrescine, and for which a satisfactory spectrophotometric assay has been developed.

Most assay methods measuring a decrease in substrate concentration, or an increase in concentration of oxidised substrate, are applicable to only a single substrate or group of substrates. They have the advantage of giving a direct measure of enzymic activity and, ~~particular~~ particularly if the oxidation product is estimated, may be completely specific for histaminase.

The comparison of histaminase activities towards several classes of substrate in parallel demands measurement of one of the common reactants or products. The general formula for oxidative deamination is :

$$R.CH_2NH_2 + H_2O + O_2 \longrightarrow R.CHO + NH_3 + H_2O_2$$

Measurement of oxygen consumption, or the production of ammonia or hydrogen peroxide, offers a more suitable basis for comparing rates of oxidation of different substrates than does a series of individual assay methods, each applied to a single substrate. Uniformity is gained at the cost of specificity; for instance, contributions to oxygen consumption may be made by other enzyme systems present in crude placental extracts, and careful controls may have to be applied for each enzymic assay.

2.1.1 Methods measuring decrease in histamine concentration

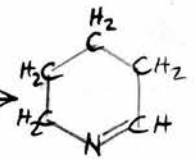
Biological assay of residual histamine, after incubation with histaminase, was widely used in early studies on the enzyme (e.g. Ahlmark, 1944; Anrep, Barsoum and Ibrahim, 1947). Histamine was extracted from the assay solution and estimated by the contraction produced in the guinea-pig ileum. The method is sensitive, but it is not specific for histamine, is vulnerable to interference by other enzymes metabolising histamine, and depends on animal material. The use of a biological assay was not seriously contemplated in the present work.

The most sensitive chemical method of determining histamine is the fluorimetric assay of Shore, Burkhalter and Cohn (1959). This method was used in the present work for a special purpose, but its routine application could not be considered on account of three factors: (a) the possibility of interference from placental imidazole N-methyl transferase, which also metabolises histamine, (b) the necessity of assaying, in parallel, histaminase activity on the diamines, by some completely dissimilar method, and (c) the small number of assays (about twenty) which could be performed carefully in a normal working day.

Schayer, Kobayashi and Smiley (1955) have described an isotopic dilution method for the micro-estimation of histamine, applicable to histaminase assays. In the context of the present study, this method would have been subject to the same limitations as the fluorimetric method, and would have required expensive equipment and reagents.

2.1.2. Methods measuring increase in product concentration

There is no method in general use for the quantitative estimation of imidazoleacetaldehyde. Oxidative deamination of certain aliphatic diamines is followed by the spontaneous formation of cyclic azomethines. e.g.

$NH_2\ (CH_2)_5\ NH_2 \xrightarrow{\text{histaminase}} NH_2\ (CH_2)_4\ CHO \longrightarrow$ 

diaminopentane (cadaverine)

Δ^1-piperidine

Okuyama and Kobayashi (1961) found that while putrescine and cadaverine are insoluble in toluene, their cyclic oxidation products, Δ^1-pyrroline and Δ^1-piperidine, may be extracted into toluene. They measured the radioactivity of a toluene extract after incubation of histaminase with ^{14}C-labelled putrescine or cadaverine. Their sensitive method could conceivably have been used in the present study in parallel with a histaminase assay, but the expense of equipment and substrates prohibited this.

Holmstedt and Tham (1959) have developed a colorimetric method based on the yellow 1,2-dihydroquinazolinium derivatives formed by condensing o-aminobenzaldehyde with the cyclic azomethines which result from the oxidative deamination of putrescine, cadaverine or 1,6-diaminohexane. Their method is fairly sensitive and specific, and is extremely convenient for the rapid assay of many enzyme fractions. However, it is not applicable to all diamines, nor to histamine.

2.1.3. Measurement of oxygen consumption.

Manometric estimation of oxygen uptake has been extensively used in recent years, notably by Buffoni and Blaschko (1964), Hill and Mann (1964) and Mondovi et al. (1964). Catalase must be added to the assay in order to break down hydrogen peroxide produced in the enzymic reaction. The abundance of haemoproteins in placental extracts introduces a danger of non-specific oxygen uptake. Born (1953) found that after histamine had been oxidised to imidazoleacetaldehyde, further oxidation to imidazoleacetic acid, catalysed by cupric ions, resulted in secondary uptake of oxygen. This non-specific oxygen uptake did not occur when aliphatic diamines were used as substrates. Manometry was considered too insensitive and laborious for the assay of numerous dilute enzyme fractions, and offered no special advantages for the present study.

2.1.4. Measurement of ammonia production.

Estimation of ammonia in Conway units (Cotzias and Dole, 1952) (Reif, 1960) is complicated by the diffusibility of the cyclic products of putrescine or cadaverine oxidation, which interfere also with the Nessler reaction (Zeller, 1963). Detection of ammonia by enzymic amination of α-oxo-glutaric acid, utilising glutamic dehydrogenase and NADPH, was proposed by Zeller (1963), but the method had not been published in detail when the present work had been completed. If the experimental difficulties implied by this delay can be overcome, Zeller's specific method would be the most appropriate for comparing the substrate specificities of different varieties of DAO.

2.1.5. Measurement of hydrogen peroxide production.

Several assay methods depend on the detection of hydrogen peroxide produced in the enzyme-substrate reaction. In principle, all such methods are unsuitable for the assay of crude placental histaminase, since abundant haemoglobin present in crude preparations competes with the detection system for the hydrogen peroxide. Indeed, Mondovi et al (1964) have used a semi-quantitative test for hog kidney histaminase which depends on the oxidation of added haemoglobin to methaemoglobin by hydrogen peroxide.

Zeller (1963) mentioned a "spot-test" for DAO, utilising the oxidation of dianisidine by hydrogen peroxide in the presence of peroxidase, and this method has recently been fully described by Aarsen and Kemp (1964).

Keston and Brandt (1965) have developed a method for the estimation of ultramicro quantities of hydrogen peroxide, depending on an increase in fluorescence of diacetyldichlorofluorescein in the presence of hydrogen peroxide and peroxidase. Since about 10^{-11} mole hydrogen peroxide/ml. can be detected, this method might provide the most sensitive test for histaminase activity, provided that other components of the assay system (including haemoprotein contaminants of the enzyme solution) do not interfere in the fluorimetric reaction.

Of the assay methods based on hydrogen peroxide production, Kapeller-Adler's microvolumetric indigo test (1951) has been the most fully described, and applied in the widest range of investigations. This method promised to be quantitatively valid for all substrates of histaminase, free from interference by imidazole N-methyl transferase, and especially convenient for numerous samples. When a choice had to be made, no other assay method had been described which fulfilled all the latter requirements.

Kapeller-Adler's indigo test was therefore adopted throughout the first year of this project, with only minor changes in procedure (Section 2.2).

A number of anomalies arising from the routine application of Kapeller-Adler's indigo test led to a re-examination of the method, and the development of a new, sensitive spectrophotometric indigo test (Section 2.3).

The fluorimetric histamine assay of Shore et al., the dianisidine test of Aarsen and Kemp, and the method of Holmstedt and Tham were occasionally used in the present work, and will be described in Section 2.4. The materials used in the work described in this chapter are listed in Appendix 1, and the pull-out Table in Appendix 2 briefly describes the purification Stages by which the various enzyme preparations were obtained.

2.2. Kapeller-Adler's indigo test for histaminase.

2.2.1 History of the test.

During the early series of experiments which sought to establish the identity of DAO and histaminase, Zeller (1938, b) noted that indigo disulphonate was decolourised by oxidation in the presence of DAO and substrate. The decolorisation of indigo was made the basis of a test for serum histaminase, and pregnancy, published by Zeller and Birkhäuser (1940).

Koloszynski (1945) introduced a more elaborate form of indigo test for pregnancy sera, estimating residual indigo after 72 hr. incubation of serum, histamine, and indigo. Indigo was extracted from the assay mixture with acetone, and estimated colorimetrically.

In one of the earliest attempts to apply an indigo test to serum histaminase in pregnancy disorders, Kapeller-Adler (1944) made only visual assessments of residual indigo colour. This technique was superseded by Kapeller-Adler's micro-volumetric indigo test (1951), estimating residual indigo in the reaction mixture by direct titration with N/500 permanganate solution. Essentially the same method has been used by Kapeller-Adler and her associates for fifteen years, in spite of vigorous opposition from Zeller since its inception. Objections to the indigo test will be discussed in Section 2.2.15.

2.2.2. Standard assay conditions (I) adopted in the present work.

The form of indigo test initially adopted in this project was identical to the method quoted by Kapeller-Adler and MacFarlane (1963) for hog kidney histaminase, except for three changes:

(i) Rather than adding a drop of chloroform to each assay, which often resulted in formation of a film of denatured protein around the drop of preservative, the assay buffer was saturated with chloroform before use.

(ii) Substrate concentrations were changed to approach more closely the optimal concentrations for placental histaminase.

(iii) 0.1 mM EDTA was added to the assay buffer to prevent inhibition of histaminase by metal ions. The reasons for this decision will be discussed in Section 4.3.4.

Reagents: Assay buffer. Sodium phosphate buffer 0.175 M, pH 6.8 containing 0.1 mM EDTA; preserved at room temperature over chloroform.

Indigo disulphate Analar, 0.667 mg./ml. distilled water; preserved at 4° in a brown bottle. (Referred to as "indigo").

Cadaverine dihydrochloride, 10 mg./ml. in assay buffer.

Putrescine dihydrochloride, 10 mg./ml. in assay buffer.

Histamine dihydrochloride, 1.25 mg./ml. in assay buffer.

0.1 N potassium permanganate solution; stored at room temperature in a brown bottle.

Procedure: The reaction mixture consisted of 0.2 ml. enzyme solution, 0.2 ml. substrate solution, 2.0 ml. indigo solution, made up to 10.0 ml. with assay buffer. Components were present in the following final concentrations:

Indigo	1.333 mg.	2.86 μ mole in 10 ml.
Cadaverine	2.0 mg.	11.4 μ mole in 10 ml.
or Putrescine	2.0 mg.	12.4 μ mole in 10 ml.
or Histamine	250 μg.	1.36 μ mole in 10 ml.

Control assays were set up containing all the above components except substrate. When purified enzyme solutions were employed, enzyme could be omitted from the controls without appreciable effect on the control titres. Oxygen was bubbled at a moderate rate through a manifold connecting three assays in series (first through a control, then through duplicate tests) for 60 seconds, and the assay-tubes closed with a rubber bung. The contents were mixed by gentle inversion, and the tubes placed in an incubator at 37° for 24 hr.

After incubation, residual indigo, not oxidised by the enzyme-substrate reaction, was titrated in the assay tube with 0.002 N permanganate solution, freshly diluted from the stock 0.1 N solution. The reaction mixture was vigorously agitated during the titration, which proceeded over a period of about two minutes to an equivalence point where all trace of blue colour had disappeared; the end-point colour was greenish, gold, or grey according to the substrate used. A 5 ml. auto-zero micro-burette was used for maximum precision. On the scale of this burette, 0.1 ml. titrant occupied about 5 mm. length. The difference in titres between a control and a test assay was a measure of the indigo oxidised as a result of the enzyme-substrate reaction. A titre difference of 1.0 ml. was expressed as an enzymic activity of 10 P.U. (permanganate units).

Stage 1 : $R.CH_2NH_2 + H_2O + O_2 \longrightarrow R.CHO + NH_3 + H_2O_2$.

Stage 2 : $2H_2O_2$ + Indigo $\longrightarrow$ 2 Isatin + $2H_2O$.

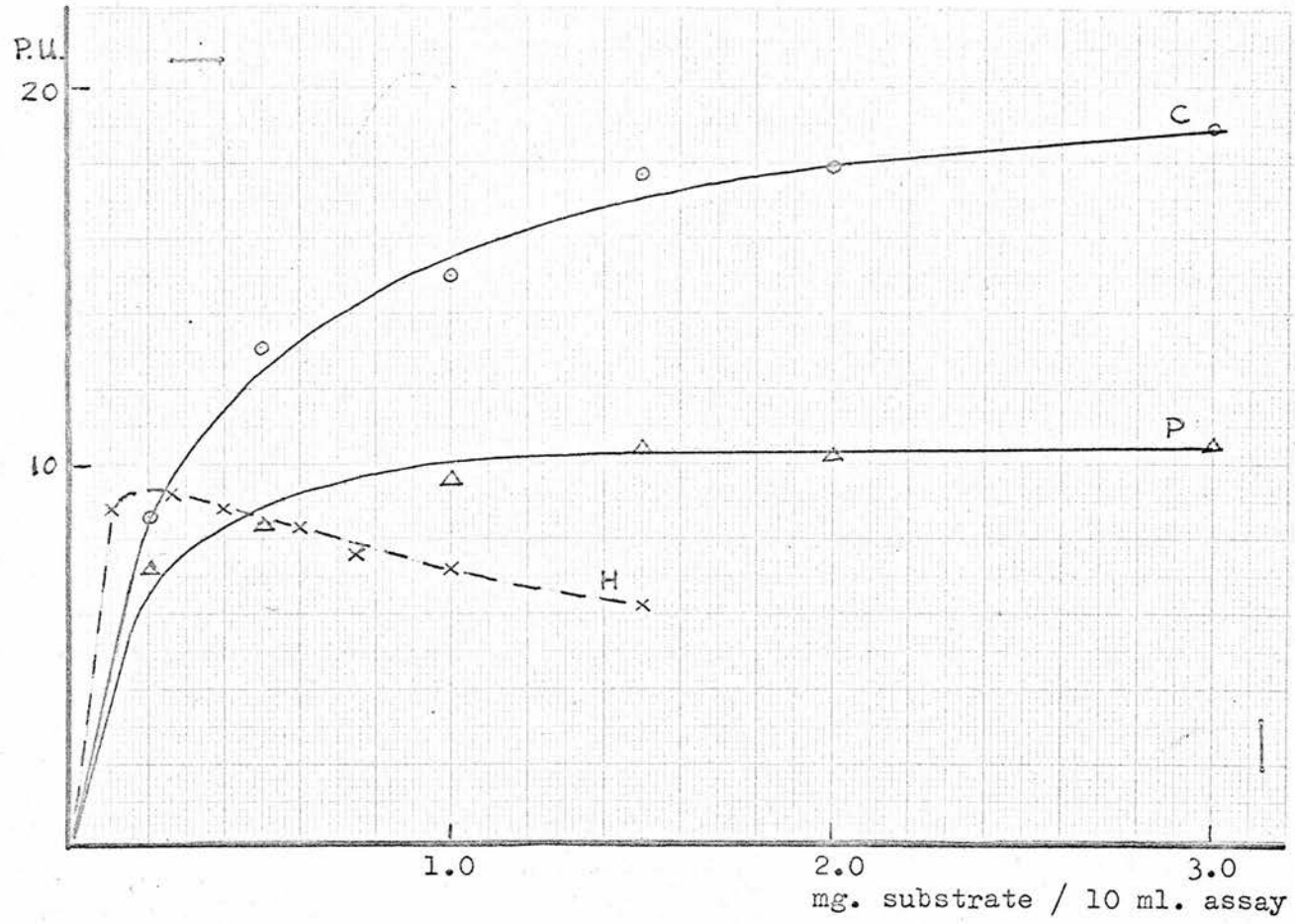


Fig. 2.2.1. Optimal substrate concentrations for Method I.

Stage 5 enzyme solution was used. Assays were incubated at 37° for 24 hr., under standard conditions (Method I), except for variations in substrate concentration.

o——o cadaverine dihydrochloride.
Δ——Δ putrescine dihydrochloride.
×- -× histamine dihydrochloride.

2.0 ml. 0.002 N $KMnO_4$ ≡ 1 μ mole Indigo ≡ 2 μ mole H_2O_2 ≡ 2 μ mole substrate.

Hence 1 P.U. ≡ 0.1 μ mole substrate oxidised per 24 hr.

≡ 6.95×10^{-5} μ mole substrate oxidised per min.

Substrate concentrations.

Histaminase activities were determined at a wide range of substrate concentrations, using the same amount of purified enzyme, and the substrates histamine, cadaverine and putrescine. Assays were incubated for 24 hr., and the substrate concentrations resulting in highest activities were determined from Fig. 2.2.1.

Very nearly maximal activity over 24 hr. was achieved in the presence of 2 mg. putrescine or cadaverine dihydrochloride per 10 ml. assay, and this concentration of putrescine and cadaverine was adopted routinely in the standard assay Method I. Inhibition of histaminase activity by excess histamine was observed; this behaviour is typical of DAO (cf. Section 5.2). 0.25 mg. histamine dihydrochloride per 10 ml. assay was chosen as the standard histamine concentration in Method I.

Since single measurements of residual indigo after 24 hr. incubation gave no reliable indication of initial rates of indigo oxidation, especially at low substrate concentrations, these data were not suitable for the calculation of kinetic constants.

2.2.3. Spectrophotometric adaptation of Kapeller-Adler's method.

Instead of estimating residual indigo by titration with permanganate, the final concentration of indigo in the same assay solution could be measured by its extinction in the region = 500 - 700 mμ. At the extinction maximum, 610 mμ, $= 1.73 \times 10^4$ mole^{-1} cm.$^{-1}$

The concentration of indigo in Kapeller-Adler's indigo test was too high to permit accurate extinction measurements at 610 mμ. The simplest alternative to dilution of the assays was to read the extinctions at 510 mμ, at which wavelength the extinctions of controls and tests, read against water in the reference cuvette, fall within the range 0.6 - 0.4, a region of high precision on the Unicam S.P. 500 spectrophotometer. Isatin and other products of the indigo test have negligible extinction above 500 mμ. Very crude enzyme solutions had appreciable $E_{510\ m\mu}$, but controls incubated with enzyme, but without substrate, corrected adequately for coloured or turbid enzyme preparations.

This spectrophotometric modification simply replaced the final titrimetric stage of Kapeller-Adler's test. In all other respects, the methods were identical.

2.2.4. Kapeller-Adler's investigations of the indigo test.

Kapeller-Adler has modified her original indigo test several times to meet the demands of different enzyme systems. Kapeller-Adler's indigo test was initially adopted in the present work on the basis of evidence presented by Kapeller-Adler (1951) and Kapeller-Adler and Renwick (1956), and after discussion with Dr. Kapeller-Adler.

Kapeller-Adler and Renwick (1956) observed a linear relationship between P.U. measured in the indigo test and histamine inactivation measured in a parallel biological test. They demonstrated linear progress of enzymic

activity, measured in P.U., over a 24 hr. incubation period; the activities of more than 30 P.U. quoted by Kapeller-Adler and Renwick give rise to confusion, since a maximum of 15 P.U. could be detected using the stated amount of indigo in the assays. Diagrams in the publications of Kapeller-Adler (1951) and Kapeller-Adler and Renwick (1956) suggest that P.U. measurements are very precise and reproducible. Titre differences of 0.04 ml. to 0.18 ml. are quoted with an accuracy of ± 0.01 ml. (Kapeller-Adler and Renwick, 1956). Kapeller-Adler (1951) shows a linear dependence between P.U. measured and enzyme concentration, up to 30 P.U. - that is, virtually complete exhaustion of the indigo present.

2.2.5. Practical application of Kapeller-Adler's indigo test in the present work.

Kapeller-Adler's micro-volumetric indigo test was used satisfactorily in the early months of the present work. The test was particularly convenient for large numbers of chromatographic effluents, each test requiring a total of about three minutes bench time. The test was used at all stages of purification, from the saline extract to purified enzyme solutions containing few contaminants. Yields of enzymic activity were usually between 80 - 120% at each stage; well-defined patterns of enzyme eluted from chromatographic columns were obtained; and the ratio of enzymic activities towards cadaverine and histamine (called the C/H ratio) remained fairly constant at about 2 throughout purification, if precautions were taken to avoid selective inhibition by metal ions.

A number of minor difficulties assumed greater importance when increased reproducibility was demanded of the technique. Firstly, the end-point of the permanganate titration was not easily discerned (cf. Burkard, 1954); the sequence of colour changes near the end-point was different for each substrate used, and for the controls without substrate. Even when a single substrate

Table 2.2.1.

Standard deviation of Kapeller-Adler's microvolumetric indigo test.

Replicate assays.	(a) P.U.	(b) P.U.
	5.5	13.1
	5.8	13.9
	4.4	13.6
	6.8	13.0
	4.7	11.4
	5.9	12.5
	5.7	12.8
	5.7	12.3
	5.8	10.8
	6.0	12.6
Mean -	5.5 P.U.	Mean - 12.6 P.U.
S.D. -	0.6 P.U.	S.D. - 0.9 P.U.

was used in a series of experiments, the speed of titration and the choice of end-point colour had to be closely controlled. The colour of a titration nearing completion could not be compared with the previous completed titration, since the end-point colour changed to rose-red within about 30 seconds. With long practice, consistent titres could be obtained within the same system, but any change in substrate, or the presence of coloured contaminants of the enzyme, put a heavy premium on experience.

Since a major object of this work was the comparison of enzymic activities towards several substrates at different stages of purification, end-point difficulties were a source of anxiety.

The standard deviation of the titrimetric method was determined at two dilutions of highly-purified enzyme. Ten assays were performed at each dilution.

Table 2.2.1 shows a mean value of 5.5 ± 0.6 P.U., and a mean value of 12.6 ± 0.9 P.U. These results were reflected in the standard of reproducibility demanded during routine application of the test. Agreement of duplicates within 0.5 P.U. was achieved in about 90% of assays, and a difference of 1.0 P.U. between duplicates led to repetition of the assay. The S.D., more than 10% at 5.5 P.U., became relatively more important at lower enzymic activities, and 5 P.U. was adopted arbitrarily as the lower limit of activity measurable to an acceptable degree of reproducibility. After careful use of the test under the most favourable conditions the author could not achieve the degree of accuracy obtained by Kapeller-Adler (Section 2.2.4).

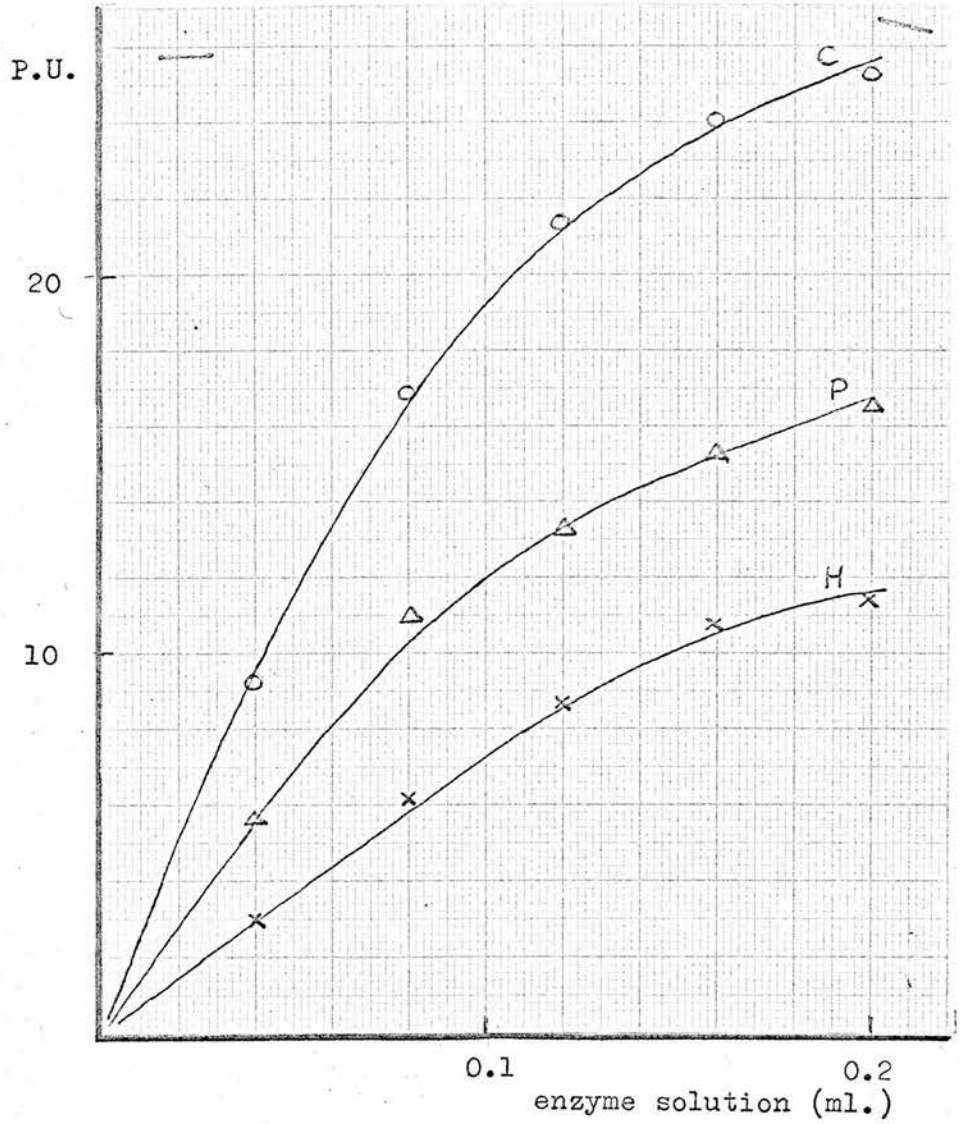


Fig. 2.2.2. Effect of increasing enzyme concentration in Method I (titrimetric form).

Stage 5 enzyme was used. Assays were carried out under standard conditions (Method I).

Substrate: o—o cadaverine
Δ—Δ putrescine
×—× histamine

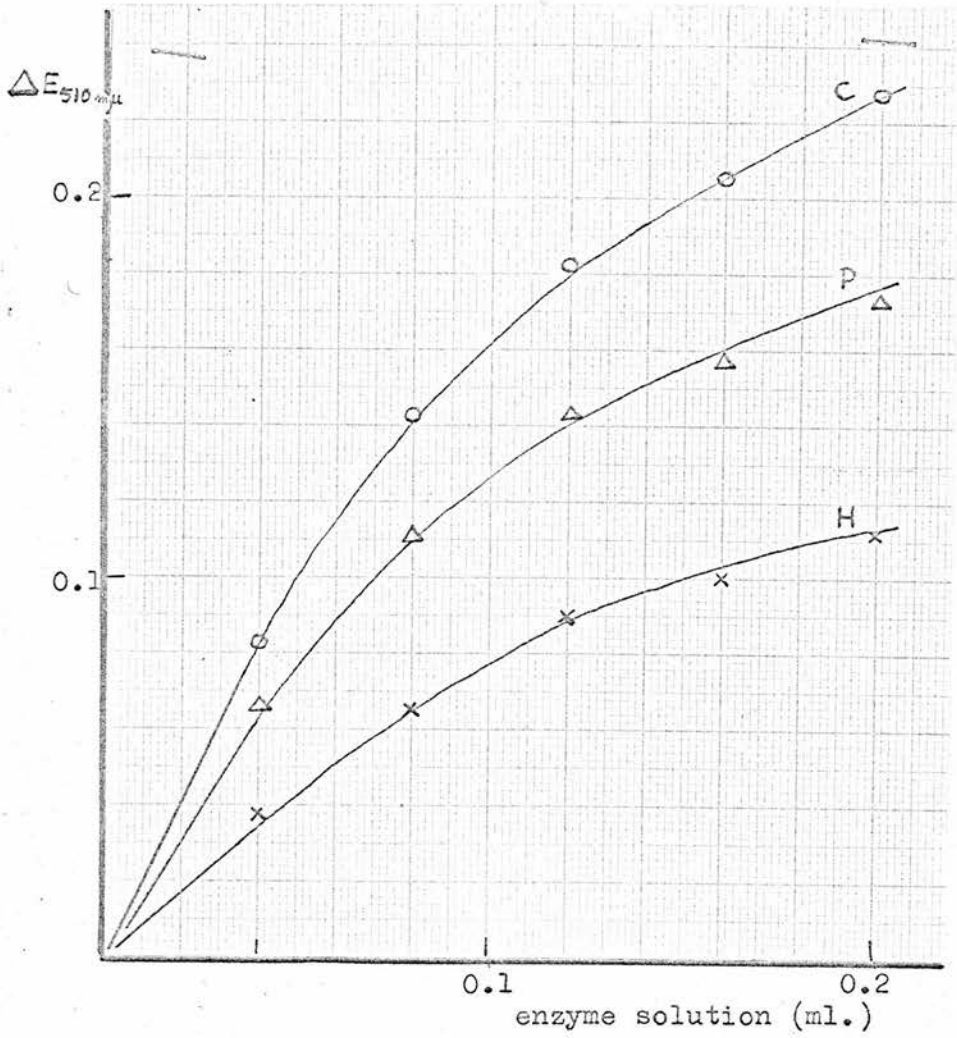


Fig. 2.2.3. Effect of increasing enzyme concentration in Method I (spectrophotometric form).

Assays were incubated exactly as in Fig. 2.2.2.

Substrate: o—o cadaverine
△—△ putrescine
×—× histamine

2.2.6. Range of Kapeller-Adler's test.

More serious inconsistencies were encountered when several dilutions of an enzyme solution of unknown activity had to be assayed in parallel. It was repeatedly observed that indigo oxidation was not linearly related to enzyme concentration throughout the range 0 - 25 P.U. quoted by Kapeller-Adler and Renwick (1956) for placental histaminase.

The variation of indigo oxidation with enzyme concentration is shown in Fig. 2.2.2 for Kapeller-Adler's microvolumetric method (I), and in Fig. 2.2.3 using the same reaction system followed by spectrophotometric estimation of residual indigo. These results were obtained using a solution of enzyme highly purified by salt fractionation and ion-exchange chromatography, but the pattern was similar at all stages of purification of the enzyme. The upper limit of validity of Kapeller-Adler's method was seen to be about 15 P.U., using cadaverine as substrate. As shown above, the poor reproducibility of the method sets a lower limit of measurement at about 5 P.U. This range of reliability, 5 - 15 P.U., is thus not as wide as that reported by Kapeller-Adler (1951).

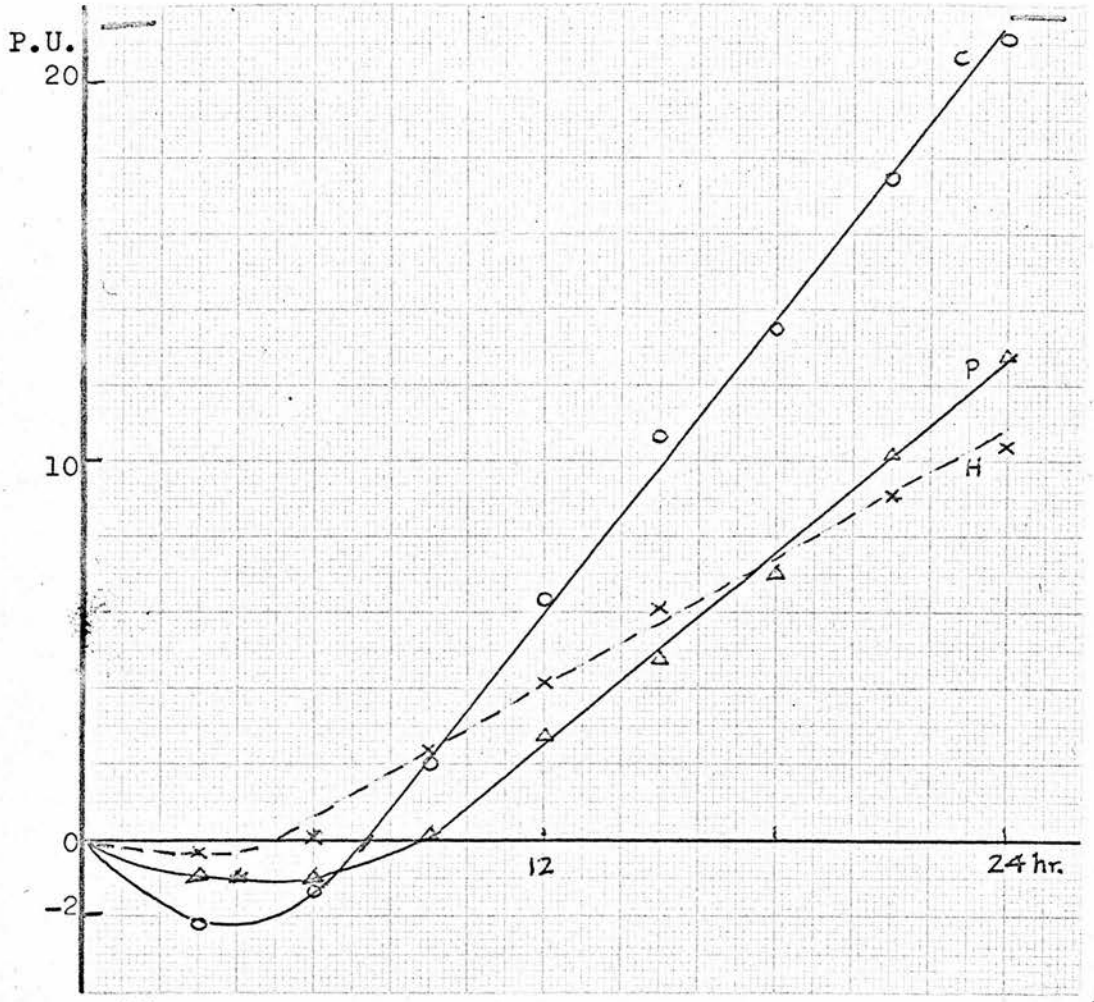


Fig. 2.2.4. Progress of indigo oxidation in Method I.

Stage 5 enzyme was used. Assays were carried out under standard conditions (Method I), except for variation of the incubation period. Activity expressed in P.U., obtained by subtracting test titre from control titre.

Substrate: o—o cadaverine
△—△ putrescine
×--× histamine

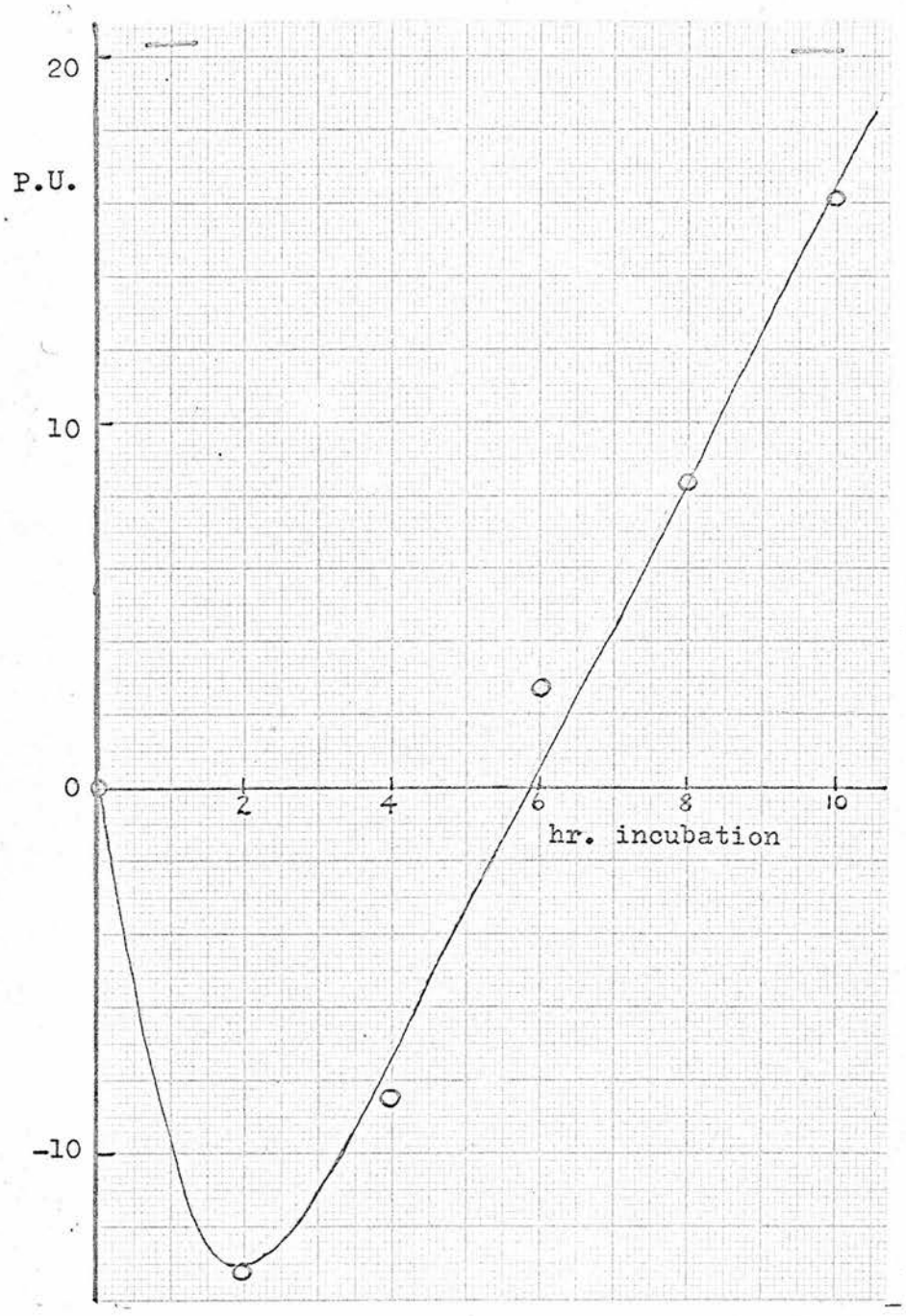


Fig. 2.2.5. Titrimetric estimation of indigo in the early hours of incubation of assay Method I.

Stage 6 enzyme was used under standard conditions (Method I). The substrate was cadaverine.

2.2.7. Progress of indigo oxidation in Kapeller-Adler's test.

For these reasons it was considered important to check that indigo oxidation increased linearly with the period of incubation of the indigo test, as indicated by Kapeller-Adler and Renwick (1956).

A number of replicate assays were set up in parallel, using Kapeller-Adler's method (I), and a highly purified solution of placental histaminase. At intervals during incubation, "test" and "control" assays were withdrawn for titration of residual indigo, and the titre difference expressed in the usual way - "control" titre (ml.) minus "test" titre (ml.) x 10 = P.U. Progress curves are shown in Fig. 2.2.4 for the substrates histamine, cadaverine and putrescine. A period during which indigo oxidation is truly linear is preceded by a period during which no indigo appears to be oxidised, and indeed more reducing substance is present in the test solution than in the control. Similar "lag periods" were observed when the experiment was repeated using enzyme solutions at different stages of purification.

The early progress of indigo oxidation was re-examined, using a larger amount of enzyme, in an attempt to obtain more significant titre differences. Fig. 2.2.5 shows that an increased amount of enzyme magnified the excess of reducing substance titratable in the test assay during the first few hours of incubation.

Indigo was then omitted from assays, incubated otherwise under standard conditions, (Method I). Test and control assays were titrated at intervals with 0.002 N permanganate solution to a permanent pink-brown end-point; while the technique could not be recommended for the routine estimation of such small amounts of reducing substance, the negative titre differences (up to 1.7 ml.) were certainly significant. Neither substrate

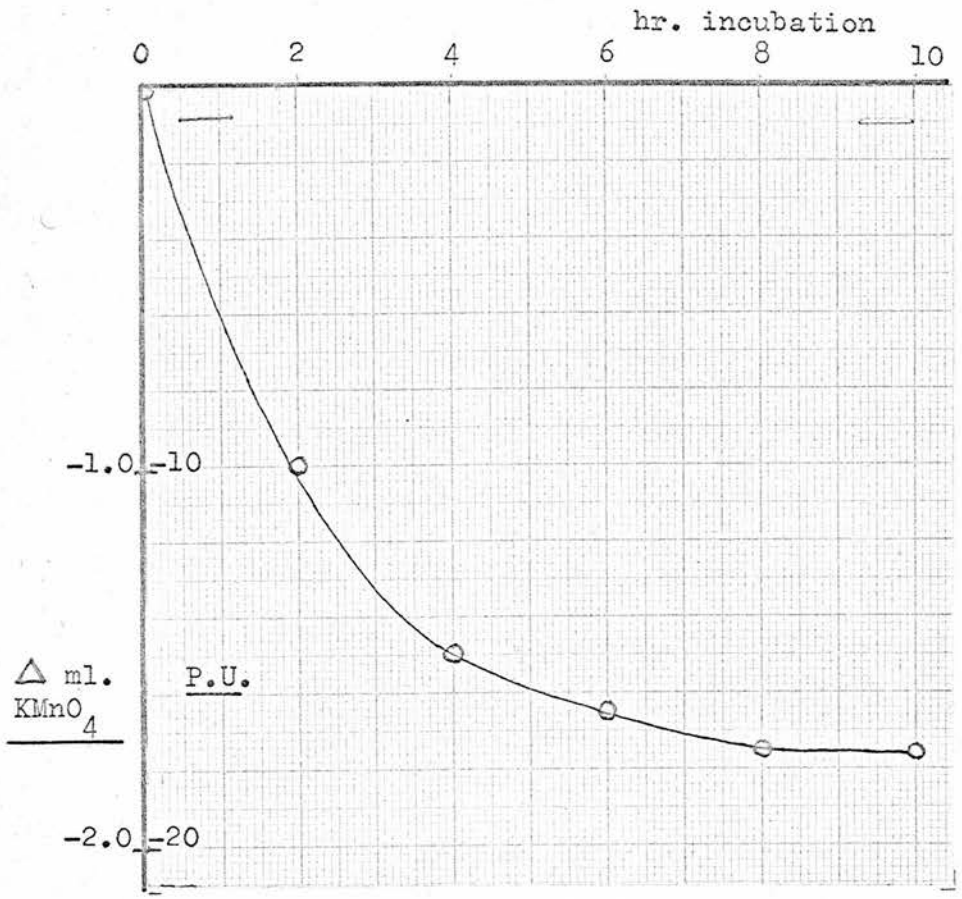


Fig. 2.2.6. Titration of tests incubated without indigo.

Tests and controls were incubated as in standard Method I, except for the omission of indigo. The production of reducing substances was expressed in the usual way :-

(Control titre (ml.) - Test titre (ml.)) x 10 = P.U.

nor enzyme alone was oxidised in this medium by permanganate, so that the excess reducing substance must have been a product of the enzyme-substrate reaction. Fig. 2.2.6 shows that the excess reducing substance, accumulating in the absence of indigo, reached a constant level after some 8 hr.

It was concluded from these experiments that the negative titre differences of Kapeller-Adler's indigo test were due to a delay in indigo oxidation by hydrogen peroxide formed by the enzyme-substrate reaction. In the early hours of the test, accumulated hydrogen peroxide was titratable with permanganate, according to the reaction:

$$2\ MnO_4^- + 5H_2O_2 + 6H^+ \longrightarrow 2Mn^{++} + 5O_2 + 8H_2O.$$

a reaction commonly used to standardise hydrogen peroxide (Vogel, 1961, p. 295). Test solutions containing both indigo and hydrogen peroxide therefore had a higher permanganate titre than controls, containing indigo only. To explain the constant level of hydrogen peroxide reached in the absence of indigo, it could be suggested that the accumulated hydrogen peroxide may (a) denature the enzyme, (cf. Section 2.3.7), (b) increase the rate of the reverse enzymic reaction, or (c) be removed by a bimolecular reaction, such as:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2.$$

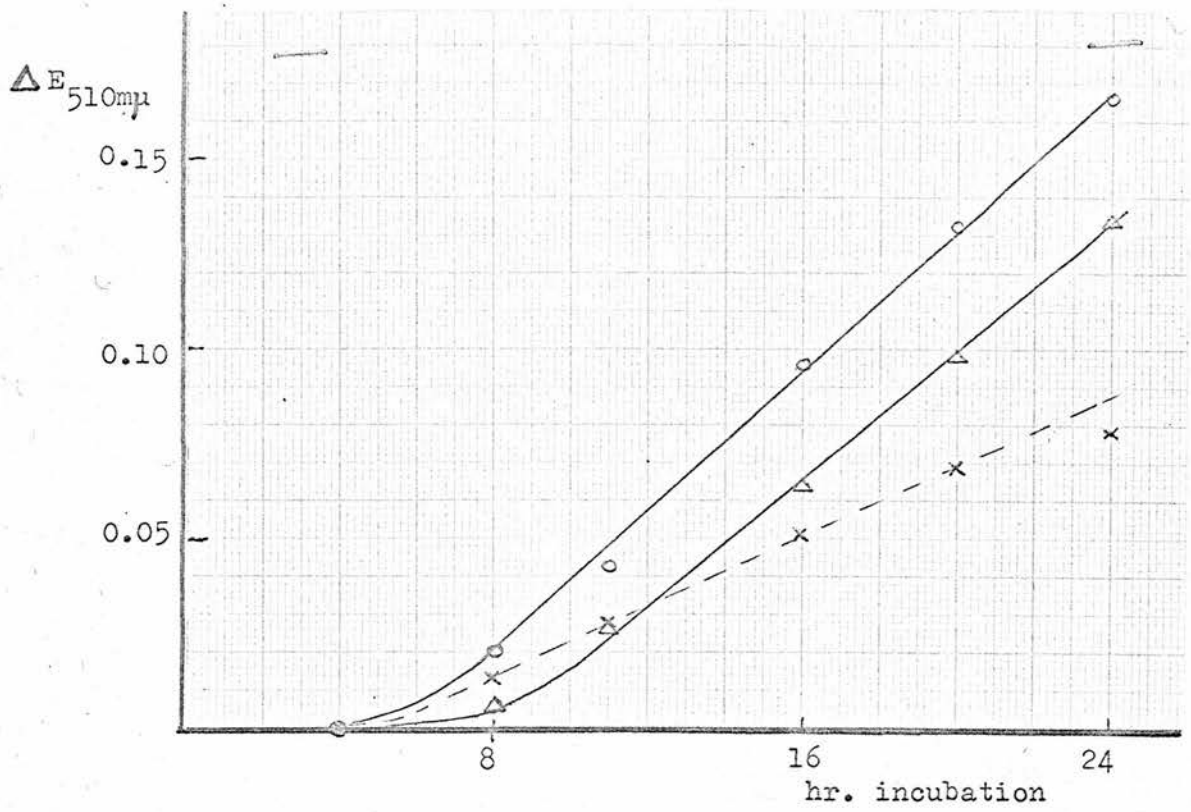


Fig. 2.2.7. Progress of indigo oxidation in assay Method I (spectrophotometric form).

Stage 5 enzyme was used. Assays were carried out under standard conditions (Method I) except for variation of incubation period.

Substrates: o——o cadaverine
Δ——Δ putrescine
× - - × histamine

2.2.8. Spectrophotometric investigation of the lag period.

When the progressive oxidation of indigo was followed spectrophotometrically rather than titrimetrically, the lag period was confirmed. Fig. 2.2.7 shows the progressive decrease in $E_{510m\mu}$ of assays set up and incubated under standard conditions (Method I) for the indigo test. For a considerable period, only very slow indigo oxidation took place - but no "negative" extinction differences were observed. This observation supported the belief that negative titre differences in the early hours of the titrimetric test were due to hydrogen peroxide, and not to any change in the indigo. The lag periods were of a similar order for all three substrates used.

A series of experiments was carried out to test the hypothesis that indigo was not oxidised by hydrogen peroxide in the initial stages of incubation, and to find some explanation of this anomaly. The enzyme used in these experiments was the purest available at that time, but contained appreciable amounts of haptoglobin-methaemoglobin complex (HpMHb), haemoglobin (Hb) and methaemoglobin (MHb). Multiple assays were set up, essentially according to the standard Method I, single or duplicate tests and blanks being withdrawn from incubation at intervals for extinction measurements at 510 mμ. Lag periods were defined as the intercepts of the linear portion of the progress curves on the time axis. Considerable scatter of experimental points often made extrapolation uncertain; a difference in lag periods of one hour could not be regarded as significant in these experiments. The scatter was due to the high S.D. of replicate assays, and the small extinction differences observed, especially in the early hours of incubation.

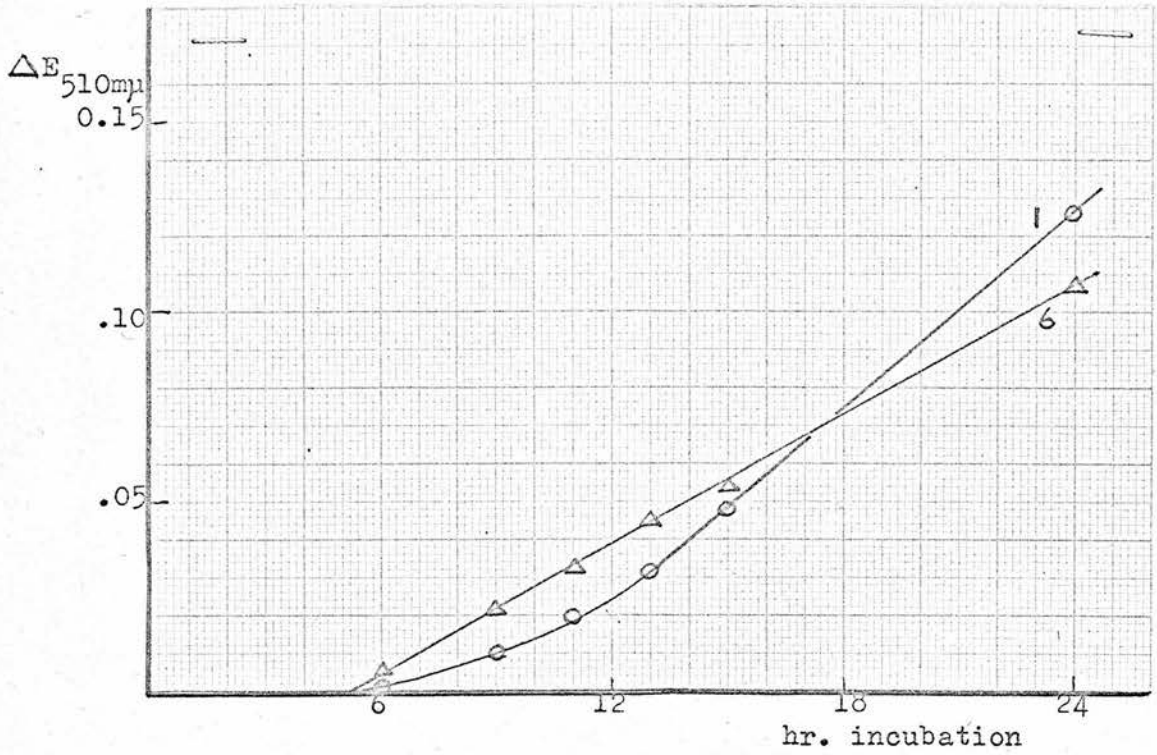


Fig. 2.2.8. Effect of enzyme purity on delay in indigo oxidation.

Standard assay conditions (Method I) were used, with cadaverine as substrate.

o —— o Crude enzyme; Stage 1, dialysed.
Δ —— Δ Purified enzyme; Stage 6.

2.2.9. Is the lag period an artefact of the purification process?

The preceding experiments on the delay in indigo oxidation might be misleading if the purified enzyme solutions employed had been deficient in a histaminase cofactor, or some catalyst of indigo oxidation, lost during the purification process. To check this possibility, progress curves for the indigo test were obtained using, in parallel, the purest enzyme solution available, and the crude, dialysed placental extract: undialysed placental extract could not be used, since it was always severely inhibited by dialysable contaminants.

Fig. 2.2.8 shows that the saline extract displayed a rather longer lag period than the purified enzyme. This comparison disposed of the only obstacle to the use of purified enzyme preparations in the investigation of the lag period, and discouraged further arbitrary attempts to "reactivate" the purified enzyme with hypothetical cofactors or activators.

2.2.10. Is an inhibitor present in one of the assay components?

If one of the components of the assay system contained an oxidisable contaminant capable of (a) inhibiting the enzyme-substrate reaction, or (b) competing with indigo for hydrogen peroxide formed by the enzymic reaction, an initial period of incubation during which hydrogen peroxide was consumed by the inhibitor, rather than by indigo, could be succeeded by a period of linear indigo oxidation, when the inhibitor had been completely oxidised to a harmless product. Such behaviour would be consistent with the pattern of indigo oxidation in the assay, although it would not explain the accumulation of hydrogen peroxide in the early hours of incubation (Section 2.2.7).

The possible presence of an oxidisable inhibitor was checked by varying the concentration of each assay component in succession. The design

of these experiments and the conclusions drawn from them may be clarified by an analysis of the possible sources of inhibition.

In this discussion, A and B represent contaminants of the assay system, which could cause a delay in indigo oxidation by different mechanisms:

A inhibits the action of histaminase on its substrate, but not completely, and A may be oxidised by hydrogen peroxide to a substance which does not inhibit the enzyme.

B is more readily oxidised than indigo by hydrogen peroxide formed in the enzymic reaction, but its oxidation product cannot compete with indigo for hydrogen peroxide.

(i) Contamination by a histaminase inhibitor, A.

If A is a contaminant of any component of the assay system, other than the enzyme solution, an increase in concentration of that component in the assay would result in an increase in inhibition of enzymic activity, and an increase in the delay in indigo oxidation. The lag period might not vary linearly with increase in concentration of the contaminated component; it is conceivable that a minute amount of A might be sufficient to "saturate" the enzyme's active centres almost completely, so that further increments of A would have little effect on the lag period. However, such potent inhibition would be inconsistent with the mechanism proposed above, which requires that the enzyme should rapidly produce sufficient hydrogen peroxide to destroy the inhibitor, A.

If A is a contaminant of the enzyme solution, an increase in enzyme concentration and activity would be offset by increased inhibition by A. Depending on the type of inhibition involved, the affinity of the enzyme for the inhibitor, and the relative concentration of the inhibitor in the enzyme

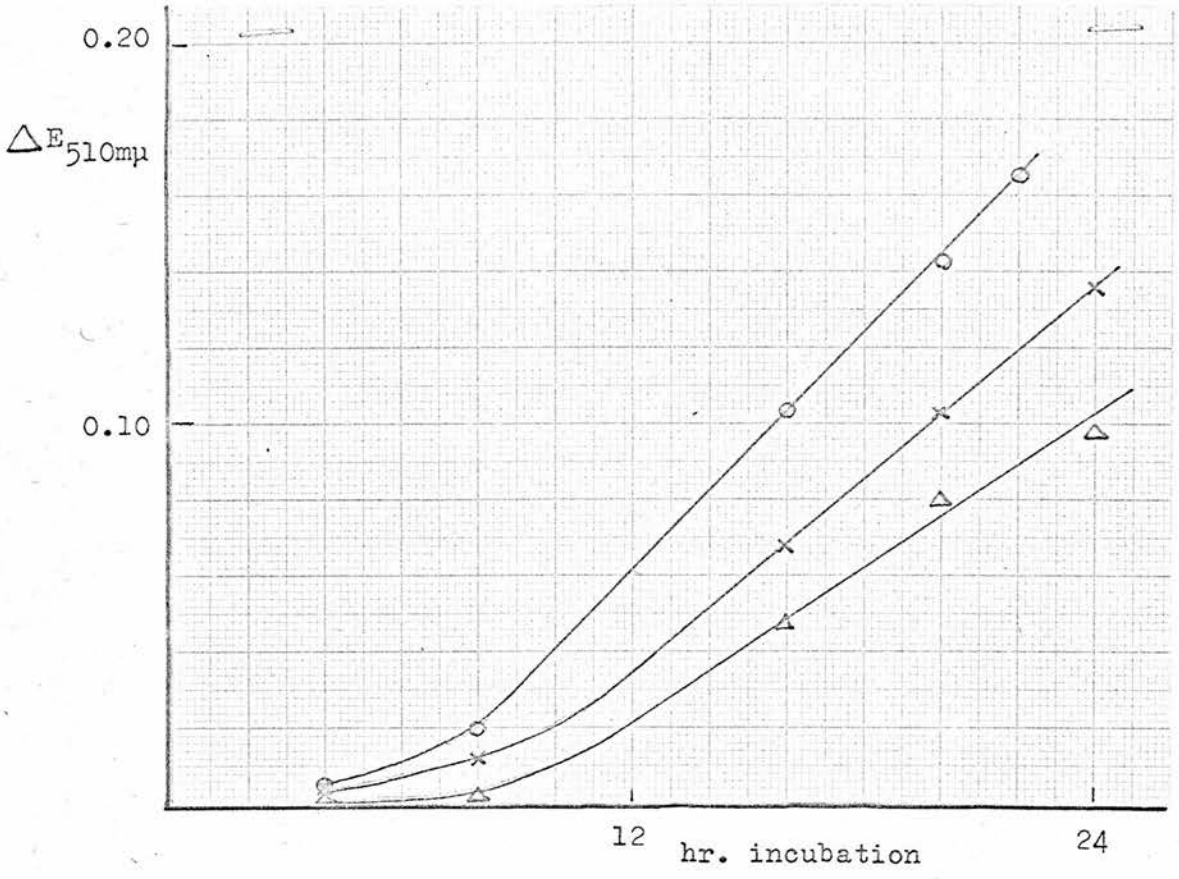


Fig. 2.2.9. Effect of cadaverine concentration on delay in indigo oxidation.

Standard assay conditions were used, except for variations in cadaverine concentration.

2 mg.	cadaverine. 2HCl	(final conc.	1.14 mM)
0.5 mg.	"	"	0.29 mM
0.2 mg.	"	"	0.114 mM

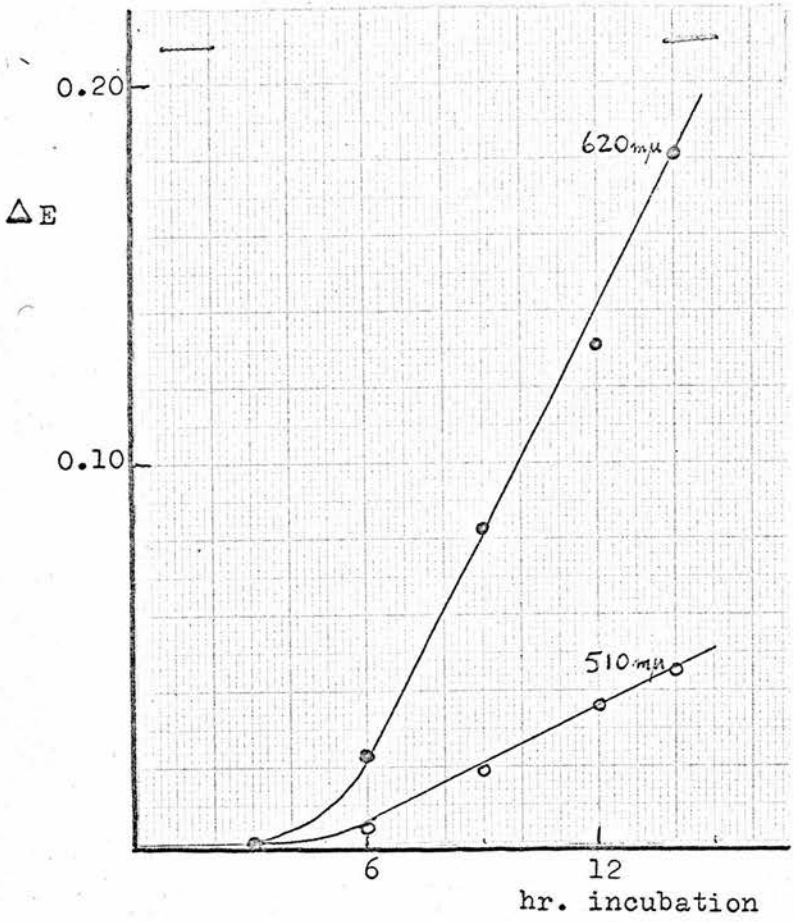


Fig. 2.2.10. Effect of initial indigo concentration on delay in indigo oxidation.

Both series of assays were incubated under standard conditions (Method I), except for variation of indigo concentration. The extinction of assays at the lower concentration of indigo was read near the wavelength of maximum extinction, rather than at 510 mμ.

o—o 2 ml. indigo (final conc. 286 μM); decrease in $E_{510m\mu}$

●—● 0.2 ml.indigo (final conc. 28.6μM); decrease in $E_{620m\mu}$

solution, the lag period might be increased or remain constant with increased concentration of contaminated enzyme in the assay. However, the type of inhibition proposed above would not be consistent with a decrease of lag period with increased concentration of enzyme.

(ii) Contamination by a competitor of indigo oxidation, B.

If B is a contaminant of the enzyme solution, the increased inhibitor concentration should be precisely offset by an increase in enzymic production of hydrogen peroxide; no change in the lag period would be expected on increasing the concentration of contaminated enzyme in the assay.

If B is a contaminant of any other component of the assay, the lag period due to preferential oxidation of B by hydrogen peroxide should vary directly with the concentration of contaminated component in the assay.

Experimental observations and conclusions.

In the experiment illustrated in Fig. 2.2.9, the concentration of cadaverine was varied tenfold. Dilution of the substrate markedly decreased the rate of indigo oxidation, as a result of the lower enzymic activity, but did not substantially alter the lag period. Fig. 2.2.10 shows the effect of a tenfold variation of indigo concentration in the assay. Reduction of indigo concentration did not significantly reduce the lag period. It was further confirmed that the assay buffer, made from Analar salts and glass-distilled water, contained no oxidisable inhibitor of the required characteristics; five-fold reduction of buffer concentration, or the omission of EDTA, did not alter the lag period.

These experiments strongly suggest that the delay in indigo oxidation in Kapeller-Adler's indigo test is not due to the presence of oxidisable contaminants in the assay reagents.

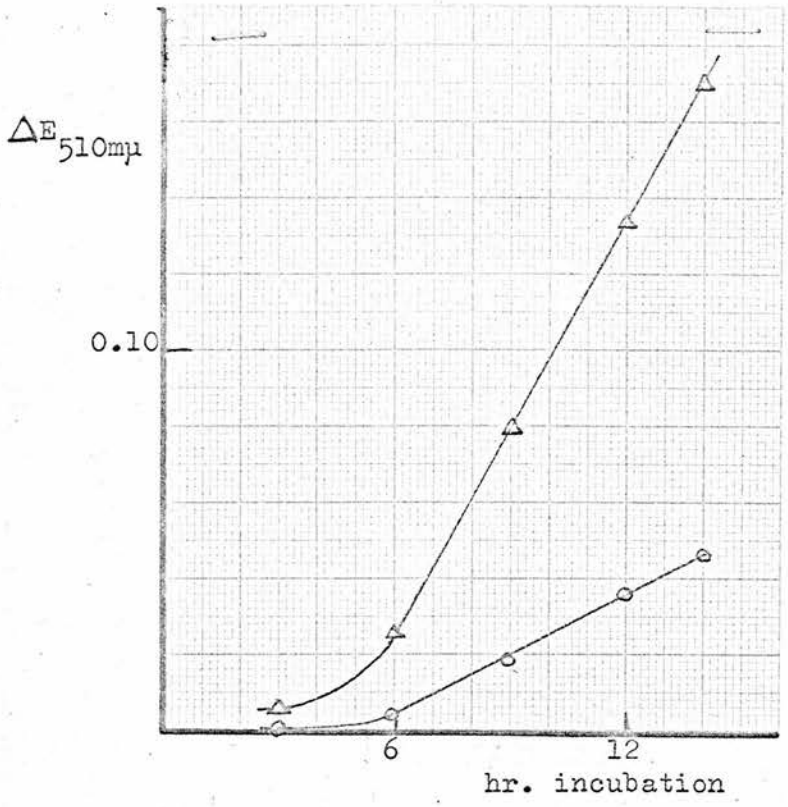


Fig. 2.2.11. Effect of enzyme concentration on delay in indigo oxidation.

Stage 5 enzyme was assayed under standard conditions, (Method I). 0.2 ml. enzyme solution was appropriate for a standard 24 hr. assay.

o——o 0.2 ml. enzyme solution
Δ——Δ 1.0 ml. enzyme solution

In the experiment illustrated in Fig. 2.2.11, a five-fold decrease in enzyme concentration failed to reduce the lag period. This was considered fair evidence that no inhibitor of the enzyme-substrate reaction, present in the enzyme solution, was responsible for the lag period; but this experiment did not exclude the possibility that the enzyme solution contained an oxidisable substance competing with indigo for hydrogen peroxide. This point will be recalled in Section 2.2.13 below.

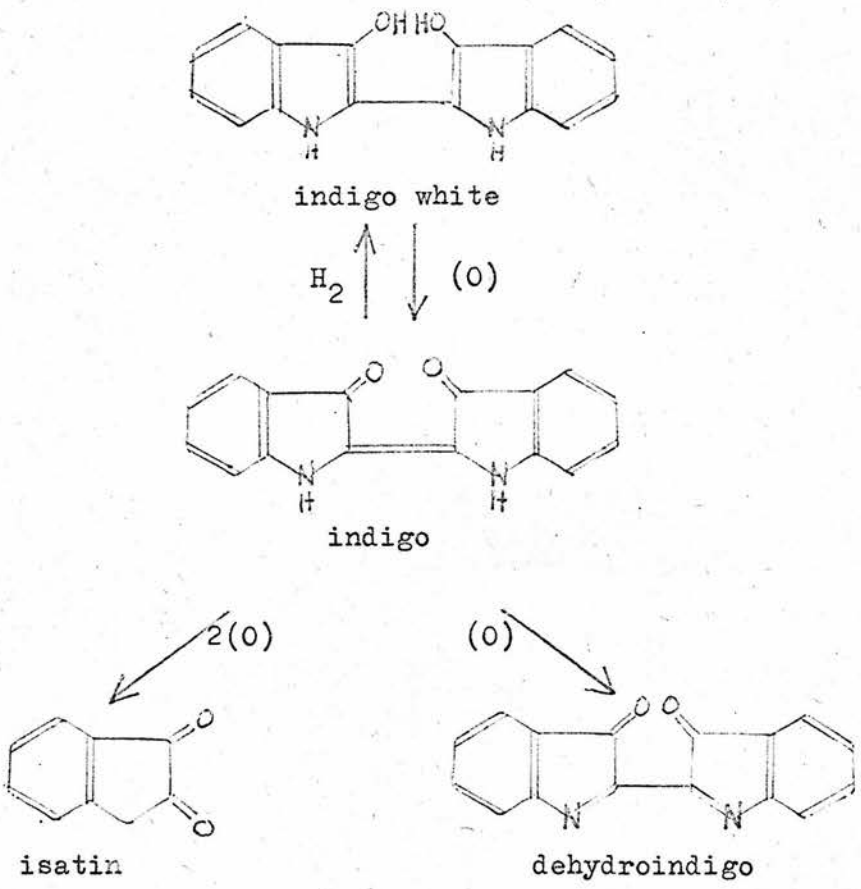
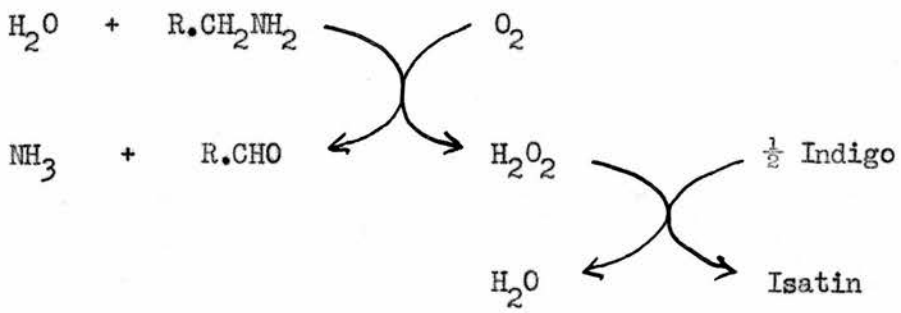


Fig. 2.2.12. Oxidation and reduction of indigo.

2.2.11. A possible alternative mechanism of indigo decolorisation.

Indigo, besides being oxidisable to isatin, may be decolorised by reduction to "indigo white" (Fig. 2.2.12).

Zeller (1938b) found that the decolorisation of indigo in the presence of DAO and substrate was increased by oxygen and inhibited by nitrogen. Kapeller-Adler and MacFarlane (1963) have claimed that indigo tests carried out without prior oxygenation may lose 65% of the activity in oxygen. The accepted mechanism of indigo oxidation in the presence of histaminase and substrate may be represented as follows:



It is conceivable that, contrary to the accepted oxidative mechanism, indigo decolorisation may be due to a reductive mechanism, indigo being used to re-oxidise a co-factor, F, reduced in the initial enzyme-substrate reaction. Oxygen present in the assay might compete with indigo for the reduced co-factor. When the oxygen was exhausted, decolorisation of indigo by reduction might proceed. If the competing oxygen formed hydrogen peroxide with the reduced co-factor, the free hydrogen peroxide detectable in the early hours of the indigo test might be explained, if it were further assumed that hydrogen peroxide did not oxidise indigo. Interference with the reductive decolorisation of indigo would be increased by increased oxygen tension in the assay solution.

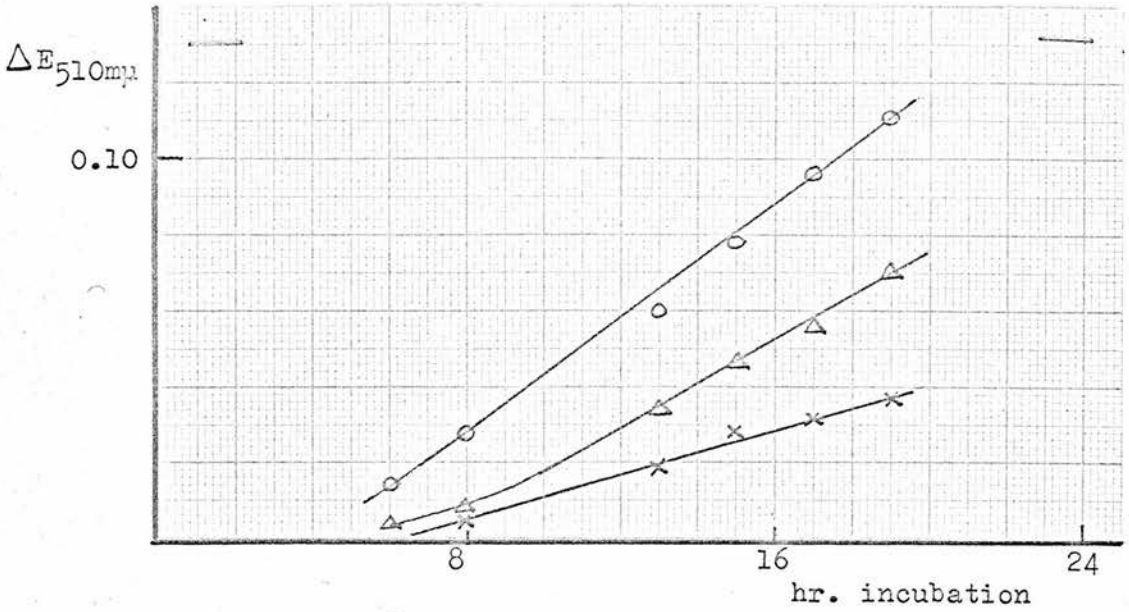
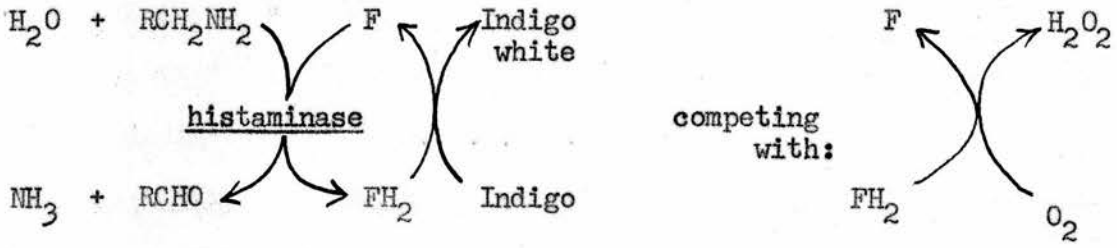


Fig. 2.2.13. Investigation of a possible reductive mechanism of indigo decolorisation.

Stage 5 enzyme was assayed under standard conditions, except for incubation under different oxygen tensions.

o——o oxygen passed for 1 min.
Δ——Δ no gas passed.
×——× nitrogen passed for 1 min.



To test this hypothesis, assays were carried out in varying concentrations of oxygen (1) after bubbling oxygen for one minute, (2) without oxygenation (i.e. in air) and (3) after bubbling nitrogen for one minute. The results are illustrated graphically in Fig. 2.2.13.

Indigo oxidation decreased with decreasing oxygen tension in the assays, and the reductive mechanism discussed above is inconsistent with this experimental result. Further, the lag period was not increased by increasing the concentration of oxygen. The U.V. and visible spectrum of an indigo test, in which part of the indigo had been decolorised by the enzyme-substrate reaction, showed peaks characteristic of isatin as well as of unchanged indigo.

2.2.12. Possible autocatalytic mechanisms.

The shape of progress curves for the indigo test is partially consistent with an autocatalytic mechanism; an initially very slow enzymic reaction may release into the assay system a catalyst which can accelerate the enzymic reaction. Ideally, this mechanism should result in indigo oxidation varying exponentially with time, but a rectilinear pattern might be imposed by some rate-limiting factor in the overall reaction; for instance, some component of the assay system, e.g., a minor contaminant of the enzyme solution, might combine with one of the products of the enzyme reaction to form a catalyst which accelerates either the enzymic reaction or the oxidation of indigo by hydrogen peroxide. When all the contaminant had been used to form the catalytic compound, the rate of indigo oxidation would cease to be exponential, and become linear.

If indigo oxidation in the test were catalysed directly or indirectly by a product of the enzymic reaction, deliberate addition of this reaction product to the assay before incubation should hasten the onset of the period of linear indigo oxidation. The chief overall products of the indigo test are ammonia, hydrogen peroxide, isatin sulphonate and Δ^1-piperidine (when cadaverine is used as substrate). Ammonia inhibits the indigo test, (Section 5.3) and was not considered a likely catalyst; synthetic hydrogen peroxide could easily be added to the assay; although isatin sulphonate was not readily available, isatin, which should be similar in most reactions to the sulphonate, could be added to the assay; Δ^1-piperidine was not readily available in pure form. All these products could be tested for catalytic effect by adding to the assay an aliquot of a completed indigo test. Since it was necessary, however, to boil the completed indigo test to destroy its

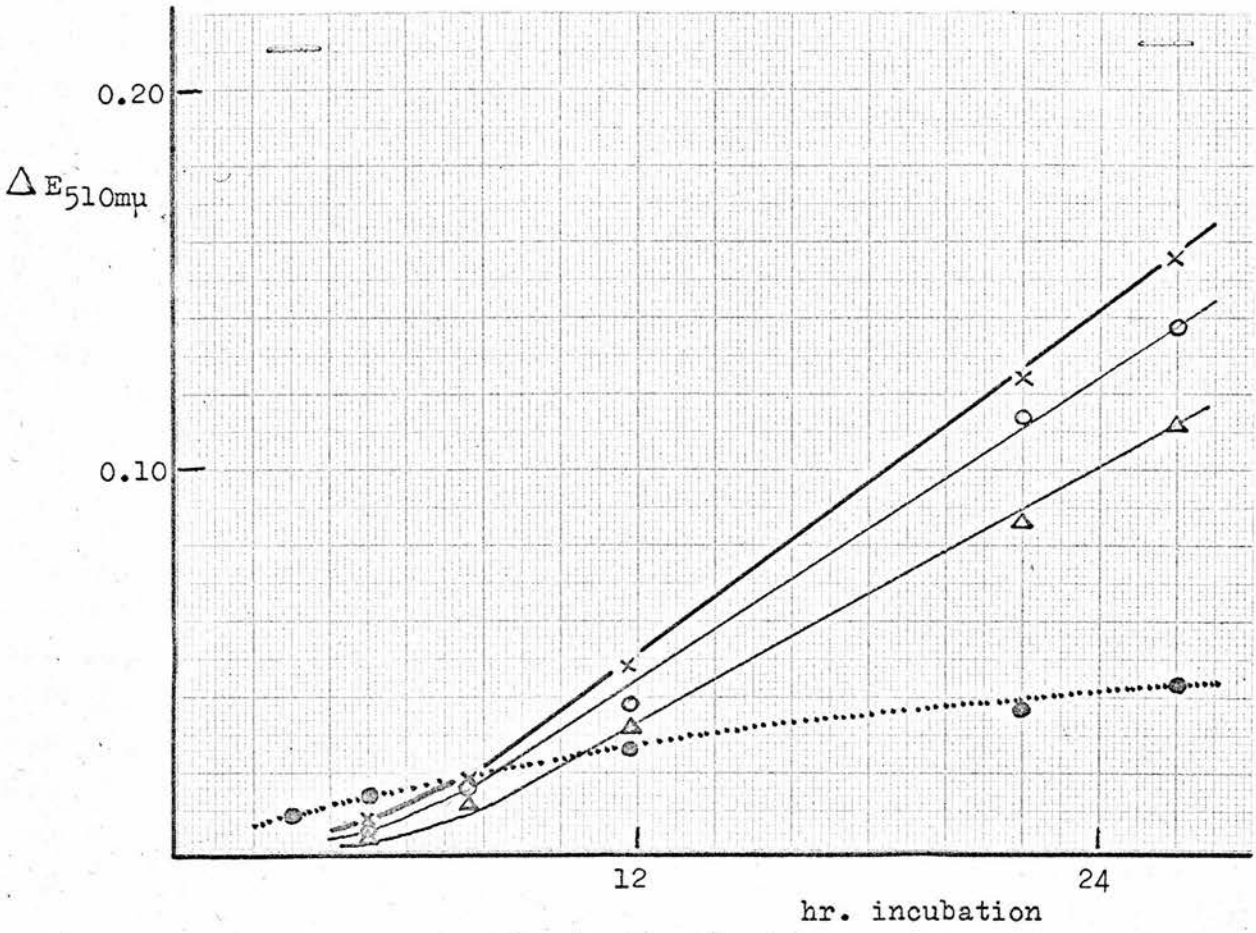


Fig. 2.2.14. Investigation of possible autocatalytic mechanisms.

Stage 5 enzyme was assayed under standard conditions, (Method I), except for the addition of possible catalysts of indigo oxidation.

×——× No addition.
o——o "Boiled test" added.
Δ——Δ Isatin (84 µg) added.
●·····● H_2O_2 (2.4 µmole) added.

enzymic activity, the possibility could not be dismissed that a thermo-labile product of the indigo test might be absent from the aliquot added to the test assay.

Three potential catalytic agents were added to the indigo test (Method I) and the progress of indigo oxidation followed in each case. (1) Isatin was added to the test in an amount approximately equivalent to the amount of indigo oxidised in six hours in a 10 P.U. indigo test. (2) Hydrogen peroxide (Analar), diluted in assay buffer, was added in a concentration calculated to result in an extinction change of 0.24 units at 510 mμ, assuming the equation:

$$2\ H_2O_2 \ + \ \text{Indigo} \longrightarrow 2\ H_2O \ + \ 2\ \text{Isatin}$$

(3) A complete indigo test was allowed to proceed for 48 hours, until the histaminase-cadaverine reaction had oxidised about two-thirds of the indigo initially present. This completed test solution was placed in boiling water for ten minutes, and 0.5 ml. of "boiled test" solution added to a series of assays. "Boiled test" was enzymically inactive, but should have contained indigo, isatin, cadaverine, Δ^1-piperidine and any heat-stable co-factors of histaminase.

Fig. 2.2.14 shows the results of adding these possible catalysts to a standard indigo test. Isatin and "boiled test" inhibited the rate of indigo oxidation, but had no significant effect on the lag period. Synthetic hydrogen peroxide inhibited histaminase, and the added hydrogen peroxide failed to oxidise indigo stoichiometrically, even after 26 hr. incubation in the presence of histaminase. These experiments did not support any theory of autocatalysis of indigo oxidation by one of the products of the reaction -

Table 2.2.2.

Indigo µmole	Enzyme ml.	Cadaverine µmole	Histamine µmole	H_2O_2 µmole	E 510 mµ Exptl.	Theor.
2.86	-	-	-	-	0.631	0.631
2.86	-	-	-	0.25	0.631	0.606
2.86	-	-	-	0.5	0.628	0.581
2.86	-	-	-	1.0	0.616	0.531
2.86	0.1	-	-	-	0.631	0.631
2.86	0.1	-	-	0.25	0.631	0.606
2.86	0.1	-	-	0.5	0.628	0.581
2.86	0.1	-	-	1.0	0.616	0.531
2.86	0.2	-	-	-	0.633	0.633
2.86	0.2	-	-	0.25	0.631	0.608
2.86	0.2	-	-	0.5	0.627	0.583
2.86	0.2	-	-	1.0	0.616	0.533
2.86	-	11.4	-	-	0.629	0.629
2.86	-	11.4	-	0.5	0.592	0.579
2.86	-	-	1.36	-	0.630	0.630
2.86	-	-	1.36	0.5	0.598	0.578

but the possibility of autocatalysis by a thermolabile reaction product could not be eliminated.

The second stage of the indigo test has been stated to depend on the peroxidatic properties of histaminase, or the presence of some ubiquitous contaminant with peroxidatic properties (Zeller, 1956; Kapeller-Adler, 1956b). The experimental demonstration, in Fig. 2.2.14, of slow indigo oxidation by synthetic hydrogen peroxide did not agree with this view, and led to further experiments in which synthetic hydrogen peroxide was used as a substitute for enzymically-produced hydrogen peroxide in the second stage of the indigo test.

On the basis of the equation:

$$2\ H_2O_2 \quad + \quad \text{Indigo} \longrightarrow 2\ H_2O \quad + \quad 2\ \text{Isatin}$$

it was estimated that 1 μmole H_2O_2 was equivalent to an extinction decrease: $\Delta E_{510m\mu} = 0.1$ in the indigo test (Method I).

The catalytic effect of purified histaminase (Stage 6) in the indigo-synthetic hydrogen peroxide reaction was tested, using two concentrations of enzyme and several concentrations of synthetic hydrogen peroxide. Similarly, the catalytic effects of cadaverine and histamine in the oxidation of indigo were compared. The components of these tests and their extinctions at 510 mμ after 24 hr. incubation, are presented in Table 2.2.2.

These results confirmed that the oxidation of indigo by synthetic hydrogen peroxide is not stoichiometric according to equation 1, and that the purified enzyme had no peroxidase-like activity in this system (although it was contaminated with haptoglobin-methaemoglobin, a potent peroxidase in many systems). Substrates of histaminase ~~had some peroxidatic activity,~~ increased the rate of indigo oxidation but the

theoretical amount of indigo oxidation did not occur even in the presence of these substrates. Moreover, cadaverine ~~was, if anything, a better peroxidase~~ accelerated indigo oxidation better than histamine, which does not agree with Zeller's claim (1963) that histamine is especially active in this respect.

It is difficult to avoid the conclusion that synthetic hydrogen peroxide and hydrogen peroxide produced by the action of histaminase on its substrate do not have the same properties in the second stage of the indigo test. The hydrogen acceptor which oxidises indigo in the enzymic test (and which can be titrated with permanganate in the early hours of such a test) may not be identical with hydrogen peroxide. The term "nascent hydrogen peroxide" must be eschewed as a vitalist heresy, but it is scarcely more informative to suggest that, in the enzymic test, hydrogen peroxide is produced in a form "specially active" towards indigo. In the absence of any explanation of this postulated "activation", it remains impossible to decide whether hydrogen peroxide or some precursor is the active principle in the second stage of the indigo test.

Two further theories, involving autocatalysis of the indigo test by hydrogen peroxide, were examined. (1) The lag pattern of indigo oxidation suggested the possibility that the concentration of hydrogen peroxide in the assay had to reach a critical or threshold level before linear indigo oxidation commenced. (2) It was conceivable that histaminase, or some contaminant of the enzyme solution, became an active peroxidase in the oxidation of indigo only after oxidation by hydrogen peroxide formed in the enzyme-substrate reaction. Either of these autocatalytic mechanisms would result in a delay in indigo oxidation, followed by linear indigo oxidation, but the second theory would not explain the accumulation of hydrogen peroxide during the lag period. (Section 2.2.7).

Despite the discrepancies between the behaviour of synthetic and enzymic hydrogen peroxide in the oxidation of indigo, these theories could only be tested using synthetic hydrogen peroxide as a substitute for enzymic hydrogen peroxide.

Purified histaminase (Stage 5) was pre-incubated with synthetic hydrogen peroxide in assay buffer for six hours before the addition of substrate and indigo, and the progress of indigo oxidation followed thereafter. The lag periods for these tests were compared with the lag periods for tests in which the enzyme was pre-incubated in assay buffer without hydrogen peroxide, or in which hydrogen peroxide was added at the same time as substrate and indigo. The results are tabulated below:

Pre-incubation period (hr.)	Hydrogen peroxide added (μmole)	Lag period (hr.) after addition of indigo and substrate
6	0	6
6	0.3	6
6	0.6	6
0	0	5
0	0.6	5

Pre-incubation of the enzyme with hydrogen peroxide did not significantly alter the delay in indigo oxidation when the enzyme-substrate reaction was initiated. Had either of the above theories been applicable, the lag period would have been reduced by pre-incubation of histaminase with hydrogen peroxide, provided synthetic hydrogen peroxide was an appropriate substitute for enzymic hydrogen peroxide. Since the latter reservation was never adequately proved, these model experiments using synthetic hydrogen peroxide cannot be regarded as conclusive disproof of a reaction mechanism involving autocatalysis by hydrogen peroxide produced in the enzymic reaction.

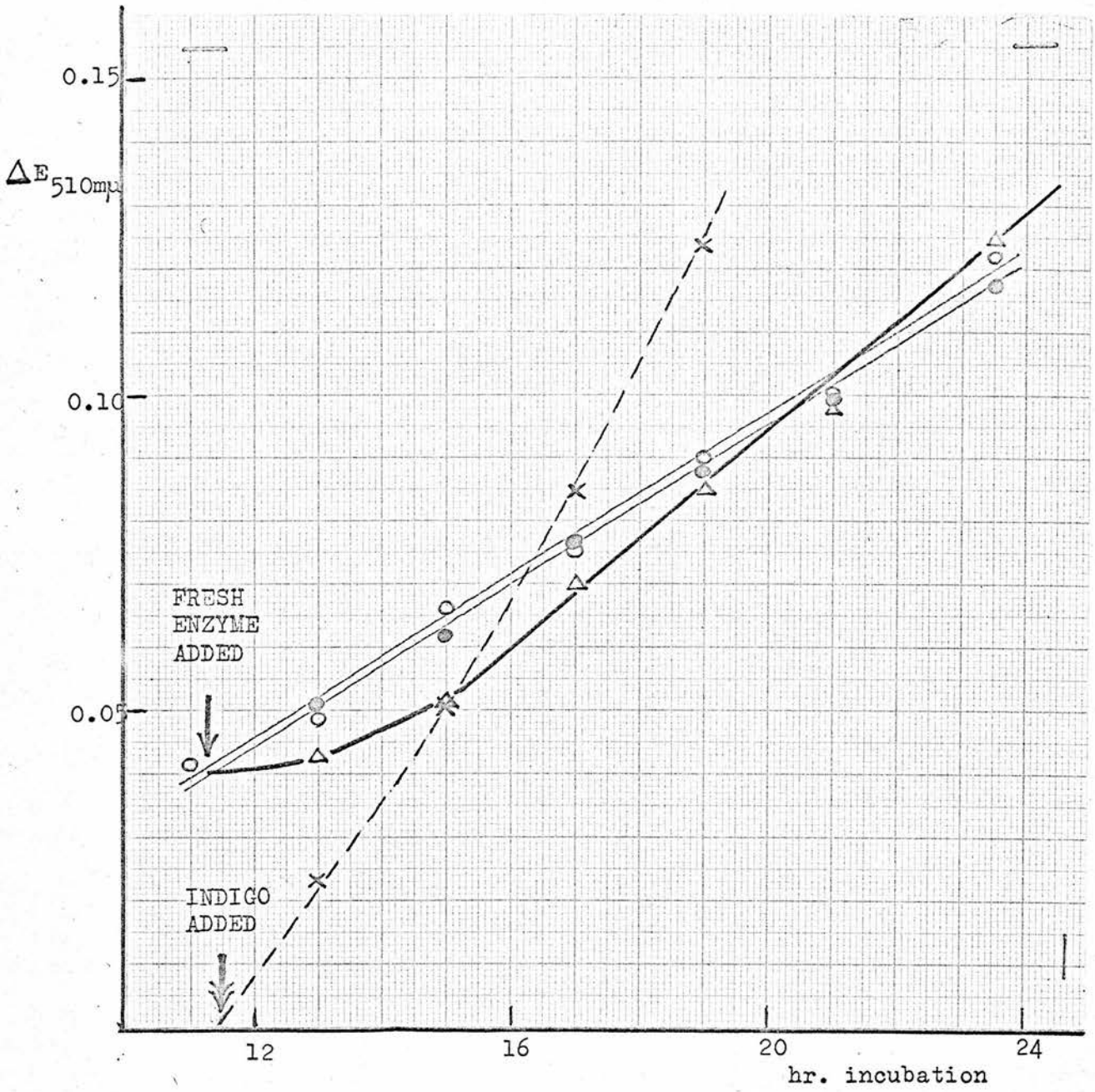


Fig. 2.2.15. Interruption of active tests.

o—o Standard indigo test, not interrupted.

●—● Standard indigo test, with 0.2 ml. buffer added after 11.25 hr. incubation, and re-oxygenated.

△—△ Standard indigo test, with 0.2 ml. enzyme added after 11.25 hr. incubation, and re-oxygenated.

×--× Standard test with indigo omitted; indigo added after 11.5 hr. incubation, and re-oxygenated.

2.2.13. Interruption of the indigo test after the lag period.

Assays were set up, as in Method I (Section 2.2.2), except for the omission of indigo, and incubated for a period of 11½ hr., that is, until the usual lag period would have elapsed. Indigo was then added to controls and tests, and the assays re-oxygenated. The subsequent progress of indigo oxidation is shown in Fig. 2.2.15. Indigo oxidation proceeded immediately, without the intervention of a lag period, implying that an ~~hydrogen acceptor~~ oxidising agent had accumulated in the absence of indigo. This experiment reinforced the earlier conclusion that an impurity in the indigo was not responsible for the delay in indigo oxidation.

Similarly, complete standard assays were incubated, as in Method I, containing enzyme, substrate and indigo. After 11 1/4 hr ., a second addition of a small volume of enzyme solution was made to one series of tests, and the same volume of buffer added to a control series. Both series of assays were re-oxygenated and returned to the incubator. The progress of indigo oxidation in these assays was compared with an uninterrupted series. As can be seen from Fig. 2.2.15, addition of buffer did not significantly deflect the progress of indigo oxidation from its previous course. However, the addition of fresh enzyme re-imposed a lag period on the progress of indigo oxidation; not only was the fresh enzyme inactive towards indigo during this period, but indigo oxidation due to the original enzyme present appeared to be suppressed, and it was some 8 hr. before the interrupted test had attained as much indigo oxidation as the control tests. This result is consistent with the hypothesis suggested in Section 2.2.10, that the enzyme solution contains a contaminant more easily oxidised by hydrogen peroxide than is indigo. It is unlikely, however, that this is the main cause of

the lag period in indigo oxidation. The lag period resulting from the introduction of fresh enzyme solution was only half as long as the usual initial lag period of some 6 - 8 hr. Also the effect of contamination by an oxidisable component in the enzyme, haemoglobin for instance, would be expected to vary considerably with the state of purity of the enzyme preparation tested; no such large variation was observed. (Section 2.2.9).

2.2.14. Conclusions.

The conclusions to be drawn from this examination of Kapeller-Adler's indigo test may now be presented.

1. Kapeller-Adler's claim that indigo oxidation varies linearly with enzyme concentration, at least from 0 - 30 P.U., could not be substantiated. The useful range of activities measurable by Kapeller-Adler's test was found to be only 5 - 15 P.U., using cadaverine as substrate, and within this range the S.D. of the method was rather high, in the hands of a worker familiar with the method. Difficulties were encountered in judging the end-point of the permanganate titration in the presence of different substrates and enzyme solutions contaminated by coloured proteins. These difficulties could be controlled by a spectrophotometric procedure for the estimation of residual indigo.

2. Kapeller-Adler's claim that indigo oxidation proceeds linearly with incubation time could not be confirmed. A latent period of approximately six hours, during which hydrogen peroxide accumulated and indigo was only slowly oxidised, was encountered in tests using enzyme solutions at many stages of purification.

3. This delay in indigo oxidation was not due to impurities in the buffer, substrate or indigo, and could not be explained by loss of co-factors of histaminase.

4. The decolorisation of indigo was shown to be an oxidative process, and a reductive mechanism which might have explained the lag period was discarded.

5. Although synthetic hydrogen peroxide proved to be a poor substitute for hydrogen peroxide formed in the enzyme-substrate reaction, model experiments did not support the hypothesis that hydrogen peroxide altered histaminase to

a specially active form, or that some threshold concentration of hydrogen peroxide had to be attained before indigo oxidation commenced. Discrepancies between synthetic and enzymic hydrogen peroxide in the second stage of the indigo test raised the possibility that some precursor or analogue of hydrogen peroxide might be responsible for indigo oxidation in the enzymic test, rather than free hydrogen peroxide.

6. None of the postulated autocatalytic mechanisms of indigo oxidation was supported by experimental evidence.

7. It appeared possible that a contaminant of the enzyme solution might contribute to the lag period, by being preferentially oxidised by hydrogen peroxide formed in the enzyme-substrate reaction; but this effect could not fully account for the lag period.

2.2.15. Discussion of Kapeller-Adler's indigo test.

Burkard (1954) applied Kapeller-Adler's indigo test to the assay of low histaminase activities in animal tissues and fluids, and experienced difficulty in discerning the equivalence point of the permanganate titration. After some attempts at modifying the titration, Burkard abandoned the indigo test in favour of the more sensitive biological assay of histamine. The present author also found the permanganate titration to be poorly reproducible in coloured or turbid enzyme solutions. This difficulty, however, could be avoided by spectrophotometric estimation of residual indigo after incubation of the assays. The spectrophotometric adaptation is also more specific for indigo than is the permanganate titration, which is disturbed at alkaline pH or in the presence of reducing agents added, e.g., as inhibitors of histaminase. However, the titrimetric method of Kapeller-Adler (1951) can give quite reproducible results using purified enzyme solutions in closely similar media.

Zeller (1956) has claimed that the rate of production of ammonia does not parallel the rate of indigo oxidation, and the discrepancy varies with the substrate used. The limited data presented by Zeller (1956) suggest that the discrepancy is not due to any lag period in indigo oxidation, as encountered in the present work, and Zeller attributed it to the variable efficiency of different substrates in catalysing indigo oxidation. Data presented in Section 2.2.12 do not support Zeller's view (1963) that histamine is a better catalyst than the diamines in the oxidation of indigo by hydrogen peroxide. Zeller's view does not account for the fact that, as judged by Kapeller-Adler's indigo test, histamine is attacked more rapidly

than cadaverine by hog kidney DAO, but more slowly than cadaverine by placental histaminase.

Zeller (1956) has objected to Kapeller-Adler's indigo test on the ground that a 24 hr. incubation period cannot give a measure of initial velocity of the enzymic reaction. Kapeller-Adler and Renwick's (1956) demonstration of linear indigo oxidation over 24 hr. incubation time effectively answered Zeller's objection. However the lag period in indigo oxidation encountered in the present work does not agree with Kapeller-Adler and Renwick's observations. It must be concluded that in its present application, Kapeller-Adler's indigo test using a single indigo measurement after 24 hr. may be an unreliable index of initial velocity of enzymic reaction. The evidence presented in Sections 2.2.7 - 2.2.13 suggests that there is a dislocation between the immediate production of hydrogen peroxide by the enzyme-substrate reaction, and the oxidation of indigo used to measure hydrogen peroxide production.

The advantages of Kapeller-Adler's method are its simplicity and applicability to all classes of substrate of histaminase. It is a suitable method for the assay of many chromatographic fractions. Except when purified enzyme and a single substrate are used, however, the comparison of enzymic activities by the indigo test is only semi-quantitative. In particular, the test is not suitable for kinetic measurements.

2.3. A new spectrophotometric indigo test for histaminase.

2.3.1. Re-designing the indigo test.

Kapeller-Adler's indigo test having proved unsatisfactory for quantitative assays over a wide range of histaminase activities, an improved assay was sought which might exploit the validity of the indigo test for all substrates of histaminase, yet minimise the defects of Kapeller-Adler's test.

Experience with the simple spectrophotometric adaptation of Kapeller-Adler's indigo test had demonstrated the rapidity and convenience of measuring residual indigo by its extinction in the visible range. It was realised that a more sensitive test would have further advantages;

(a) Less enzyme solution would be used, permitting a greater number of tests on the same enzyme solution.

(b) The assay system would be contaminated with a lower concentration of inhibitors introduced in the enzyme solution.

(c) A lower concentration of possibly inhibitory reaction products would accumulate in the assay during incubation.

Sensitivity was achieved by reducing the concentration of indigo in the assay, and making extinction measurements at 610 mμ, the extinction maximum of indigo disulphonate. Ideally, the indigo concentration should have been so low that, read against a water blank, the extinction of the assay fell within the range, 0.4 - 0.7, optimal for accurate spectrophotometric measurements. However, when such a low indigo concentration was used, the linear increase in indigo oxidation was not maintained over a convenient range of histaminase activities. Optimal sensitivity was retained by doubling the "ideal" indigo concentration, and reading the extinctions of all assays, at the extinction maximum, against a reference solution containing half the concentration of indigo in the assays.

Thus:

Indigo 0.287 μ mole /4 ml., read against Indigo 0.144 μ mole /4 ml. had an extinction at 610 mμ. : $E_{610 m\mu}$ = 0.620.

Indigo 0.144 μ mole / 4 ml., read against water, had an extinction at 610 mμ. : $E_{610 m\mu}$ = 0.620.

The concentration of cadaverine was considerably increased in comparison with Kapeller-Adler's (1951) test; and the concentration of histamine was comparatively decreased to a level found to give optimal histaminase activity in the new test. The determination of these optima of substrate concentration will be described in Section 5.2. 0.067 M phosphate buffer (Sorensen, 1909) was adopted for the new assay.

The process of oxygenation was a potential source of large errors in Kapeller-Adler's (1951) test. Appreciable amounts of indigo were sometimes removed with the manifold, and scrupulous care was required to minimise these losses. Moreover, when rubber tubing was used in the manifolds, it was suspected that some inhibitor was carried into the assays during oxygenation, even when a wash-bottle was used. Fortunately, it was found that sufficient oxygen was present in the new assay system almost to "saturate" the small amounts of enzyme used, and no worthwhile increase in indigo oxidation resulted from oxygenation of the test solutions prior to incubation. Much time was saved by omitting the oxygenation stage.

2.3.2. Standard procedure for the new spectrophotometric indigo test. (Method II).

Reagents: Indigo disulphonate Analar, 133 mg./ml. distilled water.

This reagent was stored in a brown bottle at 4°; under these conditions, autoxidation of indigo was negligible over two weeks.

Phosphate buffer (Sorensen) 0.067 M, pH 6.8, containing 0.1 mM EDTA.

This assay buffer was stored at room temperature over chloroform.

Histamine dihydrochloride	125 µg./ml. assay buffer
Cadaverine dihydrochloride	8 mg./ml. assay buffer
Putrescine dihydrochloride	7.35 mg./ml. assay buffer

A complete assay contained:

0.287 µ mole indigo

9.14 ~~9.94~~ µ mole cadaverine or putrescine

0.136 µ mole histamine

The final volume of each assay was 4.0 ml. 10 - 200 µl. enzyme solution was added to the calculated volume of assay buffer in 10 ml. Pyrex test-tubes, and 1.0 ml. indigo added. The assays were warmed to 37° in a water-bath, and 0.2 ml. substrate solution added. The tubes were closed with a rubber bung and the contents mixed by gentle inversion. Control assays, containing substrate but no enzyme, were incubated along with the test assays. After 24 hr. incubation, the extinction at 610 mµ of control and test assays was measured in 10 mm. glass cuvettes, against a reference solution containing

0.144 μ mole indigo/4 ml. (i.e. half the indigo concentration of the assays). The extinction of the test was subtracted from that of the control, and the enzymic activity expressed in arbitrary spectrophotometric units (S.U.).

1 S.U. was defined as the amount of enzyme required to produce an extinction decrease of 1.0 units in the standard form of the test. In practice, activities of about 0.1 S.U. were measured. Assays showing activity greater than 0.150 S.U. were repeated using appropriately diluted enzyme solution.

The molar extinction coefficient of indigo disulphonate in assay buffer was $\epsilon = 1.73 \times 10^4$ mole^{-1} cm.$^{-1}$. Hence a decrease in indigo extinction, $\Delta E_{610\ m\mu} = 0.1$, was equivalent to the oxidation of 23.1 μm mole indigo, under standard assay conditions (Method II).

2.3.3. Choice of controls.

It was found that incubation of purified enzyme (Stage 3 and after) with indigo, in the absence of substrate, did not result in measurable indigo oxidation. However, if substrate was incubated with indigo, in the absence of enzyme, appreciable indigo autoxidation did occur. Consequently, the usual control adopted for the new indigo test was a solution containing the standard concentrations of substrate and indigo, but no enzyme.

When highly coloured or turbid enzyme solutions were assayed (Stages 1 and 2) a correction for the extinction of the enzyme solution was supplied by incubating additional controls with indigo. These controls contained no substrate, but contained the appropriate volume of enzyme, or buffer.

For example,

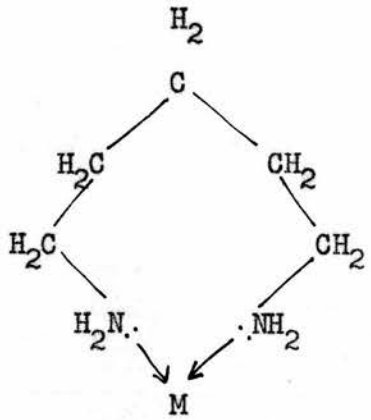
	Enzyme ml.	Buffer ml.	Cadaverine ml.	E_{610} mμ.	
	0	0.2	0	0.620	) correction for extinction
	0.2	0	0	0.650	) of enzyme: 0.030 S.U.
Normal control:	0	0.2	0.2	0.580	) uncorrected enzymic
Normal test:	0.2	0	0.2	0.480	) activity: 0.100 S.U.

Enzymic activity corrected for extinction of enzyme: 0.130 S.U.

It might be argued that an assay containing substrate and the appropriate volume of boiled enzyme would constitute an ideal control. It was found that when purified enzyme solutions were assayed, the addition of boiled enzyme to a control containing substrate had no effect on indigo oxidation - i.e. boiled enzyme had no enzymic activity, and negligible extinction. Boiling a crude enzyme solution produced a suspension differing in colour and turbidity from the unboiled enzyme; a suspension or centrifuged supernatant of boiled enzyme was therefore not considered to be a proper control for the extinction of unboiled enzyme solutions. There appeared to be no justification for using boiled enzyme with substrate as a control at any stage of enzyme purification.

The slight autoxidation of indigo which occurred on incubation with substrate alone, particularly cadaverine, was not explained. It was considered that, since this autoxidation was not observed if EDTA were absent, some complex between diamine and EDTA was capable of catalysing the autoxidation of indigo by atmospheric oxygen. It was conceivable that such a catalytic complex could be formed at neutral pH, either by salt formation (incompletely dissociated) between amino and carboxylic groups, or, more probably, by the

intervention of a metal ion. In the latter case, the N atoms of the diamine would be capable of donating electrons to a co-ordinate link with a metal ion, M, already chelated by EDTA, and the catalytic effect of the complex might be analogous to that of haem-bound iron.



$[EDTA]_n$

The catalytic complex was not further investigated, but will be recalled in Section 2.3.6, in connection with experiments using synthetic hydrogen peroxide to oxidise indigo.

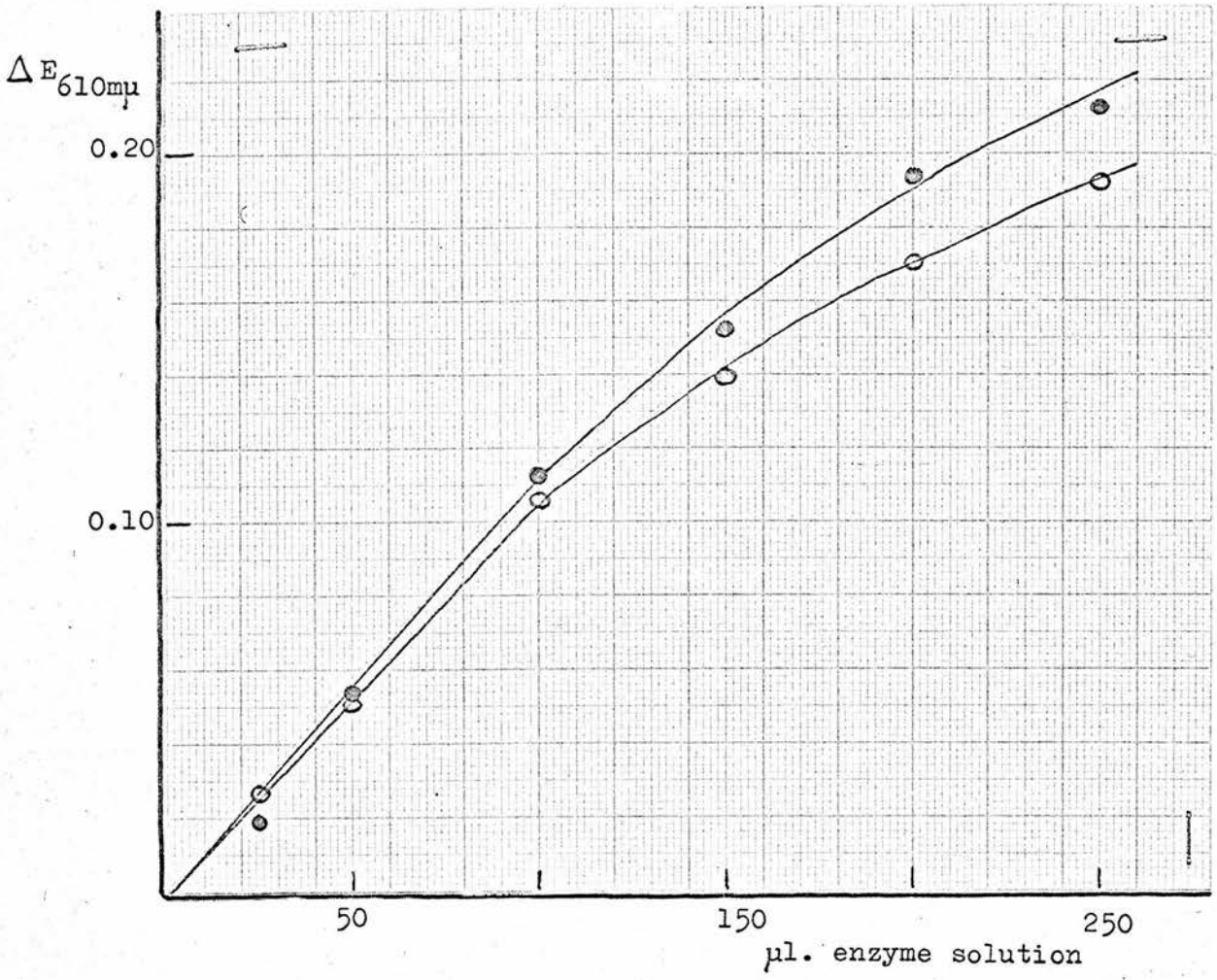


Fig. 2.3.1. Enzyme dilution curves for assay Method II, with and without oxygenation.

Dilutions of Stage 6 enzyme were assayed under standard conditions, (Method II).

●——● Incubated after oxygenation for 1 min.

○——○ Incubated without oxygenation.

Table 2.3.1. Comparison of indigo tests.

Condition	Kapeller-Adler's test Method I, Section 2.2.2	New spectrophotometric test Method II, Section 2.3.2
Assay buffer	sod. phosphate 0.17 M, pH 6.8, containing 0.1 mM EDTA.	(Sorensen) sod.-pot. phosphate 0.067 M, pH 6.8, containing 0.1 mM EDTA.
Indigo (final conc.)	286 μMolar	69.3 μMolar
Cadaverine "	1.14 mMolar	2.28 mMolar
Putrescine "	1.24 mMolar	2.28 mMolar
Histamine "	136 μMolar	34 μMolar
Relative amounts of purified enzyme used	50	1
Final volume	10 ml.	4 ml.
Gas phase	oxygen	air
Usual control	Complete test, without substrate.	Complete test, without enzyme.
Incubation	24 hr., 37°	24 hr., 37°
Measurement	Titration with 0.002 N-$KMnO_4$ (or decrease in E_{510} mμ, read against H_2O)	Decrease in E_{610} mμ (extinction maximum) read against diluted indigo.
Range of activities measurable	5-15 P.U.	0.010 - 0.150 S.U.

2.3.4. Range of the new indigo test.

Indigo oxidation in the new test was found to vary directly with enzyme concentration over a wide range of histaminase activities. Fig. 2.3.1 shows the increase of indigo oxidation with enzyme concentration, with and without oxygenation of the assays, using highly purified enzyme (Stage 5). Both graphs are linear to more than 0.100 S.U., and no increase in the linear range could be gained by oxygenating the assays for 1 min. before incubation. The linear range in this experiment was unusually short. Almost every other enzyme solution examined gave linear indigo oxidation up to at least 0.150 S.U., although the range tended to decrease as more highly purified enzyme was tested. This effect suggests that non-linearity of indigo oxidation at higher enzymic activities is due to lability of the enzyme to one or more of the products of the reaction. Certainly, oxygen concentration does not appear to be the limiting factor, and less than 0.5% of cadaverine should be used up by 0.100 S.U. activity. 0.100 S.U. does, however, represent approximately 12% of indigo initially present, and the rate of indigo oxidation depends on indigo concentration.

Table 2.3.1 opposite summarises the standard assay conditions for Kapeller-Adler's indigo test and the new spectrophotometric indigo test.

2.3.5. Errors of the spectrophotometric assay (Method II).

The method suffered from the defects inherent in all assays depending on an extinction decrease - the concentration of absorbing component in each assay had to be reproduced very exactly. In assays depending on extinction increases, the chief source of error lies in the addition of enzyme solution. This was a minor source of error in the present assay; overall reproducibility depended mainly on the reproducible addition of indigo and buffer to the assay.

An error of 1% in the addition of indigo led to an extinction error of 0.012. An error of 1% in the addition of buffer led to an extinction error of 0.005. The addition of small volumes of substrate and enzyme were less critical, and constriction micro-pipettes proved satisfactory. Errors of 1% in the addition of each reagent to a control and a test could lead to an extinction error of about 0.035 S.U. in the assay of 0.100 S.U. enzymic activity. Plainly, the first requirement for satisfactory application of the assay was the attainment of reproducible pipetting technique; the standard deviations quoted below are a measure of what could be achieved with great care - the "probable error" of casual pipetting was clearly prohibitive.

Six replicate assays were carried out on purified enzyme at each of several dilutions. The extinction difference required to deflect visibly the galvanometer needle of the spectrophotometer, and the precision with which the extinction scale could be read, permitted extinction differences of 0.002 units to be distinguished. In the calculation of the standard deviations, the mean values were rounded off to three significant figures, i.e. the degree of significance of the original data.

	I.	II.	III.	IV.
	.029 S.U.	.080 S.U.	.130 S.U.	.178 S.U.
	029	088	131	184
	029	081	128	177
	030	089	130	180
	031	078	128	179
	029	086	129	180
Mean	.030	.084	.130	.180
S.D.	.001	.004	.001	.002

As expected from the above consideration of the sources of error, the standard deviation of the method was not related to the amount of enzymic activity measured.

The errors of the method could have been decreased by preparing a stock solution of indigo and cadaverine in assay buffer, and adding the same amount of this solution to each assay. This procedure would have ensured a reproducible concentration of indigo in the completed test, provided that a fixed volume of enzyme solution, or buffer, were added to each enzyme test or control. In routine use, for example in the examination of chromatographic effluents, this standardised procedure would have been advantageous: but little benefit would have been gained in experiments, frequent in this investigation, which required departure from standard conditions - for example, by changing substrate concentration, or adding various components to the assay.

Table 2.3.2. Oxidation of indigo by hydrogen peroxide in the presence of various assay components.

Test No.	H_2O_2 (μ mole)	Cadaverine (μ mole)	Additive	$\Delta E_{610m\mu}$	
1	-	-	-	0.000	
2	-	9.12	-	0.040	
3	-	-	Enzyme	+0.001	
4	-	-	Hp-MHb	+0.002	
5	-	9.12	Enzyme	0.101	
6	-	9.12	Hp-MHb	0.041	
7	0.05	-	-	0.039)	
8	0.05	-	Enzyme	0.039)	Theoretically
9	0.05	-	Hp-MHb	0.040)	0.05 μ mole
10	0.05	9.12	-	0.372)	H_2O_2
11	0.05	9.12	Enzyme	0.393)	$E_{610m\mu} = 0.108$
12	0.05	9.12	Hp-MHb	0.369)	

2.3.6. Model experiments using synthetic hydrogen peroxide in the new assay system

It may be recalled from Section 2.2.12 that synthetic hydrogen peroxide proved to be a poor model for enzymically-produced hydrogen peroxide in Kapeller-Adler's indigo test; under these conditions, less indigo was oxidised than predicted by the equation:

$$2\ H_2O_2 + \text{Indigo} \longrightarrow 2\ H_2O + 2\ \text{Isatin}$$

In contrast to these results with Kapeller-Adler's indigo test, the addition of synthetic hydrogen peroxide to the present assay system (Method II) resulted in indigo oxidation far in excess of the theoretical values, provided that substrate was present.

The oxidation of indigo by synthetic hydrogen peroxide was studied in the presence of possible peroxidatic components of the new assay (Method II).

0.05 μ mole hydrogen peroxide was equivalent to a decrease in indigo extinction $\Delta E_{610}^{m\mu} = 0.108$, on the basis of the calculation in Section 2.3.2.

The amount of haptoglobin-methaemoglobin added was estimated to be about ten times the amount contaminating the purified enzyme solution, Stage 6. The preparation of haptoglobin-methaemoglobin is described in Appendix 1.

Cadaverine was added in the standard concentration (Method II).

The standard concentration of indigo (Method II) was present in each test. The extinction decreases of the tests after 24 hr. incubation are presented in Table 2.3.2.

No significant indigo oxidation was caused by enzyme or haptoglobin-methaemoglobin alone, but measurable indigo autoxidation occurred in the presence of cadaverine alone (Section 2.3.3). Comparison of (2) with (6)

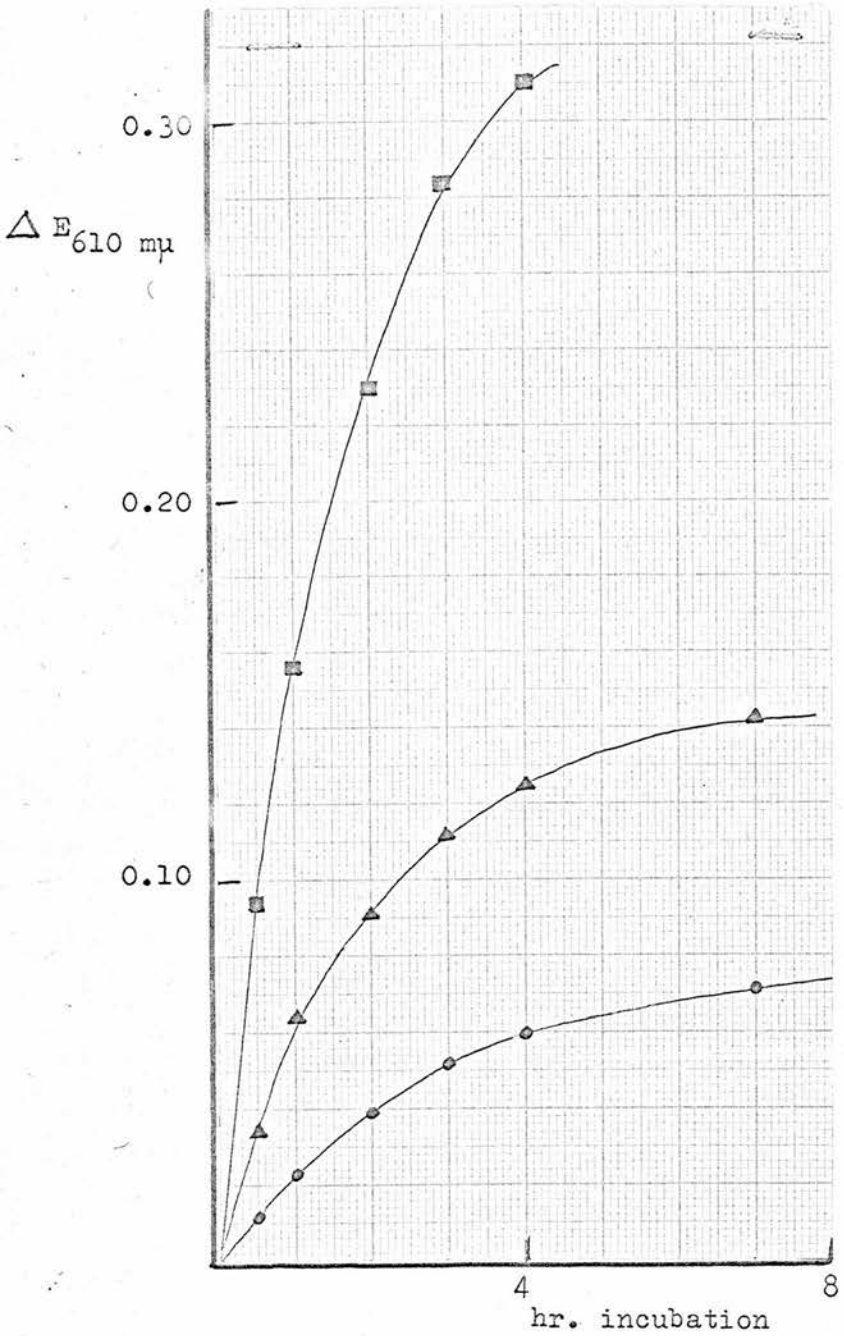


Fig. 2.3.2. Oxidation of indigo by synthetic H_2O_2 in the presence of cadaverine.

Assays were incubated as in standard Method II, with the substitution of H_2O_2 for enzyme solution.

●—● 12.5 μm mole H_2O_2

▲—▲ 25.0 "

■—■ 100.0 "

showed that haptoglobin-methaemoglobin had no histaminase activity. (7) indicated only slow oxidation of indigo by hydrogen peroxide in the absence of any catalyst. Neither enzyme (8) nor haptoglobin-methaemoglobin (9) catalysed this slow oxidation, but the addition of cadaverine resulted in much more oxidation of indigo than would have been possible by hydrogen peroxide alone, assuming a stoichiometric reaction. Haptoglobin-methaemoglobin did not further increase the catalytic effect of cadaverine in the hydrogen peroxide-indigo oxidation (cf. (10) and (12)), although haptoglobin-methaemoglobin is a potent peroxidase in other systems. Assay (11) must be compared with both (5) and (10); the simplest interpretation is that hydrogen peroxide inhibited the histaminase-cadaverine reaction, so that indigo oxidation was less than the sum of assays (5) and (10).

Further tests were carried out to compare the efficiencies of other histaminase substrates in catalysing the oxidation of indigo by synthetic hydrogen peroxide. 25 µm mole hydrogen peroxide (theoretically equivalent to $\Delta E_{610m\mu} = 0.054$ in Method II) was incubated with indigo in the presence of equimolar (2.28 mM) concentrations of substrates. The progress of indigo oxidation was similar in all cases to that shown for cadaverine in Fig. 2.3.2. The extinction decreases after 24 hr. incubation are tabulated below.

	$\Delta E_{610m\mu}$
1,2 diaminoethane	.099
1,3 diaminopropane	067
1,4 diaminobutane (putrescine)	144
1,5 diaminopentane (cadaverine)	223
1,6 diaminohexane	242
Spermine	113
Agmatine	152
Histamine	139

The peroxidatic properties were more pronounced in those substrates bearing amino-groups at the ends of long aliphatic chains, capable of approaching cyclic conformations. The side-chain amino-group of histamine can also approach the neighbouring imidazole N atom. This observation may be related to the ability of these substrates to form catalytic complexes with metal ions, or their chelates with EDTA (Section 2.3.3).

The effect of other possible components of the assay system on the peroxidation of indigo was then determined. The amount of isatin added was 28.6 μm mole, equivalent to the isatin sulphonate produced by the oxidation of one-twentieth of the indigo in a standard assay (Method II) or about 0.060 S.U. Haptoglobin-methaemoglobin and haemoglobin, free of histaminase and erythrocyte catalase, were added in excess of the concentrations contaminating assays of purified enzyme solutions.

Negligible indigo oxidation had occured in the presence of these components and 25μm mole hydrogen peroxide after 3 hr. incubation. However, when cadaverine was added to the tests, indigo oxidation started immediately in each test, following the pattern of Fig. 2.3.2. The extinction decreases after 24 hr. incubation are shown below.

H_2O_2 μ m mole	Cadaverine μ mole	Additive	$\Delta E_{610\ m\mu}$
25	9.12	-	0.223
25	9.12	Hp-MHb	0.222
25	9.12	Hb	0.074
25	9.12	Isatin	0.292

Theoretically , 25 μ m mole hydrogen peroxide $\equiv \Delta E_{610\ m\mu} = 0.054$.

As observed before, cadaverine alone catalysed the hyper-stoichiometric oxidation of indigo. Haptoglobin-methaemoglobin did not further enhance the peroxidation, and haemoglobin competed with indigo for hydrogen peroxide, with the formation of methaemoglobin. Isatin significantly increased the catalytic effect of cadaverine in the hydrogen peroxide-indigo oxidation.

The implications of these model experiments were important. If the model experiments accurately represented the peroxidatic second stage of the enzymic indigo test, it appeared possible that different substrates could markedly affect the rate of oxidation of indigo by enzymically-produced hydrogen peroxide. If the enzyme-substrate reaction were much faster than the second peroxidatic reaction, so that the latter determined the overall rate of indigo oxidation, the varying peroxidatic properties of different substrates might lead to false conclusions concerning the relative rates of the true enzyme-substrate reactions.

However, no evidence was acquired confirming that synthetic hydrogen peroxide was an appropriate model for "enzymic" hydrogen peroxide. Whereas indigo oxidation in the enzymic test was subject to a pronounced initial delay (See Section 2.3.7 below), the progress of indigo oxidation by synthetic hydrogen peroxide always followed the pattern of Fig. 2.3.2. The dependence of indigo oxidation on synthetic hydrogen peroxide concentration was examined in the presence of cadaverine; the results are illustrated in Fig. 2.3.2. Even when the amount of hydrogen peroxide added was reduced to 12.5 m μ mole (the lowest concentration resulting in accurately measurable indigo oxidation) oxidation started immediately on adding the catalysing substrate.

The only conceivable way of imitating with synthetic hydrogen peroxide the gradual release of hydrogen peroxide by the enzyme-substrate

reaction, would have involved continuous injection of minimal amounts of hydrogen peroxide, or buffer, into test and control cuvettes in a spectrophotometer, using paired motor-driven syringes, and measuring extinction differences continuously on an expanded extinction scale. The preliminary experiments with additions of decreasing amounts of hydrogen peroxide did not offer any expectation of a decisive result from this proposed "continuous injection" experiment, and it was not carried out.

It was noted above that much more than the stoichiometric amount of indigo was oxidised by hydrogen peroxide in the presence of substrate. This phenomenon was ~~not~~ now examined more closely. Indigo, cadaverine and synthetic hydrogen peroxide were incubated in air, as in Method II, and under partial vacuum in Thunberg tubes. Assays (a) in the Table below were incubated for three hours at 37° as in Method II; assays (b) were identical, except that they had been evacuated at a water-pump for one and a half hours before tipping in hydrogen peroxide from the cap of the Thunberg tubes. In each case, controls were run without hydrogen peroxide.

	$\Delta E_{610 m\mu}$		
H_2O_2 added	(a) Aerobic	(b) Anaerobic	Theoretical
25 μ m mole	.102	.071	.052
50 μ m mole	.176	.106	.104

It was found that partial removal of air from the assay system greatly reduced the hyper-stoichiometric oxidation of indigo. This observation is consistent with the hypothesis that excessive indigo oxidation is due to autoxidation of indigo in the presence of atmospheric oxygen, hydrogen peroxide and substrate.

Hyper-stoichiometric indigo oxidation was observed not only in experiments using synthetic hydrogen peroxide, but also in enzymic indigo tests. 125 µg. histamine dihydrochloride (0.68 µ mole) was incubated for 24 hr. with purified enzyme in Kapeller-Adler's indigo test (Method I); enzymic activity towards histamine was estimated titrimetrically as 19.1 P.U., which is equivalent to the oxidation of 0.98 µ mole indigo. The latter amount of indigo oxidation should require the enzymic oxidation of 1.96 µ mole substrate. Since only 0.68 µ mole histamine was initially present in the assay, indigo must have been oxidised in some other reaction.

Again, using the optimal 25 µg (136 µm mole) histamine dihydrochloride in the new spectrophotometric indigo test (Method II) the maximum amount of indigo oxidised should be 68 µ m mole, if the hydrogen peroxide-indigo reaction were stoichiometric. Hence the maximum extinction decrease in a Method II using histamine as substrate should have been $\Delta E_{610m\mu} = 0.294$. In practice, exploratory assays of "unknown" enzyme solutions often yielded activities towards histamine greater than 0.300 S.U. under standard conditions.

During the determination of optimal histamine concentration in Method II, data were collected which showed conclusively that excessive indigo oxidation occurred. For instance, complete oxidation of 15 µ m mole histamine should have resulted in the oxidation of 7.5 µ m mole indigo, equivalent to $E_{610m\mu} = .032$. The observed enzymic activity was 0.084 S.U. Other results are tabulated below.

µ m mole histamine in assay	Theoretical max. indigo oxidised (µ m mole)	Theoretical max. $\Delta E_{610\ m\mu}$	Observed $\Delta E_{610\ m\mu}$
15	7.5	.032	.084
37	18.5	.079	.199
73	36.5	.158	.267
146	73.0	.315	.294

Model experiments using synthetic hydrogen peroxide indicated that hyper-stoichiometric indigo oxidation did not occur in the absence of substrate. Nor does substrate alone (without synthetic or "enzymic" hydrogen peroxide) greatly stimulate autoxidation of indigo. It must be concluded that:

(a) hyper-stoichiometric indigo oxidation is catalysed by substrate in the presence of hydrogen peroxide, or by oxidised substrate e.g. Δ^1-piperidine.

(b) hyper-stoichiometric indigo oxidation is therefore inseparable from stoichiometric oxidation of indigo by hydrogen peroxide in the enzymic test, and no reliable correction can be applied for the hyper-stoichiometric oxidation. This in turn means that volumetric or spectrophotometric units of enzymic activity which relate to the amount of indigo oxidised cannot be translated into the agreed expression of enzymic activity (μ mole substrate metabolised per minute).

Alternatively, it is conceivable that indigo oxidation by enzymic hydrogen peroxide is the sole oxidative reaction, but is not stoichiometric according to the previously accepted equation. Such a hypothesis would require the existence of, for instance, a half-oxidised form of indigo. Dehydro-indigo (see Fig. 2.2.12) does exist, but it would immediately be oxidised to isatin by hydrogen peroxide. It seems most likely that isatin sulphonate is the sole oxidation product of indigo disulphonate in the enzymic tests; completed tests show absorption maxima in the U.V. and visible range typical of isatin.

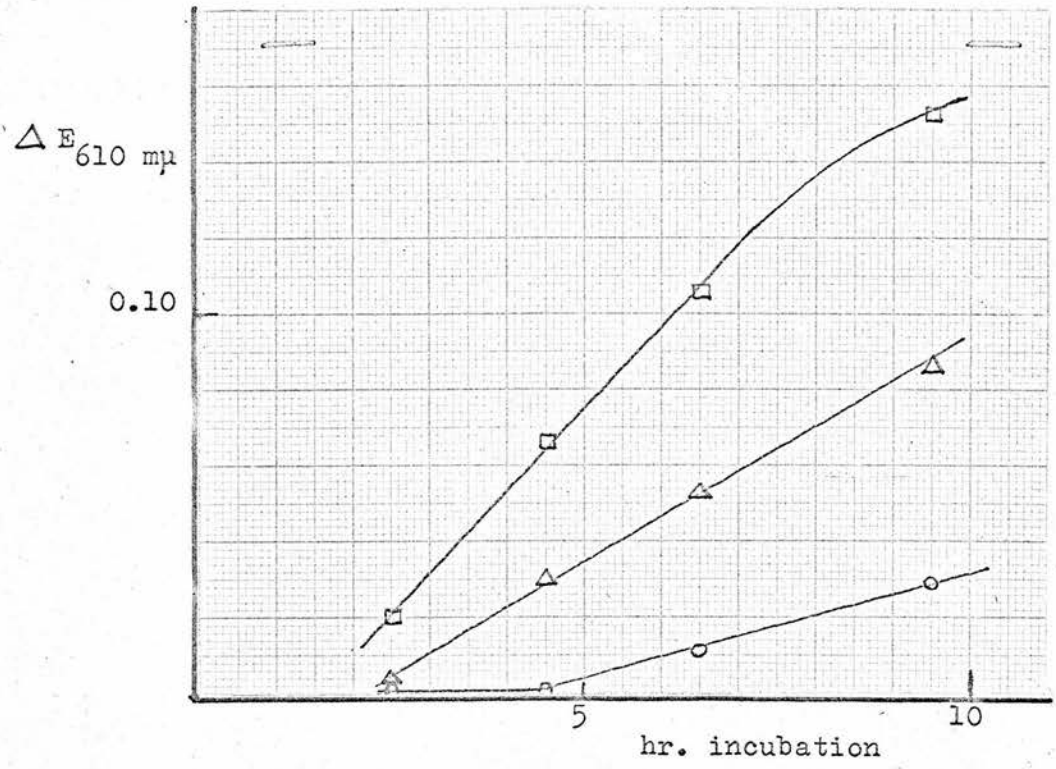


Fig. 2.3.3. Variation of lag period with enzyme concentration.

Stage 6 enzyme was assayed under standard conditions, Method II.

o——o 0.1 ml. enzyme solution.
Δ——Δ 0.2 ml. "
□——□ 0.4 ml. "

2.3.7. Delay in indigo oxidation in the new spectrophotometric assay (Method II).

Since the new spectrophotometric assay did not require oxygenation or titration, the course of indigo oxidation in a test could be followed much more easily than in the titrimetric or spectrophotometric forms of Kapeller-Adler's indigo test, which required many replicate assays for each progress curve.

Standard assays were set up and incubated at 37°, as in Method II. Test and control assays were withdrawn at intervals, extinctions measured within one minute, and the solutions returned to the incubation bath. Great care was exercised to avoid contamination of the assays during these operations.

It was found that assay Method II was subject to the same lag effect as Kapeller-Adler's test (Method I) although the delay in indigo oxidation was reduced to 2-4 hr. from about 6 hr. in Kapeller-Adler's indigo test. The lag period of the new assay was investigated, following the same lines of reasoning as in the study of Kapeller-Adler's test (Sections 2.2.7 - 2.2.13). Again, changes in indigo concentration had no effect on the lag period. Increasing concentrations of substrate slightly decreased the lag period. These results lent no support to the theory that reagent contamination caused the lag in indigo oxidation.

As shown in Fig. 2.3.3, increasing the concentration of enzyme resulted in a slight decrease in the lag period, but, within the range of histaminase concentrations useful in a standard 24 hr. assay, the variation in lag period would not have been significant; in the progress experiments, relatively large amounts of enzyme were often used to provide appreciable extinction differences in the first 12 hr. of incubation.

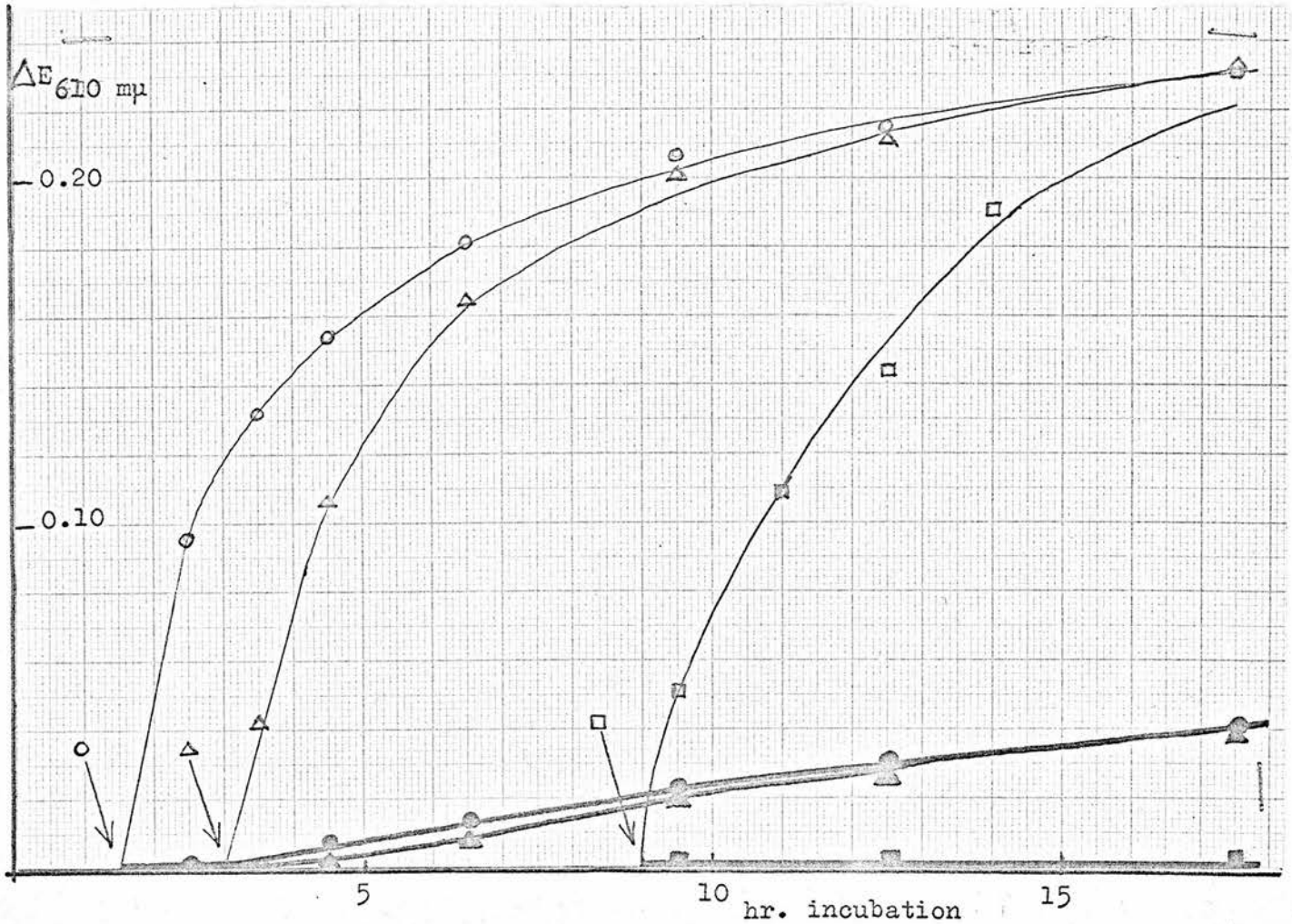


Fig. 2.3.4. Pre-incubation of histaminase with synthetic H_2O_2.

Stage 6 enzyme was incubated with 50 μm mole synthetic H_2O_2 for increasing periods before the addition of indigo and cadaverine. Controls (open symbols, fine lines) without enzyme measured indigo oxidation due to H_2O_2 alone. Subtraction of these control results from those obtained in the presence of enzyme and H_2O_2 yielded the extinction changes due solely to histaminase activity towards cadaverine (solid symbols, bold lines). Indigo and cadaverine were added to tests and controls at ↘.

●	○	Pre-incubation for 1.5 hr.
▲	△	" 3 hr.
■	□	" 9 hr.

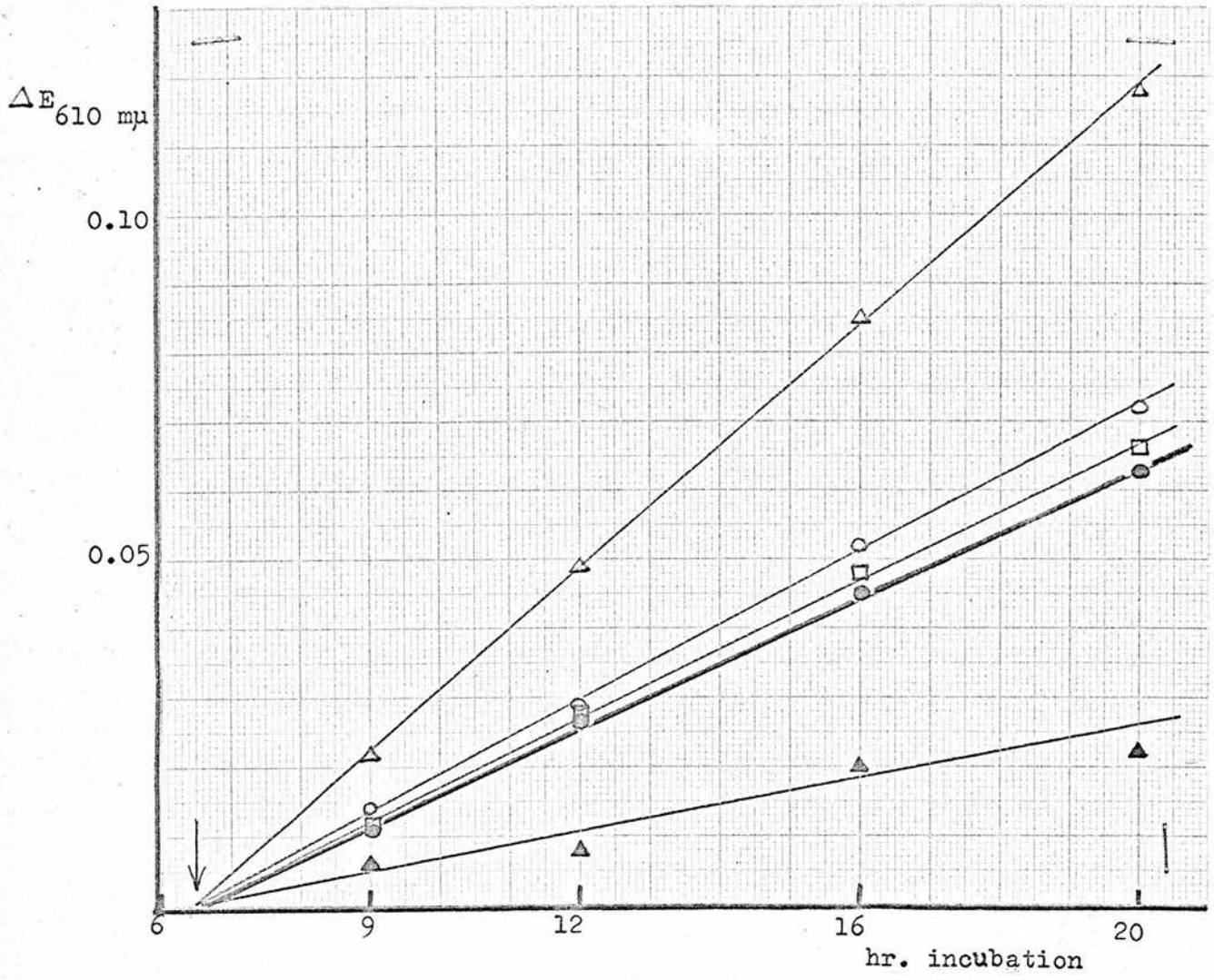


Fig. 2.3.5. Addition of substances to assays after the lag period.

Stage 6 enzyme was assayed in replicate under standard conditions, (Method II) for 6.5 hr., and one of the following substances was then added to one of the replicate assays and controls.

- Buffer
- Fresh enzyme
- Hb
- HpMHb
- Isatin

In the experiments illustrated in Fig. 2.3.4, the enzyme was pre-incubated with synthetic hydrogen peroxide before addition of substrate and indigo. The enzyme appeared to be strongly inhibited by pre-incubation; indigo oxidation was little greater than in control assays containing only hydrogen peroxide and substrate. Pre-incubation with hydrogen peroxide for 9 hr. completely destroyed the activity of the enzyme, but the hydrogen peroxide was still fully active in the oxidation of indigo. This experiment helped to prove that the enzyme did not require to be "modified" by hydrogen peroxide, before reaching full activity in the indigo test. (cf. Section 2.2.12).

Several assays and controls were incubated for 6½ hr. by which time the lag period was expected to have elapsed; the effect of adding various substances to the assay at this point was then examined. To one enzyme assay buffer only was added, serving as a control for the other additives. Results are illustrated in Fig. 2.3.5. The addition of fresh enzyme resulted in immediately increased indigo oxidation - no further delay occurred, in contrast to the effect of adding fresh enzyme to Kapeller-Adler's test (see Section 2.2.13). The addition of isatin has no appreciable effect on the rate of indigo oxidation; the increase in oxidation rate following the addition of haptoglobin-methaemoglobin was only about 10% of the original rate, and was probably not significant. The rate of indigo oxidation was greatly reduced by the addition of haemoglobin, but no lag period was observed. These results do not support the theory that a component of the purified enzyme solution competes overwhelmingly with indigo for hydrogen peroxide in the early hours of the indigo test. This explanation had been suggested by similar interruption experiments with Kapeller-Adler's indigo test (Section 2.2.13) which required much greater amounts of enzyme solution; and

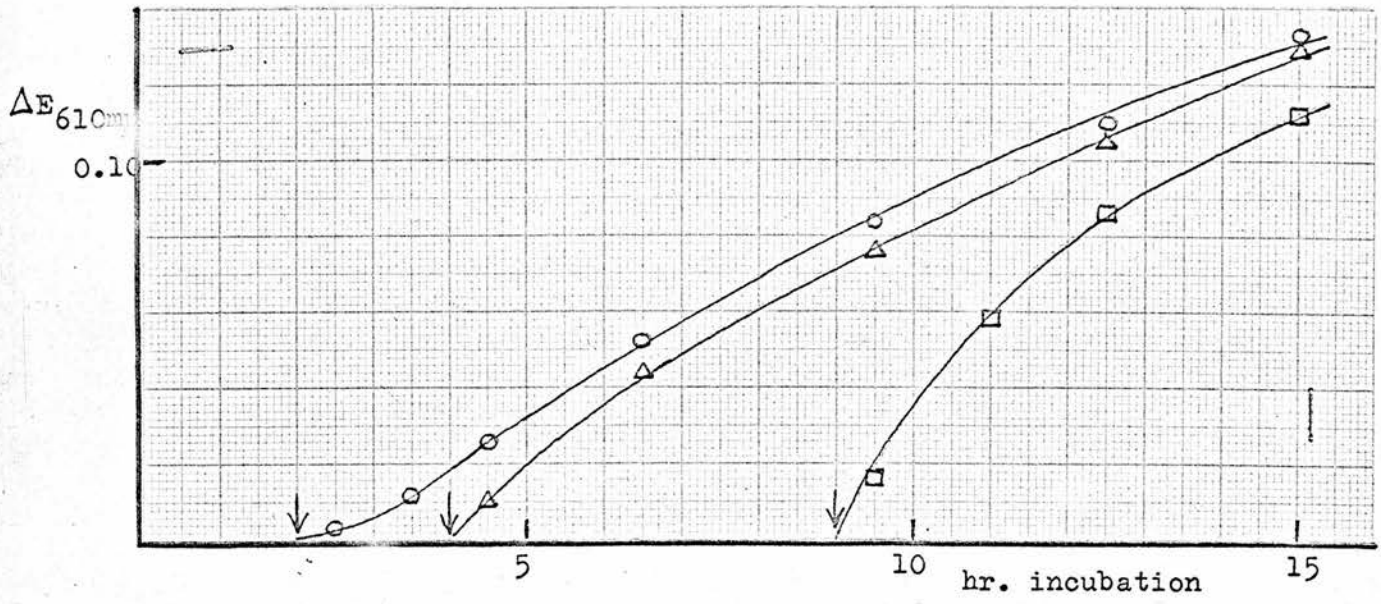


Fig. 2.3.6. Pre-incubation of histaminase and cadaverine before adding indigo.

Stage 6 enzyme was assayed under standard conditions, except for the omission of indigo, which was added after several hours' incubation.

o—o Indigo added after 2 hr.
Δ—Δ " 4 hr.
□—□ " 9 hr.

consequently burdened the assay solution with a much higher concentration of contaminants. The two assay systems differ in so many factors that it did not seem profitable to pursue this distinction in their behaviour towards the addition of fresh enzyme after the lag period.

In another experiment, the enzyme was pre-incubated with substrate for increasing periods before adding indigo; the subsequent progress of indigo oxidation is illustrated in Fig. 2.3.6. The initial rate of indigo oxidation increased with the period of pre-incubation, signifying that, in the absence of indigo, an oxidising substance accumulated in the test solution as a result of the enzyme-substrate reaction; there was no evidence that this oxidising substance was inhibitory to the enzyme. Delay in indigo oxidation was noted only when indigo was added before the usual lag period had elapsed; even then, the delay lasted less than 1 hr. - i.e. indigo oxidation commences after about 3 hr. incubation of enzyme and substrate, regardless of the time of adding indigo. This experiment showed conclusively that the lag period was not due to contamination of the indigo.

Only two explanations of the delay in indigo oxidation now seem possible:

(a) that the amount of hydrogen peroxide produced in the early hours of the enzymic test is not sufficient to initiate linear indigo oxidation,

(b) that some delay occurs in the enzyme-substrate reaction for the first few hours, if indigo is present.

No experiment was designed to test the first hypothesis, since model assays using synthetic hydrogen peroxide did not seem relevant to enzymic assays. The typical lag pattern of enzymic tests was not observed in model experiments using the smallest feasible concentrations of synthetic hydrogen peroxide. The second explanation, however, was susceptible to experimental investigation.

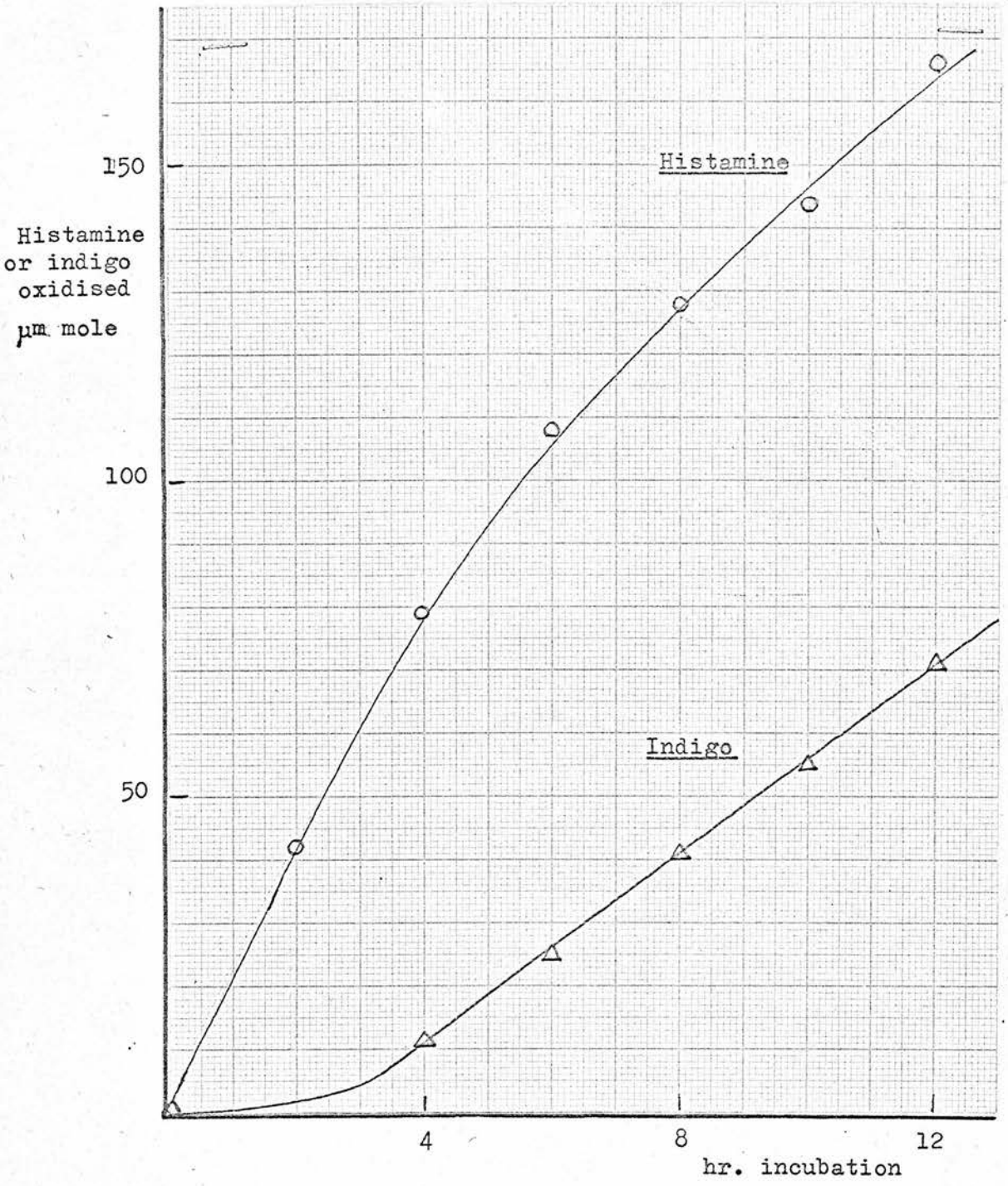


Fig. 2.3.7. Rates of oxidation of indigo and histamine in the same indigo test solution.

Stage 6 enzyme was assayed, with histamine as substrate, essentially under standard conditions, Method II. The rates of oxidation of histamine and of indigo in the same solution were compared.

o——o Histamine oxidised (calculated from decrease in histamine concentration, measured by the method of Shore et al.)

Δ——Δ Indigo oxidised (calculated from the decrease in $E_{610m\mu}$)

A relatively large amount of enzyme was incubated with histamine as substrate; the test was double the standard volume of Method II, but retained the standard concentrations of all components.

Histamine dihydrochloride	50 µg. (.264 µ mole)
Indigo	266 µg. (.574 µ mole)
Final volume	8 ml.

The assay was incubated at 37°, and the extinction change measured at 2 hr. intervals. Also at these intervals, 0.2 ml. aliquots of the same test solution were withdrawn for determination of histamine content by the fluorimetric method of Shore, Burkhalter and Cohn (Section 2.4.3). The progress of indigo oxidation is compared with the rate of disappearance of histamine in Fig. 2.3.7. Histamine oxidation started immediately on addition of substrate, and the rate of oxidation decreased as the residual histamine concentration decreased. However, linear indigo oxidation started only after a delay of about 3 hr., and the rate of indigo oxidation still remained constant when the rate of histamine oxidation (and, therefore, hydrogen peroxide production) had decreased to half the initial rate. This experiment suggests that the prolonged period during which indigo oxidation continues linearly with incubation time is a consequence of a rather slow reaction between indigo and hydrogen peroxide accumulated by the enzyme-substrate reaction.

The conclusions drawn from examining the delay in indigo oxidation in the new assay (Method II) may now be summarised.

1. The delay was probably not due to impurities in the reagents, other than the enzyme solution, and certainly not to contaminants in the indigo.

2. The delay was slightly decreased by increasing the concentration of enzyme or substrate, indicating a possible relationship between the lag period and the rate of the initial enzyme-substrate reaction. The addition of fresh enzyme after the initial lag period did not produce a second delay. Therefore the lag period is probably not due to oxidisable contaminants in the purified enzyme solution, although these may contribute to the lag period in Kapeller-Adler's indigo test, which requires much higher enzyme concentrations.
3. The lag period is not a feature of the primary enzyme-substrate reaction, but appears to be a consequence of an unusually slow reaction, between indigo and H_2O_2 formed in the enzyme-substrate reaction. The reason for this slow reaction is still unexplained, and it is not certain that free hydrogen peroxide is the substance effecting the oxidation of indigo. The difference in behaviour of synthetic and "enzymic" hydrogen peroxide in the oxidation of indigo hindered further investigation of the slow secondary reaction.
4. Any explanation of the pattern of indigo oxidation in the indigo test must take account of the following observations:
   (a) an oxidising agent, similar to hydrogen peroxide, accumulates in the early hours of the test.
   (b) this oxidising agent oxidises indigo in two ways; (i) by a slow reaction with indigo, and (ii) in conjunction with substrate, by catalysing the autoxidation of indigo by atmospheric oxygen. (Section 2.3.6).

The pattern of indigo oxidation observed in the indigo test (e.g. Fig. 2.3.3) may be interpreted in at least two ways. The curve may be exponential

(or have a linear period following an exponential period). Alternatively, the observed pattern may be the resultant of two linear reactions - a relatively slow reaction starting immediately on incubation, and a faster reaction starting only after a delay of several hours. The small extinction differences observed in the early hours of incubation do not permit a clear distinction between these two possible patterns, which may be termed monophasic and biphasic, respectively.

If the pattern of indigo oxidation is interpreted as being monophasic and exponential, this would imply an autocatalytic mechanism. None of the mechanisms suggested in Section 2.2.12 was supported by the experimental observations, but the reservation must be made that some unidentified reaction product, especially if it is thermolabile, might be responsible for autocatalysis. However, autocatalytic mechanisms do not explain the accumulation of oxidising agent in the early hours of the indigo test.

The conclusion that indigo may be oxidised in at least two distinct reactions lends some support to a biphasic mechanism. It is possible that the initial very slow phase is due to direct oxidation of indigo by an oxidising agent produced by the enzymic reaction (4(b)(i), above). The second linear phase may be due to the intervention of a second reaction (4(b)(ii), above), in which excess oxidising agent contributes to the catalysed autoxidation of indigo by atmospheric oxygen.

Experiments in Section 2.2.7 suggest that the oxidising agent is produced immediately on incubation, and that appreciable concentrations of it accumulate long before the second reaction starts. If, as proposed above, the second reaction is catalysed by the oxidising agent, it is difficult to understand why the second reaction does not start immediately on incubation.

Since synthetic hydrogen peroxide does not seem to be an appropriate substitute for the unidentified oxidising agent, it was not possible to investigate the complex reactions which may be required to initiate the second mechanism of indigo oxidation. At present, therefore, no final conclusions can be drawn concerning the number and sequence of reactions resulting in indigo oxidation, and the reasons for the delay in the second phase of indigo oxidation.

2.4. Other assay methods examined.

2.4.1. The assay of histaminase activity towards putrescine, Method III. (Holmstedt and Tham, 1959).

Reagents: 5.0 mM o-aminobenzaldehyde in assay buffer, filtered to remove insoluble residue.

20.0 mM putrescine (30.25 mg. putrescine dihydrochloride in 50 ml. assay buffer).

Assay buffer: 0.067 M phosphate buffer pH 6.8, containing 0.1 mM EDTA.

Procedure: The assay consisted of the following components:

0.2 ml. enzyme solution (at a concentration about 100 times that used in Method II).

1.6 ml. assay buffer.

2.0 ml. o-aminobenzaldehyde solution - final concentration, 2.5 mM.

0.2 ml. putrescine solution - final concentration, 1.0 mM.

After warming the first three ~~reagents~~ components to 37° in a water bath, substrate was added to start the reaction, and the extinction at 430 mμ of the assays was read at 30 min. intervals during incubation. Controls contained all components except substrate, but after Stage 2 of enzyme purification, controls without enzyme gave identical results.

Extinction differences could be converted into units of Δ^1-pyrroline formed, using the value $\epsilon = 1.86 \times 10^3$ mole^{-1} cm.$^{-1}$ for the yellow product, given by Holmstedt and Tham.

The method was that described by Holmstedt and Tham, except that the substrate concentration optimal for placental histaminase was used, 1.0 mM, rather than 10.0 mM for hog kidney DAO. The substrate optimum found in the

present work agreed with that quoted by McEwen (1964) for human pregnancy serum.

Some of the progress curves obtained during the determination of optimal putrescine concentration are shown in Fig. 2.4.1. They indicate that initial rates of product formation were not maintained beyond 30 min., the minimum incubation period giving accurately measurable extinction differences. A progressive decrease in the rate of product formation was noted at all stages of enzyme purification, and at all substrate concentrations and pH values tested. The progressive inhibition was not due to exhaustion of any of the assay components. McEwen predicted that initial velocities might not be maintained by enzyme preparations from which catalase had been removed. However, the abundance of haemoglobin in crude placental preparations should have been sufficient protection against inhibition by hydrogen peroxide formed in the enzymic reaction, even if no catalase remained.

The applications for which Holmstedt and Tham's method was considered valuable will be discussed in Section 2.5.

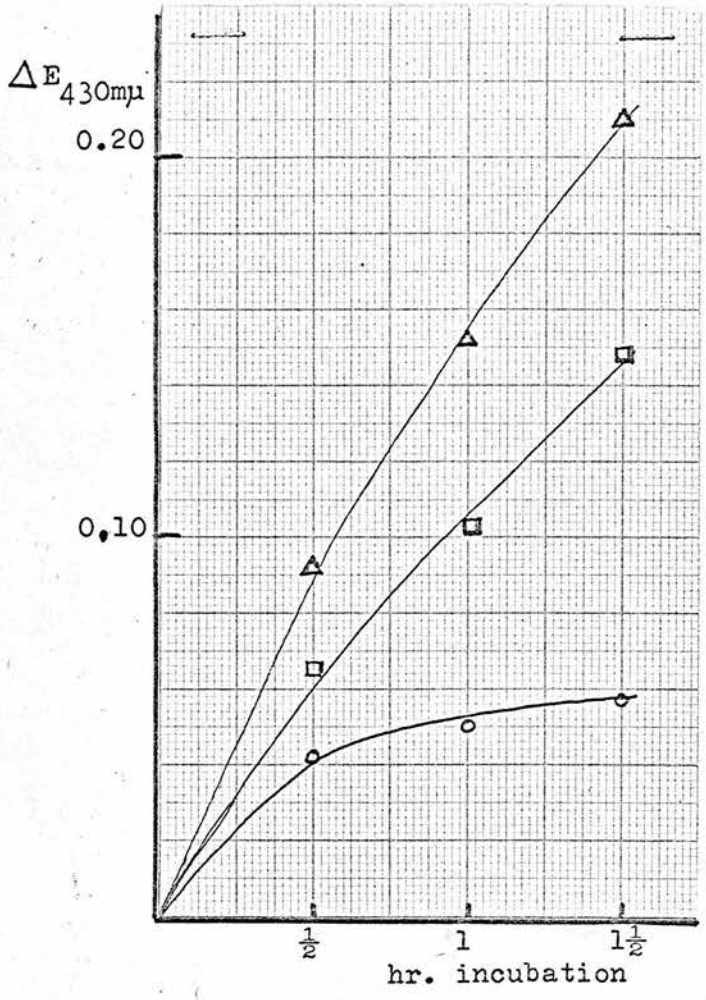


Fig. 2.4.1. Progress of Holmstedt and Tham's test (Method III) at different concentrations of putrescine.

Stage 6 enzyme was assayed under standard conditions, (Method III), except for variations in the concentration of putrescine.

o—o 0.02 mM putrescine
□—□ 1.0 mM "
△—△ 10.0 mM "

2.4.2. The dianisidine test of Aarsen and Kemp (1964).

Reagents: o,o'-dianisidine dihydrochloride, 10 mg./ml. in 0.05 N HCl.

Horse-radish peroxidase, 1 mg./ml., dialysed for 24 hr. against 5 mM borate buffer, pH 8.6.

Cadaverine dihydrochloride 1.6 mg./ml. in assay buffer (45.6 mM)

Assay buffer: 0.067 M phosphate buffer, pH 6.8, containing 0.1 mM EDTA.

Procedure: The assay consisted of the following components:

0.2 ml. enzyme solution (at a concentration 100 times that used in Method II).

3.3 ml. assay buffer

0.1 ml. peroxidase solution

0.2 ml. dianisidine solution

0.2 ml. cadaverine solution

After warming the first four reagents to 37° in a water bath, substrate was added to start the reaction, and the extinction at 470 mμ of the assays was read at intervals during incubation. Control solutions contained enzyme, but no substrate.

This procedure was broadly similar to that of Aarsen and Kemp, except for the final volume of the test, and a different dianisidine solution. The author considered that an ethanolic solution of the free base was undesirable, since the ethanol would compete for hydrogen peroxide with the dianisidine, and might also denature purified enzyme. The hydrochloride, dissolved in very dilute hydrochloric acid, was substituted for an ethanolic solution of the free base.

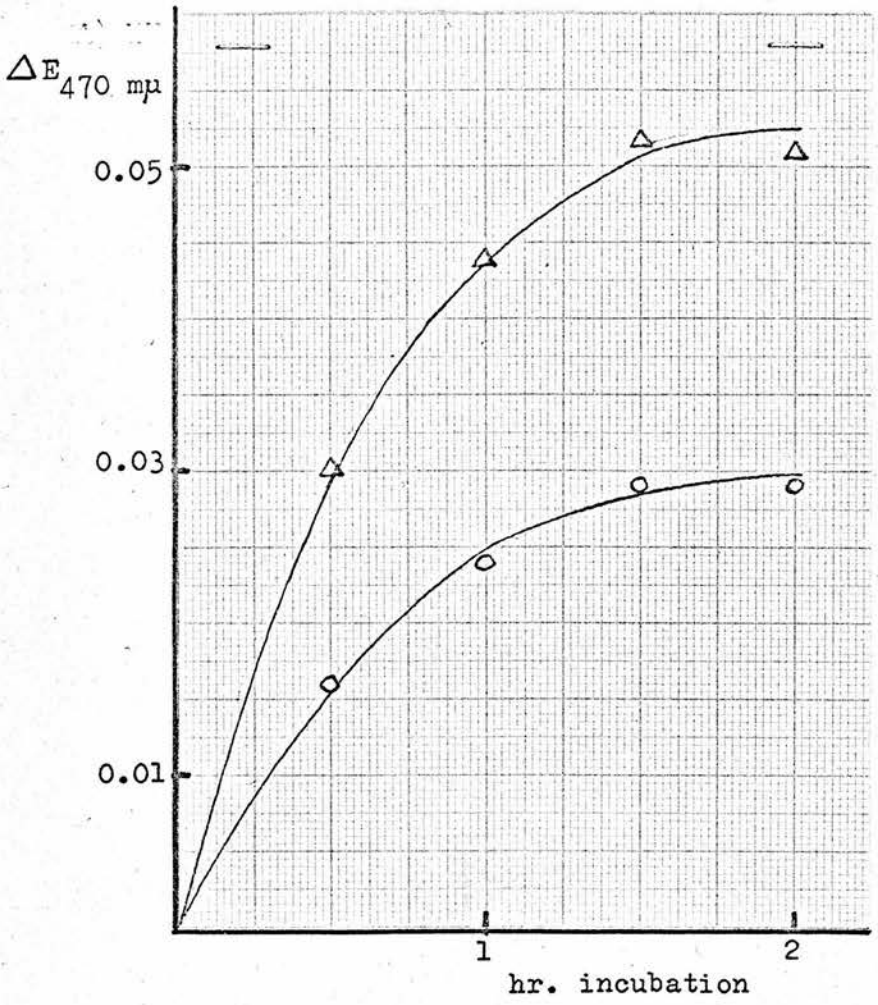


Fig. 2.4.2.
Progress of the dianisidine test for histaminase.

Stage 6 enzyme was assayed under the conditions described in Section 2.4.2.

o—o 0.1 ml. enzyme solution.
Δ—Δ 0.2 ml. "

The progress of dianisidine oxidation in the presence of substrate and purified enzyme (Stage 6) is shown in Fig. 2.4.2. Initial velocities of oxidation were not maintained for 30 mins. incubation, and the maximum extinction increases obtained were very small, using even high concentrations of histaminase. Since hydrogen peroxide cannot accumulate in the presence of peroxidase and dianisidine, hydrogen peroxide cannot be responsible for inhibition of histaminase in this case. The peroxidase used caused a very slight inhibition of histaminase in the indigo test (Method II), but the effect was not sufficient to explain the observed inhibition in the dianisidine test. The inhibition might have been due to the sensitivity of the purified enzyme to dianisidine or its oxidised product, but this was not tested.

The dianisidine test was not used in the present work, except in an attempt to stain histaminase <u>in situ</u> after starch gel electrophoresis; this was unsuccessful.

2.4.3. Fluorimetric estimation of histamine by the method of Shore, Burkhalter and Cohn (1959).

Procedure: Aliquots withdrawn from standard solutions of histamine, or from "unknown" solutions containing histamine, were made up to a final volume of 4 ml. in a 25 ml. shaking tube containing 0.5 ml. 5 N NaOH, 1.5 g. sodium chloride, and 10 ml. Analar n-butanol. The tube was shaken for 5 min. in a mechanical shaker, centrifuged, and the lower aqueous phase removed by aspiration. The butanol phase was washed by shaking with 5 ml. NaCl-saturated 0.1 N NaOH for 1 min. to remove traces of histidine. The tube was centrifuged, and the aqueous layer removed.

8 ml. of the butanol layer was added to 4.5 ml. 0.1 N HCl and 15 ml. Analar n-heptane in a 40 ml. glass-stoppered tube, and shaken for 1 min. The tube was centrifuged, and the heptane layer removed using a Pasteur pipette with a bent tip. The aqueous layer was poured into a 5 ml. test-tube and centrifuged again; the last traces of heptane were then removed.

2.0 ml. of the aqueous phase was transferred to a 10 ml. test-tube, and 0.4 ml. N NaOH added, followed by 0.1 ml. of 1% o-phthaldialdehyde in Analar methanol. 0.2 ml. 3N HCl was added after 4 min. to stop the reaction. The fluorescence of the solutions was measured within 30 mins., using the Aminco-Bowman spectrofluorimeter; fluorescence at 450 mμ was measured after activation at 360 mμ. Histamine concentration of the sample was read from a calibration graph constructed from parallel assays of histamine standards.

The assay of 18 samples (including standards) occupied a full working day, and the assays could not safely be interrupted once begun. These considerations, and the possibility of interference by imidazole N-methyl transferase, limited the application of this assay method. Several assays

were required to determine the initial velocity of each enzymic test, since the progress of histamine oxidation was linear for only a short period (See Fig. 2.3.7). A single unsuccessful attempt to compare initial velocities of histamine oxidation at different pH values illustrated the formidable logistic problems of applying this "direct" method to kinetic measurements. The fluorimetric method was used in only one experiment, where it offered unique advantages for comparing rates of oxidation of substrate and indigo in the same enzyme assay solution (Section 2.3.7).

2.5. Critical assessment of histaminase assays considered for use in the present project.

It was recognised early in this project that an insensitive assay method could rapidly use up the entire yield of laboriously purified enzyme solutions, and that comparative experiments carried out on the same enzyme solution carried much greater conviction. In practice, there were limits to the amount of placental material that could be handled in a single preparation, and the most economical use had to be made of the purified enzyme. This project would have been greatly prolonged by the repeated interruptions of experiments in order to prepare a fresh batch of placentae.

A method applicable to both the rapid assay of numerous chromatographic effluents, and the detailed study of purified enzyme, would have been ideal, and the new indigo test (Method II) was developed in an attempt to achieve this ideal. However, the validity of the indigo test was questionable in many respects. A second approach might have been to use a very rapid and convenient method for selecting the most active effluents for pooling and further purification, in conjunction with a more precise method applicable to the quantitative estimation of histaminase activity towards all substrates, at all stages of purification, and particularly to the detailed study of pure histaminase. Only Holmstedt and Tham's method was more convenient than the indigo test for numerous samples, and no quantitative method for detailed study of activity towards all substrates was any more reliable than Method II.

In the hands of the author, Kapeller-Adler's indigo test (Method I) was inferior to the new spectrophotometric test (Method II) in respect of sensitivity, reproducibility, and consumption of reagents and bench time. The new spectrophotometric test was believed to embody the maximum sensitivity

and convenience possible for the indigometric assay of histaminase activity, and also to illustrate the inherent defects of all indigo tests. These will now be discussed, with reference chiefly to Method II.

(a) Like all tests measuring hydrogen peroxide production, the indigo test is subject to interference by oxidisable contaminants of the enzyme solution, notably haemoglobin in the placental medium.

(b) The rate of indigo oxidation is slower than the rate of substrate oxidation. This anomaly probably has several major consequences. Firstly, an initial delay in indigo oxidation is always observed. This delay could not be explained completely, but several possible reasons were eliminated. Although indigo oxidation proceeds linearly with incubation time after the delay has elapsed, and although total indigo oxidation after 24 hr. is linearly related to enzymic concentration, the possibility cannot be dismissed that some change in assay conditions might alter the lag period. Such a change in lag period might not be detected by a single measurement of indigo oxidation after 24 hr. While Method II is valid for the assay of a considerable range of enzyme activities towards the same substrate in the same medium, some uncertainty entered into experiments carried out under non-standard conditions, e.g., examination of substrate and pH optima, and the effect of inhibitors. Where quantitative results were required, it was found best to measure the initial velocities of indigo oxidation, after the lag period.

Secondly, the initial delay in indigo oxidation reduced the possible advantages of setting up and reading assays on the same day. 10 hr. may be regarded as the maximum incubation period for routine assays to be completed on the same day, permitting the performance of some subsequent operation (e.g. dialysis or chromatography) overnight. If the effective period of indigo

oxidation is reduced by a 3 hr. delay, a 10 hr. assay will have only one-third the sensitivity of a 24 hr. assay. A 24 hr. incubation period was adopted in Method II, since this minimised the relative importance of the delay in indigo oxidation, and possible variations in the delay, and also fully exploited the prolonged period during which indigo oxidation progressed linearly. In practice, the 24 hr. assay permitted more convenient planning of experiments. A few series of investigations, in which the design of each experiment depended on the results of the previous one, were subject to some delay, but the great stability of placental histaminase ensured that such delays were of little consequence .

(c) A major objection to the indigo test is that indigo oxidation proceeds by at least two simultaneous or consecutive mechanisms - direct oxidation by hydrogen peroxide, and autoxidation by atmospheric oxygen catalysed by hydrogen peroxide and substrates of histaminase. No way could be found of correcting assay results for the autoxidative process. Since the rate of autoxidation probably depends on the rate of production of hydrogen peroxide, and the immediate concentration of substrate and indigo, the kinetics of total indigo oxidation are likely to be very complicated. As noted in Section 2.3.6, units of indigo oxidation cannot be translated into units of substrate oxidation. Total indigo oxidation after 24 hr. may be linearly related to enzymic oxidation of substrate, but the relationship between the two reactions is indirect and not fully understood. No serious anomalies were encountered, however, when the indigo test was compared with other assays depending on completely different principles. In particular, the method of Holmstedt and Tham gave similar results to the indigo test in most applications.

Although differences were observed in the rates at which different substrates catalysed the oxidation of indigo by synthetic hydrogen peroxide, there was no evidence that the catalytic effect of the substrates was the factor limiting indigo oxidation in the enzymic test, which appears to proceed by a different mechanism. Other components of the test, e.g. isatin, may contribute to the catalysis of indigo oxidation. There appeared to be no consistent correlation between the catalysis of indigo oxidation by a substrate in model experiments (Section 2.3.6) and its rate of oxidation as shown by the enzymic indigo test (Section 5.1.5.).

(d) Investigation of the indigo test revealed that some of Zeller's criticisms of Kapeller-Adler's indigo test are without foundation. For instance histamine exerted no exclusive catalytic effect on the oxidation of indigo (Section 2.2.12). Secondly, there was no evidence that any other enzyme had to be present in order that the indigo oxidation should proceed. It was unfortunate that histaminase was not separated completely from its last contaminant, haptoglobin-methaemoglobin, since the peroxidatic properties of this complex might be considered essential to the indigo test. The evidence against this is:

(i) Haptoglobin-methaemoglobin was enzymically inert towards diamines.

(ii) Addition of excess haptoglobin-methaemoglobin to assays did not significantly alter the rate of indigo oxidation.

(iii) In some chromatographic experiments (Section 4.4.1), partial separation of the enzyme from haptoglobin-methaemoglobin resulted in a range of effluent fractions containing widely varying ratios of enzyme and haptoglobin-methaemoglobin; the

symmetry of histaminase activity patterns in these effluents did not suggest that progressive diminution of haptoglobin-methaemoglobin content inhibited histaminase activity in the indigo test.

However, it might be argued that only a minute amount of haptoglobin-methaemoglobin is required to saturate the requirement of the indigo test for this peroxidatic component. This argument can be met only by complete isolation of histaminase, still active in the indigo test.

The advantages of the new spectrophotometric indigo test (Method II) may not appear impressive after this catalogue of its theoretical deficiencies, but the test must be seen in relation to the alternative methods available.

The test is far more sensitive than any other, except the fluorimetric assay applicable only to histamine, and the tedious and expensive methods using ^{14}C-labelled substrates. It is also specific for the oxidative deamination of substrates, and therefore possesses advantages over methods measuring changes in substrate concentration.

The test requires little bench time and is very economical. No special reagents, equipment, or technical assistance are required.

The test can be applied to any substrate of histaminase, and at least semi-quantitative comparisons of activity towards different substrates can be made.

Although the method is vulnerable to large errors, careful technique can result in precision and reproducibility which compares well with any alternative method. The theoretical deficiencies of the method have proved in practice to be no more serious than the known deficiencies of other methods in common use.

Table 2.5.1.

Alternative assay method	Advantages compared with new indigo test	Disadvantages compared with new indigo test
Fluorimetric assay of histamine	Direct measurement of substrate disappearance. Sensitive.	Laborious. Specific for histamine. Imidazole N-methyl transferase might interfere.
Estimation of histamine by isotopic dilution.	As for fluorimetric assay.	As for fluorimetric assay. Expensive substrate and equipment.
Estimation of ^{14}C-labelled products of diamine oxidation.	As for fluorimetric assay.	Many operations required. Applicable only to cadaverine and putrescine. Expensive substrates and equipment.
Dianisidine test.	No lag period.	Initial velocities not maintained. Relatively insensitive. Second enzyme (peroxidase) required.
Estimation of product of putrescine	Precise, reproducible, and convenient.	Initial velocities not long maintained. Relatively insensitive. Applicable to putrescine only.
Kapeller-Adler's indigo test.	Does not require a spectrophotometer.	Oxygenation required. Larger S.D. Longer lag period. Relatively insensitive. Reducing agents, pH changes, etc., upset final titration.
Manometric measurement of oxygen consumption.	No lag period.	Relatively very insensitive. Second enzyme (catalase) required. Possible secondary oxidation of aldehyde. Laborious.
NH_3 measurement.	Independent of hydrogen peroxide.	Relatively insensitive. Diamines cannot be used as substrates.

A brief summary of the merits and demerits of the new spectrophotometric indigo test is provided in Table 2.5.1.

The following assay strategy was finally adopted in the present study:

Activity towards cadaverine was measured at each stage of purification using standard Method II. Appropriate adjustments of dilution were then made, and the activities of the enzyme towards histamine, putrescine and cadaverine were measured in parallel at each stage, using the standard Method II. It was hoped that, at key stages of purification, enzymic activities could be checked quantitatively by independent methods, mainly to offer other workers a broader basis on which alternative purification schemes could be compared. Failure to maintain initial reaction velocities in the tests of Aarsen and Kemp, and Holmstedt and Tham, precluded the use of these assays for exact comparisons of activity as purification progressed. However, it was considered worth-while to check histaminase activities towards putrescine throughout purification, using Holmstedt and Tham's method semi-quantitatively. Activities towards all three substrates were also measured at key stages using Kapeller-Adler's microvolumetric indigo test (Method I). In this way, some insurance was provided against any future demonstration that the author had wrongly assessed the validity of the new spectrophotometric indigo test.

Chromatographic effluents were examined by the new spectrophotometric indigo test, using cadaverine as substrate. If a result was required rapidly, a greater amount of enzyme was sacrificed in the method of Holmstedt and Tham (Method III).

The purest preparations were examined using the new spectrophotometric indigo test. Despite its real and theoretical difficulties, this was considered the only sensitive method of acceptable validity for the assay of

histaminase activity towards all classes of substrate. However, initial rates of indigo oxidation were measured, rather than 24 hr. values, whenever it was important to be certain that enzymic activity was constant over the incubation period. These results were supplemented, where possible, by semi-quantitative data for putrescine alone, using the method of Holmstedt and Tham (Method III).

Chapter 3. Experimental Methods.

3.1 Estimation of protein.

3.2 Techniques in ion-exchange chromatography.

3.3 Gel filtration on Sephadex G-200.

3.4 Concentration of dilute protein solutions.

3.5 Electrophoresis.

3.1 Estimation of Protein.

The methods of estimating total protein considered for use in the present study were:

(1) Biuret methods, particularly that of Gornall, Bardawill and David (1949).

(2) Reactions using Folin's reagent, particularly the methods of (a) Lowry, Rosebrough, Farr and Randall (1951) and (b) Miller (1959).

(3) Extinction at 280 mμ (Warburg and Christian, 1941) or 210 mμ, (Tombs, Souter and Maclagan, 1959).

The sensitivity required for the most dilute column effluents was the detection of 10 μg. protein/ml., with the loss of about 0.2 ml. of the sample. The method had also to be rapid and simple enough to be applied to hundreds of effluent fractions — thus, Kjeldahl nitrogen determinations were not considered.

Proteins may be estimated using spectrophotometric methods specific for certain amino-acid residues, e.g. tyrosine residues estimated using Folin's reagent. Since proteins have variable amino-acid composition, different molar extinction coefficients are found for different proteins. Biuret methods, which essentially measure the number of peptide bonds, also give different molar extinction coefficients for different proteins. Lowry et al. (1951) reported that the ratio of equivalent molar extinctions of trypsin and gelatin was about 3, using their Folin method, and about 1.5 using the biuret reaction. Since even the percentage of nitrogen in proteins varies between about 12 and 19% (Dixon and Webb, 1964; p.30) there is no reliable standard method to which all other methods may be referred.

Calibration curves relating extinctions obtained by two independent methods may be constructed by comparing reactions with a single protein, or

a nearly-constant mixture of proteins such as normal human serum. This relationship may not remain valid when the methods are applied to other proteins. In particular, as the protein composition of an enzyme preparation changes throughout purification, the relationship between two protein estimation methods applied at each stage may undergo several changes.

Initially in this project, the biuret method was used for solutions containing at least 1 mg. protein/ml. Below this concentration, method 2(a) was used. Protein in a sample to be chromatographed was assayed by the biuret method, and dilutions of the sample were used as secondary standards to construct a calibration curve for method 2(a), applied to the column effluents. This procedure was self-consistent for each experiment, and an element of continuity was preserved by biuret measurements at successive stages of purification. However, the correlation between the two methods varied widely according to the stage of purification, and it became necessary to suffix the Specific Activities, quoted at each stage, with a reference to the protein estimation method employed.

Some inconsistencies were then eliminated by applying only method 2(a) throughout purification, making dilutions where necessary. Standard curves were constructed using pooled human serum, calibrated by Kjeldahl determinations, as standard. Since the assay reagents were prepared fresh each day, permanent standards had to be retained. Whereas standards containing about 1 - 10 mg. protein/ml. could be preserved for long periods without changing their response to the biuret reaction, it proved impossible to keep standards for method 2(a) for more than a day; possibly the protein in these very dilute standards became adsorbed to the walls of the test-tube at low temperatures. It was therefore necessary to make up fresh standards daily from frozen human serum,

or from dried bovine serum albumin. Furthermore, in the author's hands the method of Lowry et al. was subject to large errors, due to the very short reaction time, and the need to use micro-cuvettes in the spectrophotometer. A variety of mixing devices failed to improve the reproducibility of the method, and duplicate tests were resorted to; these were wasteful of material.

Miller's modified Folin method proved to be much more rapid and reproducible, and obviated the use of micro-cuvettes. However, the reagents still had to be mixed each day, and the need for calibration with permanent standards remained.

The procedure finally adopted was the measurement of the extinction of the protein solution at 280 mμ in matched 10 mm. silica cuvettes, using an appropriate buffer in the reference cuvette. A protein concentration of 1 mg./ml. was arbitrarily assigned to a solution having an extinction $E^{1\ cm.}_{280\ m\mu} = 1.0$. This relation was approximately true for human serum, standardised by Kjeldahl nitrogen determination, but may not have been true for crude histaminase preparations, e.g. Stage 3 enzyme. Certainly, there was a very poor correlation between extinction measurements and the method of Lowry et al., both calibrated using human serum, for the measurement of Stage 3 enzyme. In the absence of absolute criteria of validity, extinction measurements were preferred on the grounds of reproducibility. The method did not require reagents, and the need for standards was eliminated by the reproducibility of extinction measurements from day to day on the same instrument, and the adoption of an arbitrary concentration scale. The problem of varying extinction coefficients of the total preparation as certain elements were removed by the purification scheme was not considered important, although attempts at

"absolute" measurements of protein concentration, e.g. by nitrogen determination, might have been valuable if histaminase had finally been isolated. The wavelength of extinction measurements was also adopted arbitrarily. In practice, the enzyme solutions had a fairly broad extinction maximum in the region 270 - 285 mμ; the extinction maximum at neutral or slightly alkaline pH was approximately constant throughout purification, at 278 mμ. No attempt was made to correct results for the extinction of nucleic acids (Warburg and Christian, 1941) since these compounds were probably not retained after chromatographic fractionation, judging by the absence of extinction maxima at 260 mμ, and since exact comparisons between successive stages of purification were invalidated, in any case, by the variable extinction coefficients of the proteins alone.

Extinction measurements at 210 mμ (Tombs, Souter and Maclagan, 1959), were found to be extremely sensitive, and potentially of great value and simplicity. However, EDTA added to most of the buffers used in this project interfered with the method, and the high sensitivity was never really required.

3.2 Techniques in ion-exchange chromatography.

Principle. The theory of ion-exchange chromatography of proteins has been discussed by the originators of the technique, Peterson and Sober, in a practical and comprehensive review (1962).

A cellulose matrix is modified by reaction with polyfunctional reagents, and acts as an inert support for ionic groups, e.g. amino- or carboxylic groups, which are capable of forming electrostatic bonds, at appropriate pH values, with similar weakly dissociated residues of the protein molecules. The adsorption of a particular protein is governed by the number and strength of the bonds it can make with the adsorbent, and the extent of adsorption varies as different groups of the protein become dissociated at different pH values. The different electrical properties of proteins can thus be exploited for fractionation purposes by commencing chromatography at a pH at which almost all components of the sample are adsorbed, and subsequently changing the pH in order to reduce the attractive forces between the adsorbent and selected proteins, so that these are preferentially eluted from the column. The adsorption and elution of proteins under changes of pH is still largely an empirical procedure; a knowledge of the iso-electric points of all the components may be helpful, but these are seldom known, and in any case give little information about changes in electrical properties remote from the iso-electric pH.

The electrostatic attraction between a protein and the adsorbent may be reduced by increasing the ionic strength (I) of the medium — according to the theory of Debye and Huckel, attraction between unlike charges is diminished by an increase in I. However, proteins least strongly adsorbed travel farthest from the top of the column during development of the chromatogram, and the most strongly bound species at the top of the column are the first to encounter any

increase in the ionic strength of the eluant. Elution must therefore be carried out slowly, to promote efficient equilibration of the proteins with the adsorbent, and preferably under a very gradual, continuous gradient of ionic strength, rather than by stepwise increments which tend to elute a proportion of all proteins except those most strongly adsorbed.

In addition to separation by electrical charge, non-specific adsorption of some components to the cellulose matrix may occur. If this proves to be disadvantageous, it may be necessary to employ ion-exchangers based on matrices other than cellulose, e.g. the Sephadex exchangers (Pharmacia, Ltd.,Uppsala) based on crosslinked dextrans of varying degrees of porosity. The Sephadex exchangers, besides being less subject to non-specific adsorption, may combine ion-exchange with separation by molecular size. Exchange groups are evenly distributed throughout the gel particles but, depending on the porosity of the dextran matrix, only rather small proteins can enter the particles and equilibrate with the internal exchange groups. These smaller proteins may be retarded both by ion-exchange forces and by diffusion into the gel, but larger species (>50,000 M.W.) encounter only that fraction of exchange groups which lie near the surface of the particles, with a consequent reduction in the true exchange capacity of the adsorbent for these proteins. The use of Sephadex ion-exchangers should, in general, be postponed until preliminary separations have been carried out using the corresponding G-grade of Sephadex (without exchange groups), and the appropriate cellulosic exchanger. Only then is it possible to predict whether gel filtration and ion-exchange would occur simultaneously for most components on the Sephadex exchanger, and whether the two effects would be mutually cumulative or antagonistic.

Adsorbents used in the final purification scheme.

DEAE-cellulose (Sigma) was of medium mesh grade and had a theoretical exchange

capacity of 1 meq./g.

Cellulose phosphate (Whatman P-11) was of medium mesh grade, and had a theoretical exchange capacity of 7.4 meq./g.

These adsorbents were stored between experiments either in 0.1 N NaOH, or in dilute buffer, protected by toluene and chloroform, and in the refrigerator. All subsequent operations, including packing into columns and the chromatography proper, were effected at room temperature.

Preparation of adsorbent

Before their first use, cellulosic ion-exchangers were washed successively with 0.1N NaOH, water, 0.1N HCl, water, 0.1N NaOH and water. About 200 g. adsorbent could be efficiently washed on a 2 l. sintered glass funnel, under suction. Preliminary washing was followed by the repeated removal of fines, by stirring the adsorbent with about 5 l. of water, and decanting the material which had not sedimented within about 10 min.

The pH of the adsorbent was adjusted by slowly filtering through it a more concentrated solution, usually about 0.1 M, of the starting buffer. When the pH of the adsorbent was the same as that of the starting buffer, the adsorbent was thoroughly washed on the filter with water, followed by 10-20 l. of the dilute starting buffer.

After use, the adsorbent was removed from the column for washing and regeneration, undergoing essentially the same treatment as new adsorbent, except that the removal of fines was not repeated.

Apparatus

For small-scale work, Pyrex air condensers made suitable columns. The adsorbent bed was supported over a plastic perforated plug, wedged in the constriction of the condenser, and covered with an even layer of cleaned

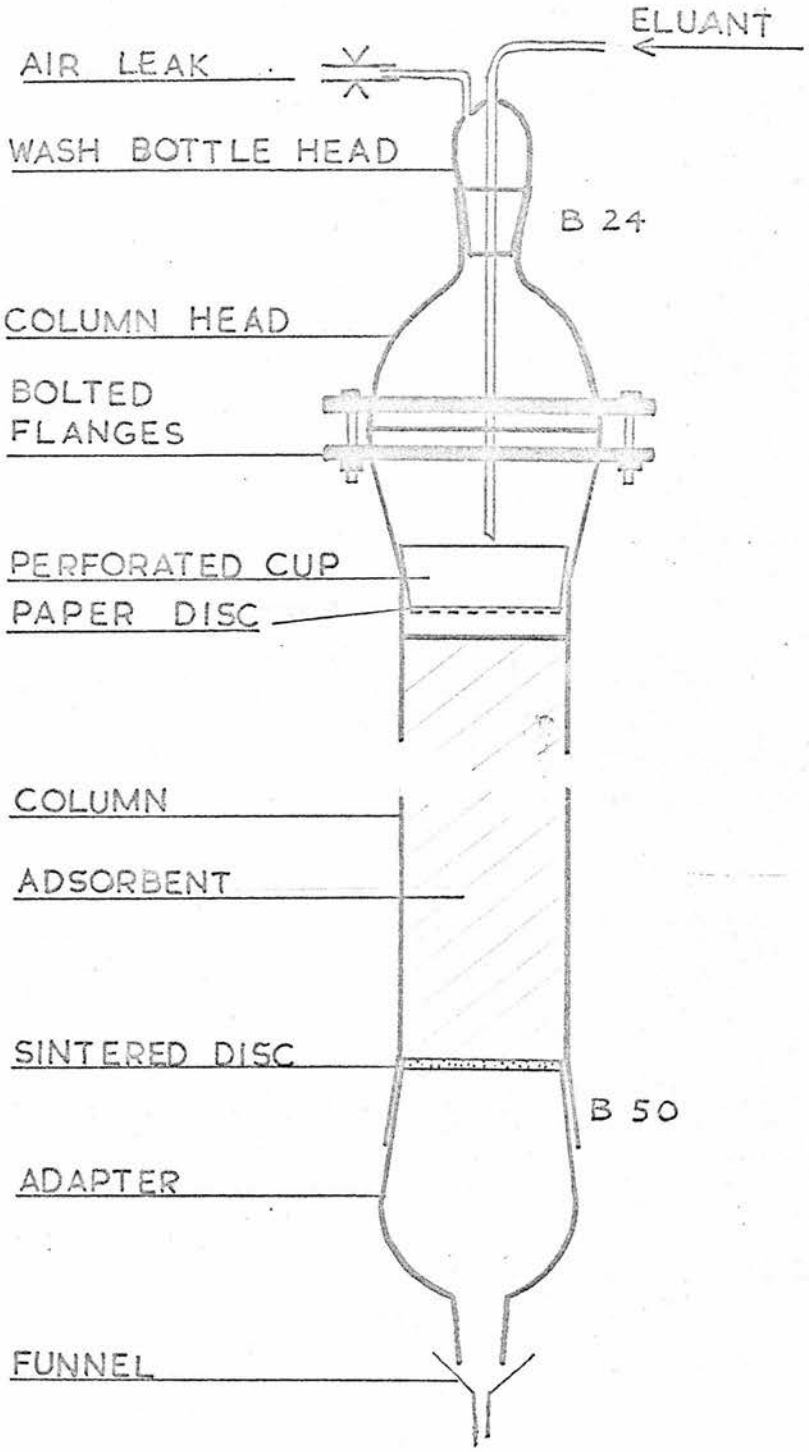


Fig. 3.2.1. Column for large-scale ion-exchange chromatography.

glass wool.

Large preparative columns, Fig. 3.2.1, were assembled from Quickfit apparatus. A heavy-walled 5 x 100 cm. Pyrex tube had a socket at the base to accept a cone fitted with a sintered glass support, and an outlet tube. The buttressed top of the column could be bolted, with the aid of brass collars, to an inlet section which accepted a wash-bottle head. One inlet of this head was used as an air leak, and the other was connected to the eluant reservoir by surgical quality plastic tubing. The effluent from the column dropped, via a small funnel, into 1 x 15 cm. Pyrex test-tubes held in a Locarte time-operated fraction collector. The adsorbent was protected from disturbance by a filter paper disc, supported just above the surface of the bed in a plastic cup with a perforated base. The sample or eluant never came in contact with any surface other than Pyrex or plastic. Care was taken to avoid contact with the Apiezon grease used to seal the joints in the upper part of the column assembly.

Packing

Unless columns were packed under pressure, the adsorbent bed tended to be easily disturbed, and to permit leakage of sample and eluant between the walls of the column and the adsorbent. On the other hand, the application of too great pressure resulted in undue resistance to flow, and the formation of pleats or folds near the bottom of the adsorbent bed. Decantation of fines and preliminary experiments with packing pressures had to be carried out on each batch of adsorbent, regardless of the manufacturer's grading, and optimum packing pressures were not the same for columns of different dimensions.

For packing, the column head was replaced by a 2 l. conical reservoir (constructed by replacing the tap of a separating funnel with a strong

tubulure), fitted flexibly to the buttressed top of the column via a large rubber bung. Oxygen pressure was applied to the top of the reservoir, and the suspension of adsorbent was swirled at intervals to keep it from settling. 200 g. ~~adsorbent~~ adsorbent was usually suspended in about 5 l. of starting buffer. Peterson and Sober (1962) recommend a higher proportion of buffer, but a compromise has to be found between a convenient speed of packing and the risk of trapping air in a thick suspension. Small columns packed with a coarse grade of cellulose required the immediate application of 5-10 p.s.i. pressure; medium grade adsorbent was usually allowed to settle into large columns under gravity until about one-third of the column was packed, then pressure was gradually increased to 2-5 p.s.i.

Sample application

The starting buffers were of such low Specific Gravity that a small sample could be layered on to the adsorbent under the buffer, giving a very sharp origin. For samples of large volume, it was found more convenient to protect the adsorbent surface with a filter paper, allow the buffer just to drain into the bed, then add the whole sample to the column from a pipette. When all the sample had drained into the bed, the walls of the column were washed with successive small aliquots of starting buffer, and elution started.

Elution

In preliminary experiments, it was usually advisable to develop the chromatogram with one bed volume of starting buffer, or until the bands had reached equilibrium positions. As experience of the particular elution system increased, it was often possible to omit development and start gradient elution immediately. During elution, the volume of eluant above the adsorbent surface was kept to a minimum in order to reduce mixing at this

point. Similarly, when stepwise changes in the composition of the eluant were used, the reservoir and delivery tubing were emptied, and the first eluant allowed to drain into the bed, before the second eluant was added; this produced the sharpest interface between successive eluants.

Gradient elution has been discussed in practical detail by Bock and Ling (1954) and Peterson and Sober (1959). The chief elution device used in the present work was a simple linear gradient of sodium chloride concentration in starting buffer. Two identical aspirators, A and B, were connected at the base by a capillary junction closed with a screw clip. Starting buffer was added to A, and eluant was continuously drawn from it to the column via a second capillary tube. Aspirator B was slowly filled with a solution of sodium chloride in starting buffer, giving a high enough concentration to elute all desired components from the adsorbent. When the weight of salt solution added to B was equal to the weight of buffer in A, the junction between them was opened and the contents of A were stirred with a magnetic stirrer. The concentration of salt solution withdrawn from A then increased linearly with eluant volume.

In the description of the purification scheme (Section 4.2), salt gradients are expressed in the following manner — "a linear gradient from 0 to 2 M over 2 l.". This means that aspirator A contained 1 l. of starting buffer, and aspirator B contained 1 l. of 2 M sodium chloride in starting buffer, in the linear gradient device described above. Salt gradients were followed by measuring the Na^+ or Cl^- content of the effluents. In the elution diagrams, e.g., Fig.4.2.2 there was a considerable interval between starting the gradient and the detection of increased salt concentration in the effluent. This was due chiefly to the large "dead volume" of the preparative columns,

and possibly partly to retardation of the appropriate counter-ion on the exchanger. Simultaneous determination of Na^+ and Cl^- would have given more information about the latter effect.

Concave gradients were obtained by decreasing the cross-sectional area of reservoir B, according to the method of Bock and Ling (1954).

3.3 Gel filtration on Sephadex G-200.

Principle

The theory of gel filtration has been discussed by Flodin (1962), Porath and Flodin (1963), and Laurent and Killander (1964). Swollen particles of cross-linked dextran form a gel bed of well-defined porosity, which excludes macromolecules greater than a certain molecular size, depending on the degree of cross-linking of the gel. Excluded macromolecules pass through interstitial spaces of the gel and are eluted more rapidly than smaller molecules, which are partly retarded within the gel particles. The elution volume of a partially-retarded species is approximately inversely proportional to the logarithm of its molecular weight (Andrews, 1964); molecular size is also important, and there are minor adsorption effects retarding solutes with, e.g., aromatic ring systems.

One common expression of the filtration rate of a solute on Sephadex, analagous in some ways to R_f values in paper chromatography, is the relative elution volume, V_r. Where V_e is the effluent volume in which the solute is eluted, and V_t is the total volume of the gel bed,

$$V_r = V_e/V_t. \qquad \text{Eq. I}$$

V_t remains constant for only a single experiment, since the gel bed may become compressed in successive separations. It is better to compare separations obtained on different columns by using an internal standard. If, in the same experiment, the elution volumes of the substance to be studied and of the reference substance are V_{e1} and V_{e2}, respectively,

$$V_{r1}/V_{r2} = V_{e1}/V_{e2}. \qquad \text{Eq. II}$$

Killander (1964) has shown that for several serum proteins V_r is quite reproducible for a given substance in a number of experiments using the same batch of gel, but less reproducible when different batches of gel are used.

His data also show that the ratio of V_r values for any two components remains almost constant, no matter what batch of gel is used.

For the purpose of the present project, the ratio V_{r1}/V_{r2} was measured as the ratio of the elution volumes of two components on the same passage through G-200. Each elution volume was defined as the volume of effluent collected from the moment the sample entered the gel until the maximum concentration of each component was reached in the effluent. This definition of elution volume differs slightly from that used by Killander, but its consistent application to all components minimises any possible discrepancies in final ratios.

Descending flow filtration

Sephadex G-200, which retards species with a molecular weight smaller than about 200,000, is the most porous of the Sephadex gels, has the lowest proportion of solid material, and is mechanically rather weak. Using descending flow, beds longer than about 100 cm. tend to become so compressed under their own weight that the flow-rate becomes unacceptably slow. When packing and running columns under descending flow, the chief aim is to minimise compression of the gel. This is done by maintaining a maximum pressure head of about 15 cm. of water, even during packing, by manipulating the levels of the buffer reservoir and the column outlet tubing. This procedure was used in the early stages of this project, until the large-scale separation of enzyme material demanded columns of a larger capacity than could conveniently be maintained by descending flow.

Ascending flow, and re-cycling gel filtration

Adequate flow-rates may be attained in columns of large dimensions if the sample and eluant are pumped upwards through the gel bed, so that pumping

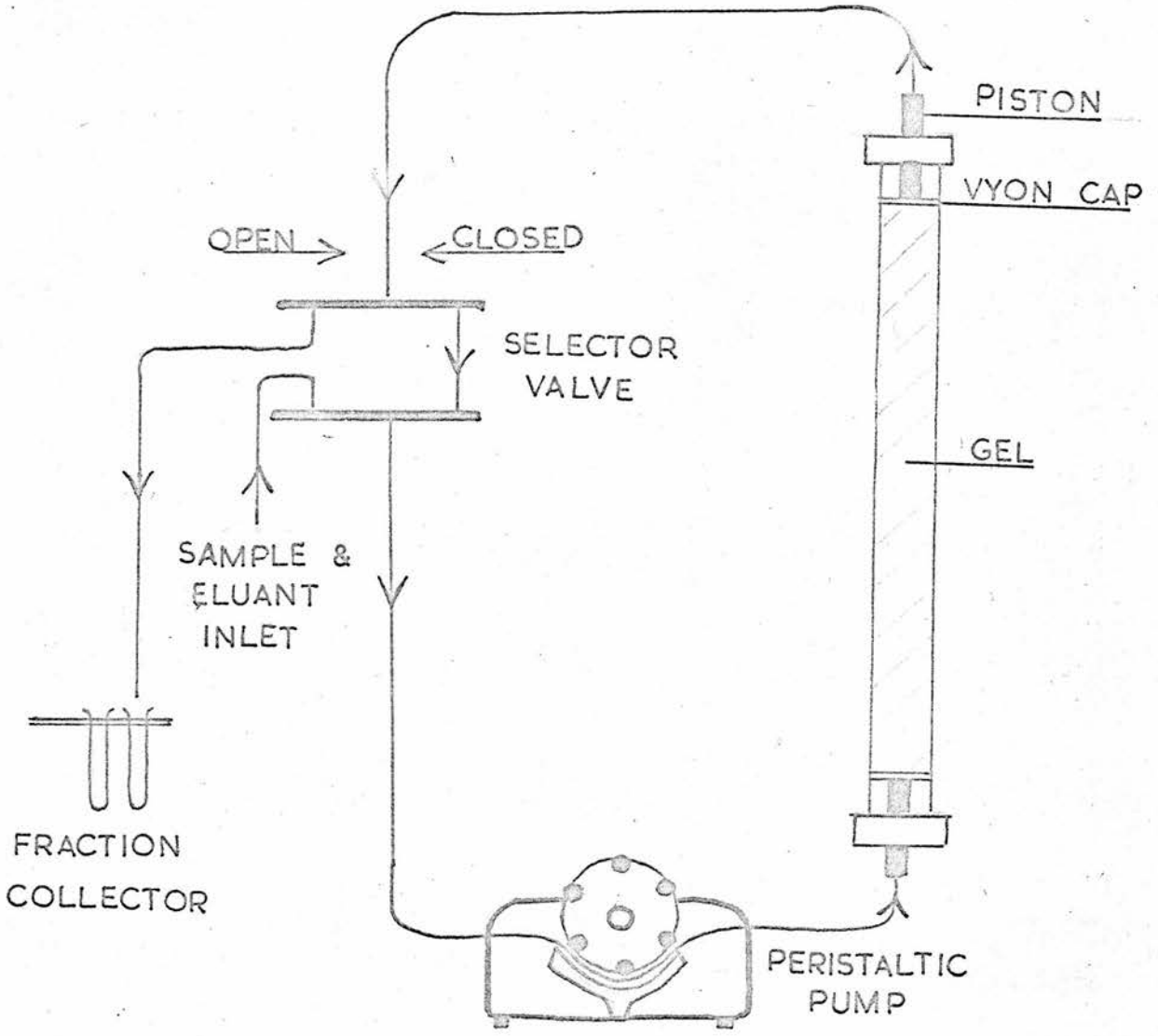


Fig 3.3.1. Diagram of system for re-cycling gel filtration.

pressure and the weight of the gel are in opposite directions, and the resultant pressure on the gel is minimised. The pump may be included in a cyclic system (Fig. 3.3.1), in which any undesired portions of the effluent may be bled out by means of a selector valve, while the desired portion may be pumped back into the bottom of the bed to undergo another cycle. Recycling chromatography (Porath and Bennich, 1962) of a desired fraction substitutes three passages through a 100 cm. column for a single passage through a 300 cm. column, which is hardly feasible using G-200.

The LKB recycling equipment was used in all Sephadex separations described in Chapter 4. Stage 6 of the purification scheme was carried out with the selector valve in the "open" position, i.e. only a single cycle was performed by all components.

It was found that solutes of high molecular weight were diluted 5 - 10 fold on a single passage through a 3 x 100 cm. column of G-200. Zone spreading limits the sample volume to about 3 ml. for two cycles, and less than 1 ml. for three cycles. Further, the sample to be recycled must be fairly homogeneous with respect to molecular weight, and should preferably be a concentrate of a narrow fraction from a previous separation on the same gel. This ensures that the smallest, and slowest, species in a mixture may be bled out on its first cycle before the largest, and fastest, species overtakes it on the latter's second cycle. A continuously recording U.V. absorptiometer, e.g. the "Uvicord" assembly, is almost obligatory for satisfactory recycling. Fitted between the top of the column and the selector valve, it should provide a continuous record of protein emerging from the gel bed, and permit accurate selection of desired fractions on the basis of previous experience. This expensive equipment was not available for the present work, and selection was carried out by following

an increasingly diffuse brown band of HpMHb.

The soluble Blue Dextran, M.W. 2,000,000, produced by Pharmacia, was found not to be homogeneous, and could not be used as an additional marker for selection of fractions to be recycled. While the bulk of the Blue Dextran was eluted ahead of HpMHb and histaminase, a small proportion tended to trail into this protein fraction. Blue Dextran was used alone to check that bands migrated evenly in the column, after each re-packing or other disturbance of the gel bed.

Packing and running G-200 columns.

5 mM borate buffer, pH 8.6, was chosen as the eluting buffer because of its known preservative effect on histaminase, and 0.1 M sodium chloride was added to increase the ionic strength of the medium and discourage aggregation of proteins (Flodin, 1962).

About 40 g. dry Sephadex G-200 was swollen in an excess of water for several months, and fines were removed by repeated settling and decantation in large volumes of buffer. Efficient removal of fines, even at the expense of much material, is crucial to the successful maintenance of either ascending or descending flow.

One of the column pistons was fitted with a deaerated porous Vyon cap, and inserted in the lower end of the column, which was immediately filled with deaerated buffer. A conical funnel was linked to the top of the column with the aid of a screw collar, and filled with a deaerated slurry of swollen G-200. The capillary leading from the lower piston was opened, and the gel allowed to settle under gravity flow. No attempt was made to reduce the pressure head, since a loosely packed gel bed tended to develop cracks when pumping commenced. When the level of packed gel had almost reached the top

of the column, the remaining gel was removed by aspiration, and buffer was passed through the column to complete settling. The reservoir was then replaced by a second capped piston, carefully inserted in the column to avoid trapping air bubbles, and pressed into the surface of the gel to a depth of about 5 mm.

Capillaries from the retaining pistons were connected to the selector valve and pump, as shown in the diagram. The remaining apertures of the selector valve were fitted with capillary tubing leading to a fraction collector and an inlet filter.

With the valve in the open position, the sample was pumped into the bottom of the bed, and the separated fractions emerged through the valve into a time-operated fraction collector. Fractions of about 10 ml. were collected by adjusting the time interval and the pump speed.

If a fraction was to be recycled, the selector valve was closed shortly before the fraction began to emerge from the column, and opened again whenever the whole fraction had re-entered the gel bed.

3.4 Concentration of dilute protein solutions

Chromatographic procedures frequently result in dilute effluents which require considerable concentration before being subjected to further analysis. Gel filtration and electrophoresis, in particular, are best carried out on small volumes of concentrated sample.

Four main principles may be recognised:

(1) Precipitation of protein.

(2) Ultrafiltration.

(3) Removal of water by evaporation.

(4) Removal of water by osmosis.

(1) Method 1 was successfully applied at Stages 2 and 3 of the purification scheme, using ammonium sulphate. The procedure is most effective when the solution contains more than about 10 mg. protein/ml. Only about 10% of histaminase activity was recovered after precipitation of dilute effluents, containing about 100 µg. protein/ml., at 65% ammonium sulphate saturation. This poor recovery may have been due to incomplete protein precipitation, failure to sediment the fine precipitate after centrifugation at 4000 x g for 1 hr., or inactivation of the enzyme. Salt precipitation was not used after Stage 3 as a method of concentrating dilute solutions.

(2) Ultrafiltration is commonly performed by supporting the protein solution in dialysis tubing in an evacuated chamber; or an evacuated "finger", formed by stretching dialysis tubing over a rigid support, may be inserted in a reservoir of the dilute solution, as in the LKB ultrafilter. These methods are rather slow, the filtration rate diminishing as the volume of the solution decreases, and they require cold-room facilities. The application of high external pressures, (Albert-Recht and Stewart, 1960) results in more rapid

filtration, but requires expensive stainless steel apparatus, and very careful technique. Peetoom and Gerald (1964) have reported simple but effective ultrafiltration of small volumes of protein solution, utilising heavy weights to exert high external pressure on narrow-diameter dialysis sacs. All ultrafiltration methods are subject to the risk of losing laboriously prepared enzyme solution through minute punctures in the dialysis tubing, and losses due to the sample drying out on the dialysis tubing as concentration progresses. (3) Water may be evaporated from a dilute solution by placing it in dialysis tubing and drying with the aid of a fan, at room temperature. Such treatment is slow, and likely to result in denaturation of proteins. The deleterious effects of drying may be reduced at low temperature, and freeze-drying is widely used for the concentration and preservation of proteins. The technique is not applicable to all enzymes and has the minor disadvantage that salts are retained in the dried sample. The author did not have access to large-scale freeze-drying equipment during the present project, and the potential value of this technique for concentrating histaminase solutions could not be investigated. (4) Protein solutions may be concentrated by osmosis against such hydrophilic substances as sucrose, Ficoll, Sephadex, polyvinyl pyrrolidone or polyethylene glycol. The solution may be contained in a dialysis sac and surrounded with the hydrophilic substance, or vice versa. Curtain (1964) has described an interesting method of removing small, controlled amounts of water by direct immersion of dried polyacrylamide rods in the solution to be concentrated.

Ficoll and Sephadex were found to be very expensive concentrating agents. Recovery of Sephadex required tedious drying with ethanol, and its low water capacity excluded its use for large volumes of dilute effluent.

Concentration against sucrose (Hsiao and Putnam, 1961; Kapeller-Adler

and MacFarlane, 1963) was attempted in the early stages of this work. Unless the ratio of sucrose to solution was about 100/1, reduction in volume of the sample was invariably accompanied by counterdialysis of sucrose into the sac. This did not appear to disturb subsequent electrophoresis of the sample, but seriously interfered with chromatographic fractionation methods. When the concentrated sample was dialysed against the dilute starting buffer prior to ion-exchange chromatography, the sac became distended under intense osmotic pressure, and sometimes burst. If a generous space was left for expansion, the resulting dilution of the sample defeated the original aim of the process. Even then, very protracted dialysis was required to remove all sucrose, which interfered with ion-exchange separations. It was soon recognised that removal of sucrose might conveniently be combined with gel filtration on Sephadex G-200. However, the sample concentrated against sucrose had such a high Specific Gravity that it sank through the gel bed when applied to the top of a Sephadex column. The viscosity of the sample was also inimical to ascending gel filtration.

Finally, the method routinely adopted for the concentration of all samples was osmosis against high-M.W. polyethylene glycol, (Carbowax, 15,000 M.W., Union Carbide.). A single length of dialysis tubing containing the solution to be concentrated was laid in a bed of shredded Carbowax in the refrigerator. As the polymer rapidly absorbed water and solutes from the sac, knots were made in the ends to keep the sac distended, and the walls continuously in contact with the solution. When the sample had been sufficiently concentrated, the sac was removed, rinsed and dialysed against dilute buffer for an hour. The contents of the sac were recovered by squeezing out the concentrate and rinsing the inside of the sac with very small aliquots of

buffer, or, if the concentrate had a very small volume, by cutting off one end of the sac and centrifuging it, open end downwards, in a small test tube. If care was taken to avoid the sample drying out on the sac, recovery of histaminase activity was virtually complete. The success of the method is attributable to its comparative rapidity, ease of operation in the cold, the elimination of high external pressures, and the inertness of the polymer. While it is extremely hydrophilic, Carbowax is not hygroscopic, and may be allowed to dry at room temperature or in the draught produced by a refrigerator fan. The dried Carbowax may be crushed and used repeatedly until the concentration of salts and other diffusible substances becomes too high.

The one disadvantage of using Carbowax is that a small amount of the polymer counterdialyses into the sac. This counterdialysed material has similar properties to "native" Carbowax remaining outside the sac (Howe, Groom and Carter, 1964). These authors found that Carbowax interferes with the determination of protein either by the use of Folin reagents, or by extinction measurements at 280 mμ. These observations were confirmed when Carbowax was used in the present project. It was initially believed that the Carbowax contained a small proportion of low-M.W. polymer and that only this fraction was capable of counterdialysis; therefore subsequent gel filtration of the concentrated sample would easily separate large proteins from the low-M.W. Carbowax. Contrary to expectations, gel filtration of counterdialysed Carbowax on G-200 did not give a sharp peak corresponding to low-M.W. species; instead, Carbowax was fairly evenly distributed throughout the effluent, as judged by extinction measurements at 280 mμ. Similar results were obtained by MacLean (1965), using G-200 in Tris-saline buffer, pH 8.6. Counterdialysed Carbowax was evenly spread throughout the effluent, whereas a higher proportion of

native Carbowax emerged in an elution volume typical of protein species of M.W. 150,000. Ryle (1965) developed a titrimetric method for estimating polyethylene glycol, based on the oxidation of the polymer with hot acid permanganate. He found that the bulk of native and counterdialysed Carbowax emerged from a column of G-100 in an elution volume similar to that of haemoglobin.

It is not immediately obvious how Carbowax can counterdialyse through Visking tubing as if it were of low M.W., yet behave on gel filtration as if it had a very high M.W. Determination of the M.W. distribution of native and counterdialysed Carbowax by osmometry might help to explain this anomaly. A possible explanation is that the low-M.W. fraction which diffuses through the sac re-aggregates to form compound molecules which are not dissociable by gel filtration.

It was recognised that effluents from Stage 6 and Stage 7 of the purification would probably contain small amounts of Carbowax, which would contribute to their extinction at 280 mμ. Control experiments in which distilled water was concentrated in place of enzyme solution and the counterdialysed material subjected to gel filtration on G-200, indicated that the extinction due to Carbowax did not exceed 0.020 units in any fraction. Most of the Carbowax tended to precipitate on storage of the concentrate in the refrigerator -- an observation which offers a little support for the above suggestion that the polymer may re-aggregate. Since there was no evidence that counterdialysed Carbowax in itself inhibited histaminase activity, or had more than a slight effect on protein measurements, no consistent attempts were made to remove the polymer from concentrated samples. This could have been done at any stage by chromatography on any of the cellulosic ion-exchangers, which do not retain the uncharged Carbowax (Howe et al., 1964).

3.5. Electrophoresis

Electrophoresis of histaminase preparations was attempted on three media, cellulose acetate, starch block, and starch gel. Only the latter medium provided interesting separations.

Cellulose acetate electrophoresis was carried out according to the method of Bodman (1960) in veronal-acetate buffer at pH 8.6, using the EEL equipment. The advantages of this medium were the rapidity of separation and the large number of samples which could be analysed in parallel. As employed by Kapeller-Adler and MacFarlane (1963), electrophoresis of column fractions gave useful information about the contaminants removed from the enzyme, but the enzyme itself could not be positively identified on the strips. Cellulose acetate electrophoresis is not, however, a very sensitive or precise analytical tool. The sample is adsorbed into the strip as a relatively broad band, and the resolution of components is consequently much less sharp than on starch gel electrophoresis. Background staining and trailing of components result in poor contrast of faint bands. Whereas Kapeller-Adler and MacFarlane used cellulose acetate electrophoresis as the ultimate test of the homogeneity of their hog-kidney preparation, the present author's experience was that minor components could easily be overlooked if this method alone were employed.

An attempt was made to separate Stage 4 enzyme by electrophoresis in borate buffer, pH 8.6, in a trough packed with starch grains, essentially according to the method of Fønss-Bech and Li (1954). After running in a cold room at a constant field strength of 5 mV/cm. for 20 hr., 1 cm. serial sections of the block were cut out and eluted on a sintered glass filter with a small volume of buffer. Determination of histaminase activity and total protein in the eluates indicated that histaminase had not been separated from its chief

contaminants, MHb and HpMHb, and that marked endosmosis had occurred. The lack of resolution obtained discouraged further attempts to develop starch block electrophoresis as a preparative method, although the recovery of activity was excellent.

Starch gel electrophoresis.

The method of Smithies (1955) was employed in preparing and using starch gels. The dilute borate buffer, pH 8.5, used to make the gel, and the more concentrated buffer used in the bridge and electrode compartments, were as described by Smithies, and the proportions of dry starch and buffer were those recommended by the manufacturers (Connaught Laboratories) for that batch of starch.

150 ml. of boiled and deaerated starch solution was poured into a demountable Perspex tray, 10.5 x 17.5 cm., to a depth of about 7 mm., and allowed to cool at 4° for an hour. With the aid of a razor-blade, slots were cut in the gel near the cathode end, and samples were inserted on small strips of Whatman 3MM paper. Four thicknesses of Whatman 3MM paper formed wicks from the gel to the bridge compartment, and from there to the electrode compartment. The apparatus was assembled in a refrigerator at 4° and connected to a power supply placed outside. A constant current of 15 mA (initial field strength, 10 V/cm.) was passed for 20 hr. and the gel removed for staining.

The tray was partly dismantled to expose the upper half of the gel, and the top 3 mm. was sliced off with the aid of a fine wire. The top slice was stained for protein, and the bottom slice for HpMHb or histaminase activity.

Protein Stain. Protein in the completed gel was detected by staining in a 1% solution of naphthalene black, or a 0.1% solution of nigrosin (Gurr, Ltd.) in methanol-acetic acid-water solvent (4/1/5 by vol.). When the former stain

was used, the gel was left in the stain for about 15 min., then washed with many changes of solvent until the background became white. When nigrosin was used, the gel was left in the stain overnight before several washings with solvent. The nigrosin stain was the more sensitive, but did not wash out quite so well as naphthalene black.

Haptoglobin Stain. A portion of the gel was placed in a solution of the following composition:

Acetate buffer, 0.3 M, pH 4.6	35 ml.
o-dianisidine	35 mg.
Ethanol	35 ml.
Water	80 ml.
H_2O_2 (100 vol.) added immediately before use	1 ml.

Adequate staining of HpMHb bands was achieved within 1 hr., after which the gel was briefly washed with water.

Detection of histaminase activity

(a) In the intact gel: As described in Section 2.4.2, it proved impossible to measure histaminase activity satisfactorily with the dianisidine method of Aarsen and Kemp (1964). Attempts were made to stain gels specifically for the enzyme by incubating at 37° with putrescine, dianisidine, and peroxidase in 0.3 M phosphate buffer, pH 6.8. This procedure was even less successful than the test-tube assay, since no bands at all were observed.

The only other assay method which appeared promising for the purpose of giving coloured bands with histaminase in the gel was the method of Holmstedt and Tham (Section 2.4.1). Gel slices were incubated at 37° in a solution having essentially the same composition as that used in the test-tube assay of histaminase.

Putrescine, 20 mM	5 ml.
O-aminobenzaldehyde, 5 mM	50 ml.
Phosphate buffer, 0.3 M, pH 6.8	50 ml.

The presence of histaminase activity in the gel was recognised by the appearance of a yellow-orange colour after 2 - 3 hr. incubation.

(b) In sections of the gel: The bottom slice of the gel, which had an even thickness, was cut into many serial sections across the path of the migrating proteins. A special cutting tool, consisting of many razor blades separated at a distance of 2.5 mm. by brass washers and held rigidly in parallel along two brass rods, was placed gently on the surface of the gel and passed through the slice with even pressure. Two long parallel cuts were then made in the gel at right angles to the serial sections, leaving a series of small gel strips, 2.5 x 3.5 x 30 mm., approximately. Each of these was removed from the surrounding gel with forceps and dropped into a test-tube bearing a number corresponding to the position of the strip from the origin.

These strips were treated as "enzyme solution" and assayed using the standard procedure for Holmstedt and Tham's test, (Section 2.4.1); before the final addition of substrate to the assays, each gel strip was macerated in the reagents with the aid of a flattened glass rod. A strip was cut from the gel at a point which had not been reached by the migrating proteins. This strip was treated in the same way as the sequential strips, and served as a control. After incubation, each assay solution was passed through a sintered glass filter to remove gel particles, and the extinction of the clear solution at 430 mμ was compared with that of the control.

Construction of Fig. 44.3

Aqueous starch gels shrink on immersion in methanolic stains. In order

accurately to compare the sections of the gel examined for total protein, HpMHb and histaminase content, the positions of the HpMHb bands in the aqueous stain, and of the gel strips removed from an aqueous slice for histaminase assay, had to be determined in relation to the positions of bands in the shrunk, methanolic gel, stained for protein. The dianisidine stain, however, was slowly soluble in the methanolic solvent. A gel slice stained with dianisidine/H_2O_2 was washed rapidly with several changes of methanolic solvent; as soon as it had shrunk to the same size as the slice stained for protein, the two slices were photographed side by side. After parallel strips had been removed from another portion of the gel for <u>in vitro</u> histaminase assays, the surrounding gel still retained the marks of the cutting tool. The surrounding gel was shrunk in methanolic solvent, and the positions of the removed strips marked against the shrunk protein-stained slice. The position of bands of water- or methanol-washed slices were thus all compared on the basis of shrunk gels in Fig. 4.4.3.

Chapter 4. Purification of placental histaminase.

4.1 Introduction.

4.2 Final purification scheme, and notes on its development.

4.3 Discussion of purification obtained.

4.4 Attempts to resolve histaminase from haptoglobin-methaemoglobin.

4.1. Introduction.

In a recent review, Hagerman (1964) has observed that, although at least seventy placental enzymes have been documented, very few have been extensively purified. He pointed out two main obstacles to the exploitation of placenta as a rich source of human enzymes -- extraction difficulties due to the tough connective tissue, and the problem of removing massive amounts of blood proteins. Since histaminase is a very soluble enzyme, the first source of difficulty was unimportant in the present work. However, the solubility of histaminase was a hindrance to the complete removal of blood from the placenta before extraction; consequently, the purification of histaminase became essentially a series of procedures to remove groups of contaminating haemoproteins.

The purification of histaminase from other sources will not be discussed here since, even if one assumes similar physical properties for, e.g., placental histaminase and hog kidney DAO, fractionation procedures depend as much on the characteristics of the contaminants as on those of the enzyme being purified.

The only attempt to purify placental histaminase, before the present work was initiated, was that of Kapeller-Adler (1956b), who succeeded in preparing a stable ammonium sulphate precipitate of placental extracts, but could not further purify histaminase in good yield on calcium phosphate gel, Recently, Kapeller-Adler (1965) has published a preliminary report of progress on the purification of the enzyme, using modern chromatographic methods. One of the weaknesses of the older fractionation methods such as precipitation with salts, organic solvents or pH changes, was that a minor component was likely to be co-precipitated along with major contaminants. It was found repeatedly in the present work that only the high resolving power of modern

techniques such as ion-exchange chromatography and gel filtration were capable of overcoming the tendency of histaminase to associate with bulk contaminants like haemoglobin. It is therefore hardly surprising that the purification of placental histaminase has met with little success until recently.

4.2 Final Purification scheme.

4.2.1. Stage 1. Extraction from placental tissue

Since there is some evidence (Kapeller-Adler, 1956b) that placentae from toxaemic subjects may be deficient in histaminase activity, such placentae were not used in the present work. Otherwise, no selection was made.

Each placenta, obtained within about 15 min. of delivery, was prepared by removing the amnion and cutting off the cord near its base. The exposed vascular system of the foetal side of the placenta was perfused with normal saline containing 0.1 mM EDTA, delivered from a raised reservoir. Blanching of the organ was continued until both the maternal and foetal sides were pale pink, or until little more blood could be removed; this process took ½ to 1½ hr. and consumed 3 to 9 l. of saline. The chorion was then cut away, and with it a thin layer of foetal placenta. Residual blood clots and poorly blanched tissue were discarded, and the remaining tissue placed in the freezer at -15°. When ten placentae had been similarly prepared, the combined tissue was left overnight at 4°; the partially-thawed mass was then broken up and passed twice through an electrically-driven butcher's mincer, in which a rotating knife forced the tissue through 3 mm. holes.

The pulp was added to 5 l. cold normal saline containing 0.1 mM EDTA, and thoroughly agitated for ten minutes using a high-speed mixer, the mixing vessel being cooled by partial immersion in ice water. The pulp was strained through several layers of surgical gauze, and re-extracted in the same way with a second 5 l. aliquot of saline-EDTA solution. The filtrates were combined and centrifuged briefly at 4° and 2000 x g to remove particulate

matter. The supernatant was called Stage 1 enzyme.

Notes: None of the previous workers on placental histaminase, including Kapeller-Adler (1956b) and Lindahl (1961), had attempted to remove much of the blood contaminating the placenta, before extracting the enzyme. Alkaline phosphatase, purified from placenta by Ahmed and King (1960) withstood lengthy perfusion with tap water; Morris (1963), prior to the extraction of choline acetylase, perfused the placenta via the cord with normal saline, to reduce haemolysis. The present author had difficulty in cannulating the cord, and preferred to remove the cord completely and perfuse the blood vessels of the foetal side of the placenta. The organ was handled gently during the perfusion to avoid cell damage and release of histaminase into the perfusate. Occasionally, it was necessary to discard part or all of a placenta because of difficulty in dissolving old blood clots.

Deep-freezing of the blanched tissue probably caused cell lysis and contributed to efficient extraction of histaminase. Various attempts to homogenise the thawed placental tissue in a hand mincer, in a Waring blender, or by grinding with sand in a mortar, were ineffective. A powerful electric mincer was required to reduce the tough, elastic tissue to a pouring consistency.

Since cold-room facilities were not available, all subsequent stages of extraction were carried out as rapidly as possible — the first ammonium sulphate precipitation was carried out within 12 hr. of mincing — and the extract was cooled at all times with ice and water, or in the refrigerator. In early preparations, straining through muslin was followed by filtration through Whatman No. 1 papers; however, the papers rapidly became coated with gelatinous material and filtration was unacceptably slow. Brief centrifugation

was more efficient, and the extract could be maintained at a low temperature.

In one preparation, the first and second saline extracts were collected and assayed separately. The first extract contained 75% of the total activity, and had a specific activity of 0.34 S.U./mg., compared with 0.24 S.U./mg. for the second extract. The omission of the second extract was considered in later preparations, but the consequent change in composition of the total extract might have invalidated the existing salt precipitation procedure, and a 25% loss in yield seemed a high price to pay for a relatively small increase in Specific Activity. Omission of the second extract, however, would have reduced the final volume of the extract, with considerable saving in ammonium sulphate and filtration time at Stage 2.

4.2.2. Stage 2. Precipitation at 65% ammonium sulphate saturation.

The Stage 1 extract was brought to 65% ammonium sulphate saturation by the slow addition of 420 g. of the powdered Analar salt per litre of extract; precipitation was effected at room temperature and under efficient stirring. When all the salt had dissolved, stirring was continued for 15 min. before filtering the suspension through Green's filter paper No. 995. The red filtrate was discarded, and the precipitate scraped from the filter papers and re-dissolved in 0.02 M phosphate buffer, pH 6.8, containing 0.1 mM EDTA. The solution was made up to 3 l. and constituted Stage 2 of the preparation.

Notes: The objects of the first precipitation were to concentrate and stabilise the enzyme, and to preserve it from the action of bacteria and proteolytic enzymes. Negligible increases in total activity were obtained by increasing the ammonium sulphate saturation to 75%, and much more haemoglobin was retained at the increased salt concentration. The weights of ammonium sulphate added to give the desired percentage saturations were calculated from the nomogram given by Dixon and Webb (1964, p.40).

4.2.3. Stage 3. Further salt fractionation.

Stage 2 solution was immediately precipitated at 35% ammonium sulphate saturation by the addition of 210 g. salt per litre of solution. The suspension was filtered through fluted papers (Green's No. 904½) and the buff, gelatinous precipitate discarded. The red filtrate was again brought to 65% ammonium sulphate saturation by the addition of 210 g. salt per litre of filtrate. The resulting suspension was filtered through Whatman No. 5 papers, and the filtrate discarded.

The red-brown precipitate was dissolved in the minimum volume of 5 mM borate buffer, pH 8.6, containing 0.1 mM EDTA, and dialysed at 4° against three 5 l. portions of the same buffer for a total of 36 hr. The contents of the dialysis sac were centrifuged briefly at 4° and 2000 x g; the supernatant was called Stage 3 enzyme.

Notes: The heavy precipitate of protein obtained by bringing Stage 2 enzyme to 35% saturation contained negligible histaminase activity, but precipitation of histaminase became appreciable at 40% saturation. The suspension formed by subsequent precipitation at 65% saturation was stable indefinitely in the refrigerator, and provided a concentrated source of crude histaminase for further purification. Where facilities are not available for the preparation of placentae on a large scale, Stage 3 is a convenient point for the pooling of several placental preparations.

The suspension was prepared for chromatography by collecting the precipitate by filtration (which proceeded quite rapidly after removal of the 35% saturation precipitate), dissolving it in the minimum volume of buffer, and dialysis. Particularly thorough dialysis was required in order to remove ammonium sulphate, which inhibits histaminase activity, and to bring

the solution to pH 8.6 with the very dilute starting buffer. Rapid gel filtration through Sephadex might have successfully replaced lengthy dialysis, but a very large gel volume would have been required to de-salt 200 ml. of enzyme solution, and considerable dilution of the sample would have occurred. Since there was no evidence that histaminase could not withstand protracted dialysis, this simpler method of buffer-changing was preferred.

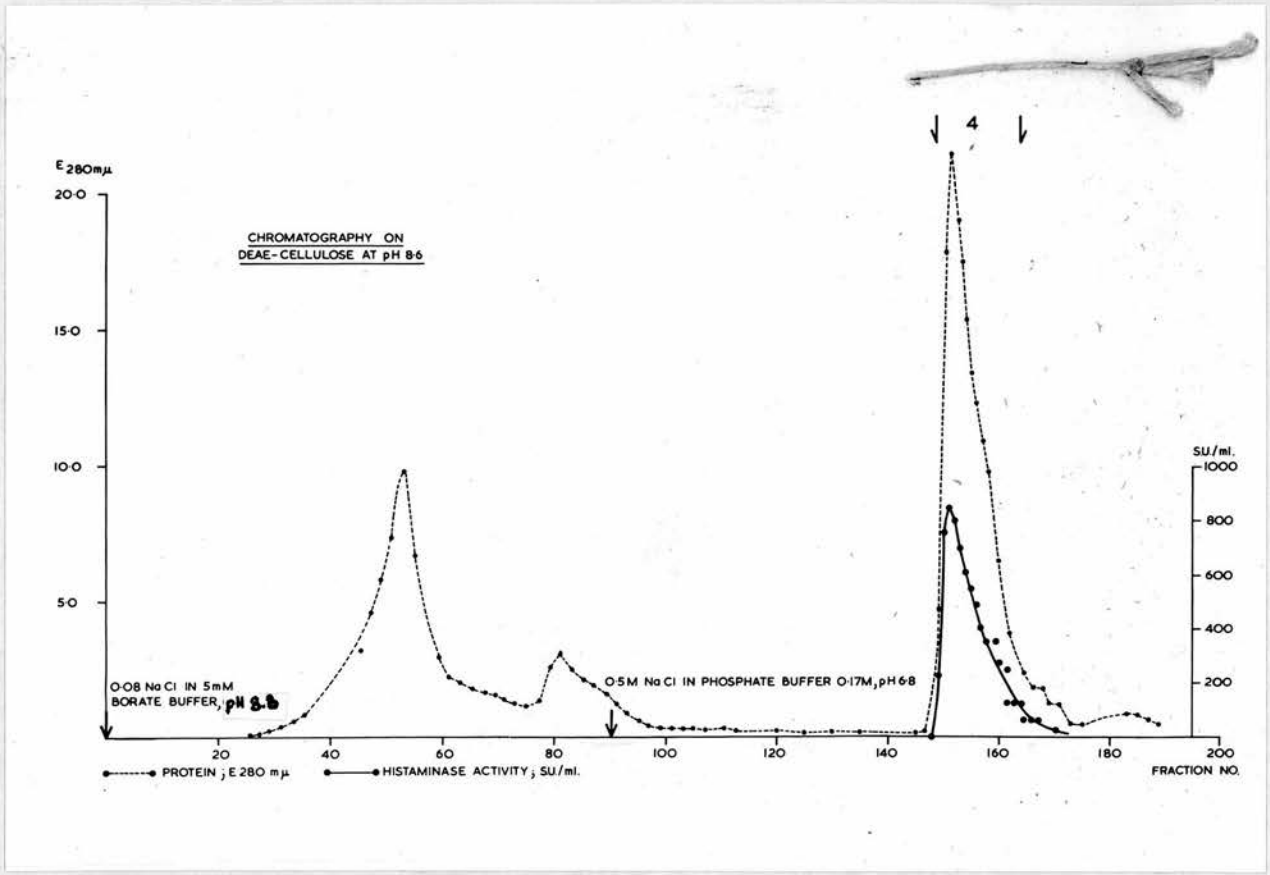


Fig. 4.2.1. Stage 4 : chromatography on DEAE-cellulose.

4.2.4. Stage 4. Chromatography on DEAE-cellulose anion exchanger.

300 g. DEAE-cellulose (0.9 meq./g., medium mesh) was equilibrated against 5 mM borate buffer, pH 8.6, containing 0.1 mM EDTA, and packed under pressure into a 5 x 80 cm. column over a sintered glass support. During the packing of the last two-thirds of the column, the excess pressure applied was increased gradually from 0 to 5 p.s.i.

The entire Stage 3 solution was applied to the top of the column, and washed in with 2 l. of the starting buffer containing 0.08 M sodium chloride, whereupon red and orange haemoprotein bands were eluted from the column. (Fig. 4.2.1).

All the histaminase activity was then eluted sharply, along with methaemoglobin, by washing the column with 0.17 M phosphate buffer pH 6.8 containing 0.1 mM EDTA and 0.5 M sodium chloride. Elution was carried out at about 5 ml./cm.^{2}/hr., and 20 ml. fractions were collected.

The dark brown effluents containing most of the histaminase activity were pooled and dialysed for 24 hr. at 4° against three 5 l. portions of 0.02 M phosphate buffer containing 0.1 mM EDTA. The contents of the dialysis sac were centrifuged briefly at 4° and 2000 x g; the supernatant was called Stage 4 enzyme.

Notes: Stage 3 enzyme contained two major contaminants, haemoglobin and methaemoglobin. The aim of the next stages was the removal of these haemoproteins on a large scale, without duplicating operations at each stage.

In column chromatography, the sample is continuously equilibrated with a fresh portion of adsorbent as it moves down the column, and separation is much more efficient than by batchwise equilibration against the adsorbent. However, much of the resolving power of chromatography is forfeited if the

sample has too great a volume or protein content; not all potentially useful adsorbents can be satisfactorily packed into columns; and column separations commonly occupy several days, in contrast to batch procedures which may be completed in a few hours.

Batch adsorption is well suited to the large-scale, crude fractionation of a mixture of proteins prior to the application of high-resolution chromatography. Unfortunately, convenient batch methods for the removal of haemoproteins from histaminase could not be devised. Just as minor components may be co-precipitated with major contaminants during salt fractionation, it was found that histaminase tended to be eluted in close association with haemoproteins, except when chromatographic methods of high resolution were applied.

Although preparative batch adsorption on alumina gel (Peanasky and Lardy, 1962; Tabor, 1951) and hydroxyl-apatite (Levin, 1962) proved unsuccessful, preliminary batch experiments were routinely used to predict adsorption and elution conditions for the more time-consuming column chromatograms. Small batches of the adsorbent to be surveyed were equilibrated against dilute buffers at several pH values, covering the range in which the enzyme was stable, and the adsorbent had appreciable exchange capacity. Aliquots of Stage 3 enzyme, previously dialysed against the appropriate buffer, were added to the batches of adsorbent in 50 ml. centrifuge tubes, and the supernatants collected, after a period of equilibration, by centrifugation. Each batch was then washed with the appropriate dilute buffer, containing successive increments in sodium chloride concentration. By comparing the Specific Activities of the supernatants with that of the starting sample, it was possible to predict favourable conditions for the adsorption of the enzyme on columns of that adsorbent, and what changes in pH and ionic strength might be used in attempts to elute the

enzyme selectively from other components. The adsorbents surveyed in this way included the cation exchangers hydroxyl-apatite, CM-cellulose, CM-Sephadex and cellulose phosphate. All these exchangers appeared to have a fairly high exchange capacity in the range pH 5.5 — pH 7. However, hydroxyl-apatite was prepared as very fine crystals which gave a poor flow-rate, even in small columns, and could not be considered for large-scale work. CM-cellulose had a gelatinous consistency, and a high resistance to flow. CM-Sephadex maintained adequate flow-rates in columns, and had a remarkably high protein capacity, despite the fact that many high molecular weight components of Stage 3 enzyme could not equilibrate with the majority of the exchange groups within the gel particles. The use of CM-Sephadex was abandoned only because of the problem of gel shrinkage: when the ionic strength of the eluant was increased, the gel shrank to about half its original volume, and cracks developed in the bed. The restricted range of ionic strength within which the Sephadex ion-exchangers could be safely used did not coincide with the range required for the fractionation of Stage 3 enzyme. Only cellulose phosphate had good enough mechanical strength to permit packing into large columns with an adequate flow-rate. The final procedure adopted for Stage 5 was developed from small-scale experiments using Stage 3 enzyme as sample. The reasons for rejecting cellulose phosphate chromatography for the preparative fractionation of Stage 3 will be discussed below.

The evolution of Stage 4 will be described briefly to illustrate progress from batch procedures, through small-scale column separations, to preparative chromatography of about 10 g. of protein.

Batch adsorption experiments using small aliquots of Stage 3 enzyme showed that all the enzymic activity could be adsorbed on DEAE-cellulose

equilibrated against 5 mM borate buffer at pH 8.6, and could be eluted at the same pH with 1 M sodium chloride; histaminase was not adsorbed at pH 5.5. The next step was to adsorb portions of Stage 3 enzyme on small (1 x 30 cm.) columns of DEAE-cellulose in 5 mM borate buffer, pH 8.6, and seek to elute histaminase selectively by increasing the ionic strength of the eluant, or decreasing the pH, or both.

(a) The sample was washed in with starting buffer, and the column eluted with 0.1 M acetate buffer, pH 5.5. Haemoglobin emerged before methaemoglobin, but almost all histaminase activity was eluted in the same fractions as these haemoproteins. Co-elution of histaminase in association with the bulk contaminants proved to be a common feature of elution patterns produced by discrete steps in pH or ionic strength, rather than by a continuous gradient. One extreme example of this was encountered during the elution of a hydroxyl-apatite column at pH 6.8, with small stepwise increments in ionic strength; a band of protein emerged at each increment, each band carrying histaminase at approximately the same Specific Activity as the starting sample. An unwary examination of the pattern of histaminase activity alone might have led to the conclusion that several isoenzymes of histaminase had been separated.

(b) After adsorption at pH 8.6, the sample was eluted at the same pH with a relatively steep gradient of sodium chloride concentration -- 0 to 2 M over 2 l. Under this steep gradient, the haemoproteins were rather better separated than in (a) above, and the peaks of histaminase activity were slightly displaced from the protein peaks.

(c) The gradient was made shallower -- 0 to 1 M over 2 l. This resulted in the elution of a well-defined peak of histaminase between the two haemoprotein peaks, and a 10-fold increase in Specific Activity was achieved in selected fractions.

(d) The device of a concave salt gradient, designed to decrease tailing of the enzyme peak into the succeeding methaemoglobin oeak, proved less successful than procedure (c).

In these exploratory experiments, the sample contained about 200 mg. protein. The final aim was fractionation of about 10 g. protein on a single column. Many experiments with shallow gradients on 2 x 40 cm. and 5 x 80 cm. columns failed to reproduce the resolution achieved using smaller columns. Histaminase tended to tail into the methaemoglobin peak, and the very shallow gradients devised to improve resolution greatly increased the dilution of the active effluents. Investigations with different column dimensions, different batches of adsorbent, and changes in pH and gradient did not reveal the reasons for failure to scale up the small chromatograms.

Finally, Stages 4 and 5 were designed as a compromise, to achieve in two steps what elution procedure (c) had achieved in one -- but on a much larger scale, (x 50), and without excessive dilution of the sample.

Stage 4 removed almost all the red haemoproteins with a very small increment in ionic strength. Histaminase and almost all other contaminants, including methaemoglobin, were eluted in a very small volume with a large change in pH and ionic strength. The pH of this eluant was chosen to facilitate equilibration of the selected effluent by dialysis against the dilute phosphate buffer to be used in Stage 5.

Figs. 4.2.1 - 3 illustrate enzymic activity towards cadaverine only. In all experiments, the pattern of activity towards histamine and putrescine coincidedwith that for cadaverine.

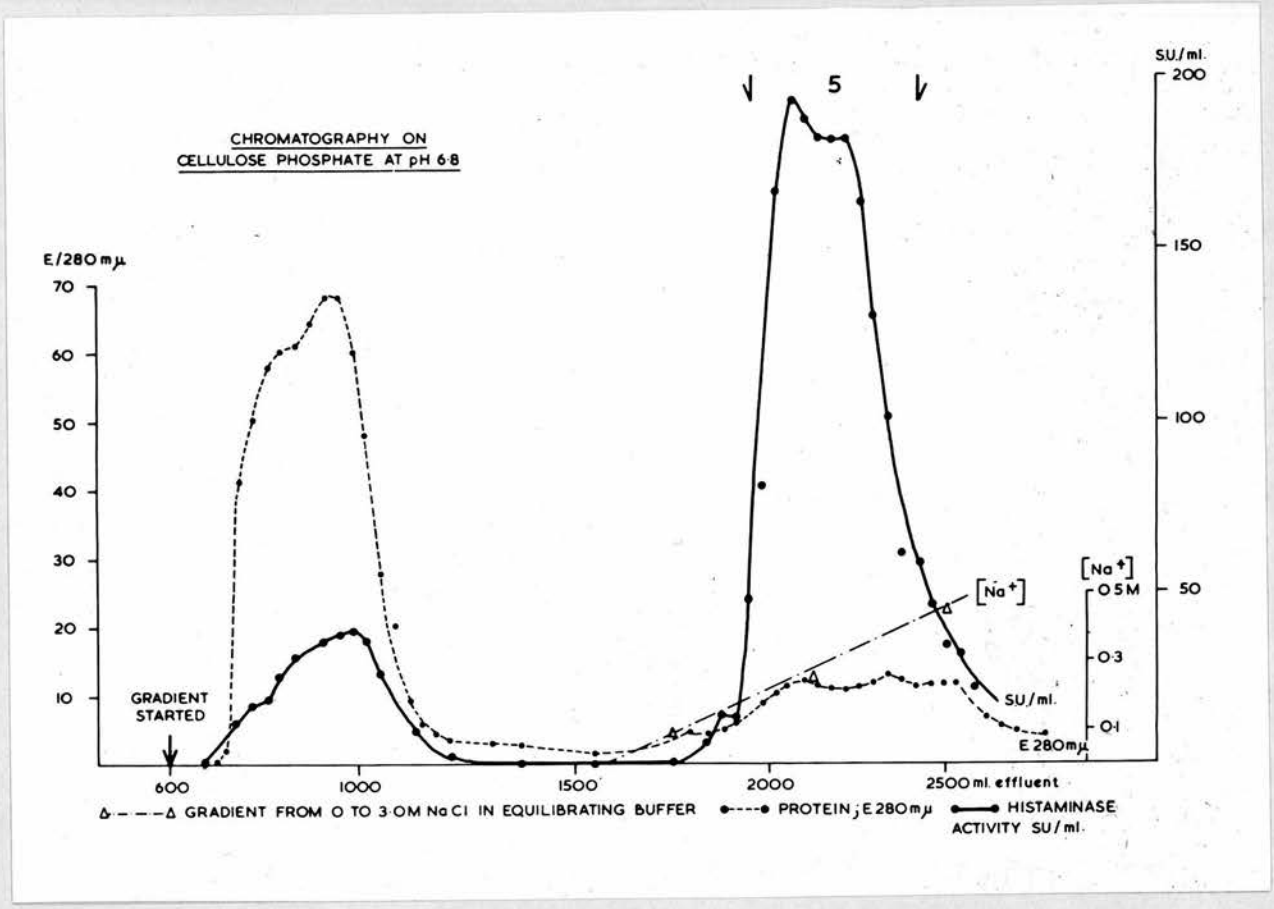


Fig. 4.2.2. Stage 5 : chromatography on cellulose phosphate.

4.2.5. Stage 5. Chromatography on cellulose-phosphate cation exchanger.

200 g. cellulose-phosphate (~~0.9~~ 7.4 meq./g., medium mesh) was equilibrated against 0.02 M phosphate buffer, pH 8.6, containing 0.1 M EDTA, and packed into a 5 x 80 cm. column over a sintered glass support. During the packing of the second half of the column, the excess pressure was increased gradually from 0 to 2 p.s.i.

The entire Stage 4 solution was added to the top of the column, and washed in with a further 300 ml. of starting buffer. Elution was then carried out with a linear gradient of sodium chloride in starting buffer, from 0 to 3 M, over 4 l.

Methaemoglobin spread down the column in a broad band, carrying with it a small amount of histaminase activity (Fig. 4.2.2). The increase in salt concentration did not become apparent in the effluents until about 1 l. had passed through the column. Under the salt gradient, a succession of orange and brown haemoprotein bands were eluted. Histaminase activity was eluted at about 0.3 M sodium chloride concentration. Fractions containing most enzymic activity were pooled to form Stage 5 enzyme.

Notes: Conditions for cation exchange chromatography on cellulose phosphate were developed from experiments with Stage 3 enzyme. These experiments showed that cellulose phosphate was not entirely suitable for the resolution of such a crude preparation, since its capacity for these proteins was lower than that of DEAE-cellulose. Methaemoglobin was not retained on cellulose phosphate at pH 6.8, and considerable amounts of histaminase emerged in non-specific association with this contaminant. When much of the protein, chiefly haemoglobin, had been removed by Stage 4, however, loss of histaminase in the methaemoglobin peak was greatly diminished as a result of the reduction in

protein load. In principle, even this loss could have been further decreased by spreading the load of Stage 4 enzyme over two or more identical columns of cellulose phosphate. In practice, however, laborious duplication resulted in negligible improvements in total yield or Specific Activity, and in an undesirable increase in the volume of effluent containing histaminase activity.

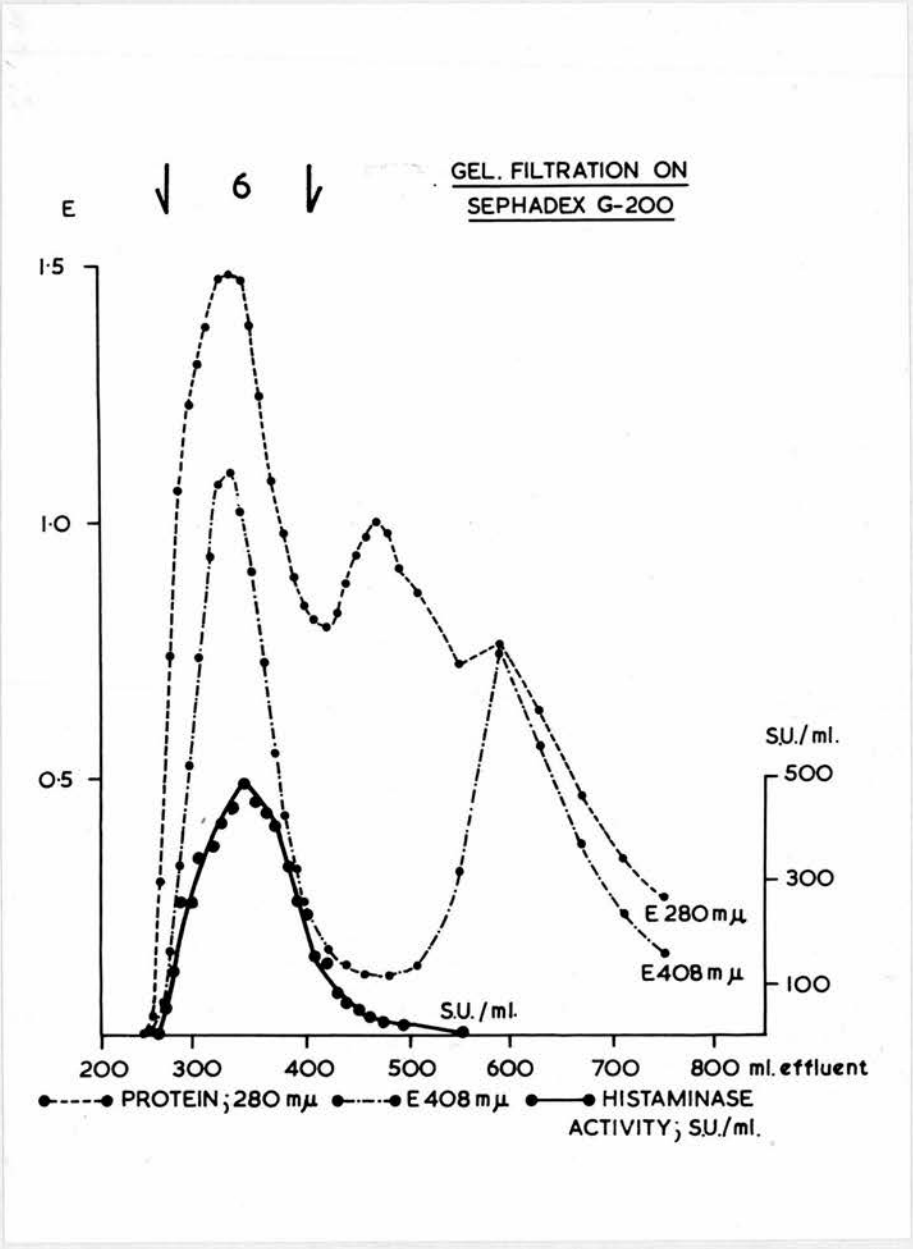


Fig. 4.2.3. Stage 6 : gel filtration on Sephadex G-200.

4.2.6. Stage 6. Concentration, and gel filtration on Sephadex G-200.

Stage 5 enzyme from cellulose phosphate chromatography was concentrated to 20 ml. by osmosis against Carbowax (polyethylene glycol, M.W. 15,000) at 4°.

A column 3 x 100 cm. of Sephadex G-200 was prepared from a batch of gel which had been swollen in buffer for several months, and decanted many times to remove fine particles. The LKB re-cycling column was used in all Sephadex separations, and the eluting buffer was 5 mM borate buffer, pH 8.6, containing 0.1 M sodium chloride.

The entire concentrated Stage 5 solution (20 ml.) was pumped upwards through the gel bed, followed by eluting buffer, at a rate of 2 ml./cm.^{2}/hr. The effluent consisted of three protein peaks, the first of which, corresponding to species having a M.W. of about 200,000 or more, contained all the histaminase activity. (Fig. 4.2.3). These high M.W. fractions were pooled, and constituted Stage 6 enzyme.

Notes: Methods of concentrating the enzyme solution, and the technique of upward-flow gel filtration, were discussed in Chapter 3.

20 ml. was the maximum volume of sample that could be separated into three distinct peaks on the 3 x 100 column of G-200. Better resolution was generally achieved by using a smaller sample, but it was inconvenient to run several identical separations, and the alternative of concentrating the whole sample to about 10 ml. resulted in an undesirable increase in viscosity. The pumping rate was kept low to permit efficient equilibration of the sample with the gel, and to minimise compression of the gel.

Histaminase emerged in the first peak, and haemoglobin and methaemoglobin in the third peak. Even using a large sample volume, these peaks were

completely separated. It therefore appears that, in principle, haemoproteins could have been removed from the preparation without the intervening ion-exchange stages. However, Stage 3 enzyme contained so much haemoprotein that it could not be reduced much below 200 ml. without the risk of precipitating protein, or unduly increasing the viscosity. The adopted procedure of preparing a sample of low volume and viscosity, suitable for a single passage through Sephadex, seemed simpler than the alternative of running ten large-scale Sephadex separations of Stage 3 enzyme, and offered additional prospects of removing other components besides the haemoproteins. Partly purified histaminase may be prepared quite simply on a small scale from Stage 3, or even Stage 1 enzyme, by immediate gel filtration on G-200.

Fraction 6 indicated in Fig. 4.2.3 was concentrated and subjected to starch gel electrophoresis at pH 8.6. The gel, stained for protein, showed that the enzyme was contaminated with haptoglobin-methaemoglobin and traces of globulins. The Stage 6 enzyme probably also contained some β-lipoprotein.

No further purification in terms of Specific Activity was achieved by subsequent attempts to fractionate Stage 6 enzyme; histaminase was partly inactivated by any process resolving it from the remaining contaminants.

Abbreviations:

H histamine

C cadaverine

P putrescine

P/C, etc.: ratio of histaminase activities towards P and towards C

Units for Method III: 1 Unit results in extinction change, $\Delta E_{430m\mu}$ = 1.0 in 1 hr., under standard conditions (Section 2.4.1).

Table 4.3.1

Stage	Volume ml.	Protein mg./ml.	Total Protein mg.	Kapeller-Adler's test: Method I — Substrate - C				Spectrophotometric indigo test: Method II — Substrate - C						Holmstedt & Tham's test: Method III — Substrate - P			
				PU/mg.	Total PU	P/C	H/C	SU/mg.	Total SU	Yield %	Purification	P/C	H/C	Units/mg.	Total units x 1000	Yield %	Purification
1. Saline extract	9,620	11.95	115,000	4.5	520,000	0.46	0.41	0.40	46,000	100	-	0.50	0.99	-	-	-	-
2. First salt pptn.	3,000	17.8	53,300	-	-	-	-	0.67	36,000	78	1.7	0.34	0.57	34.8	1860	100	-
3. Second salt pptn.	223	47.6	11,600	35.7	414,000	0.78	0.59	20.4	216,000	605	30.4	0.33	0.16	86.1	1000	54	2.5
4. Chromatography on DEAE-cellulose	344	11.05	3,800	-	-	-	-	32.3	123,000	57	1.6	0.49	0.23	154	585	59	1.8
5. Chromatography on cellulose phosphate	440	1.06	466	-	-	-	-	99.0	46,000	37	3.1	0.39	0.21	528	246	42	3.4
6. Gel filtration on Sephadex G-200	130	1.16	151	386.0	58,200	0.78	0.52	314.0	47,000	103	3.2	0.46	0.21	1530	231	94	2.9

4.3. Discussion of purification obtained.

An analysis of histaminase activity towards different substrates at several stages of purification, determined by three assay methods, is presented in Table 4.3.1.

Assay Method I was used only at key stages of the purification. Assay Method II was used at all stages. Assay Method III was used at all stages except the crude placental extract, where the extinction of the enzyme solution was too great to permit accurate determination of small extinction changes due to specific enzymic activity towards putrescine.

Before assay, enzyme solutions from Stages 1, 2 and 3 were dialysed against 5 mM borate buffer, pH 8.6, containing 0.1 mM EDTA, to remove inhibitors, including ammonium sulphate. Stage 4, 5 and 6 solutions were chromatographic effluents and did not require dialysis. All enzyme solutions were preserved at - 15° before being thawed and assayed together, using the three assay methods in parallel.

4.3.1. Yields.

The absence of reliable activities by Method III for Stage 1 enzyme makes it necessary in Table 4.3.1 to express yields at each stage as a fraction of the activity at the previous stage, rather than as a fraction of the activity of the original extract. Overall yields from Stages 2 and 3 to Stage 6 are included in Table 4.3.2.

Method III is less susceptible to interference from impurities in the enzyme solution than are Methods I and II. The latter are inhibited by haemoglobin, and by enzymes such as catalase, which compete with indigo for the hydrogen peroxide formed in the enzyme reaction. Comparison of data for Stages 2 and 3 obtained by Method II indicates that ammonium sulphate fractionation removed an inhibitor of the indigo test, present in Stages 1 and 2. Yields far exceeding 100% were always obtained after salt fractionation using Method II. Since Stage 1 activity is artificially low, owing to inhibitors, the relevant part of Table 4.3.1 expresses the yield at each stage as a percentage of the activity recovered from the previous stage. Overall yields from Stages 1 and 3 to Stage 6 are included separately in Table 4.3.2.

After Stage 3, when inhibitors had been removed, Method II and Method III indicated closely comparable yields; this observation tends to support the quantitative validity of the new spectrophotometric indigo test (Method II).

Yields at chromatographic Stages 4, 5 and 6 refer to the fraction retained for further purification, and not to total recoveries from the column. Thus, the total recovery over all chromatographic fractions was typically 80 - 120%, of which only about 50% was well resolved from major contaminants. Although increase in Specific Activity was the chief criterion for selecting fractions for further purification, the total activity of the

fraction, and the particular contaminants it contained, were also taken into account. For instance, a very dilute fraction having a Specific Activity ten times greater than that of the starting sample, but containing only 1% of the total activity, might be less useful for further purification than a fraction of lower Specific Activity containing 10% of the total. Similarly, a fraction from ion-exchange chromatography containing a contaminant, X, may have a rather higher Specific Activity than one containing a contaminant, Y; but if it is known that Y may be more easily removed by a subsequent procedure, on account, say, of its molecular size, the fraction of lower Specific Activity may be more valuable for further purification.

4.3.2. Purification factors.

Many of the points made in the preceding discussion of yields are also applicable to the comparison of Specific Activities. The presence of indigo test inhibitors in Stages 1 and 2 reduces the importance of data for the overall purification from Stage 1 to Stage 6; these are included separately in Table 4.3.2.

The numerical purification factors obtained at chromatographic stages were rather low, even for large-scale separations. This may be attributed to failure to resolve histaminase from the contaminant haptoglobin-methaemoglobin which had very similar physical properties and may have been present in much greater amounts than histaminase in the enzyme solutions. However, the attainment of some preconceived purification factor was not one of the aims of this project. The identification of placental histaminase rather demanded that the last contaminants should be identified, so that the possibility of their interfering with the characterisation of histaminase could be assessed.

4.3.3. Activities towards different substrates

In this discussion, the term "substrate ratio" means "the ratio of the rates of oxidation of these two substrates by histaminase".

Method I. P/C and H/C ratios for Stages 1 and 2 varied greatly in different preparations, possibly because of variations in the concentration of inhibitors. From Stage 3 to Stage 6, however, the ratios remained fairly constant at 0.7 - 0.8 for P/C, and 0.5 - 0.6 for H/C, in each preparation. The ratios given in Table 4.3.1 for Stages 3 and 6 support the theory that histamine and the diamines are oxidised by the same enzyme.

Method II. Substrate ratios for Method II were not comparable with those obtained by Method I. In the absence of an independent assay for histaminase activity towards all substrates, it was not possible to decide which of these methods gave the more reliable ratios. The H/C ratio found by Lindahl (1961), who assayed the activity of crude placental extracts manometrically, agrees more closely with the ratio found by Method I. However, the variation in Stage 1 ratios for a single assay method suggests that comparison of crude extract ratios, obtained by manometric and indigometric methods, may not be a sound basis for discriminating between Methods I and II.

The P/C ratio is rather variable between successive Stages, but there is no steady progression towards 0 or ∞, as would be expected if two enzymes, each responsible for the oxidation of only one of these diamines, were being separated by the purification procedure. It may be interesting to discuss at this point how much reliance should be placed on such substrate ratios in deciding whether one or two enzymes are responsible for the oxidation of two substrates.

Constant substrate ratios throughout purification do not finally

establish that only a single enzyme is active; there remains the possibility, diminishing with the variety of procedures adopted, that even methods of high resolution may be incapable of separating two distinct enzymes, each specific for one substrate. Conversely, an increase in substrate ratio cannot be interpreted as the separation of two enzymes, unless certain conditions are met:

(1) The variation in substrate ratio should be cumulative over several stages of the purification, and should not be attributable to known errors in individual activity measurements.

(2) The possibility of inhibitors acting preferentially on one of the substrates should have been eliminated; this is best done by ensuring that an increase in the substrate ratio of the purified fraction is accompanied by a decrease in the substrate ratio in the discarded fractions.

Errors of x% in the determination of individual activities may result in a maximum error of 2x% in their ratio. Indigometric measurements on the same enzyme solution, using the same substrate, are reproducible within about 5%; the variation in P/C, using Method II, corresponds to errors of this order in the individual activity measurements.

Similar variations in benzylamine/histamine ratios were observed by Buffoni and Blaschko (1964) during the purification of serum benzylamine oxidase. Mondovi et al. (1964), using a manometric assay, found variations of over 10% in the C/H ratio during the purification of hog kidney DAO. These authors considered that these variations were due to acceptable errors in the individual assays, and did not indicate the separation of two distinct enzymes, especially since the variations did not follow a regular progression throughout purification. On the other hand, Kapeller-Adler (1956b) has used

15% differences in C/H ratios for crude extracts of individual placentae as evidence that two enzymes are responsible for the oxidation of these substrates.

Dixon and Webb (1964, p.254) have reviewed a number of enzymes which act on substrates differing only in the length of an aliphatic chain; the variation of relative rates of activity with chain length of homologous substrates shows remarkably few discontinuities, especially near optimal chain length. The amine oxidases, such as hog kidney DAO (Zeller, 1938b) and liver MAO (Blaschko, Richter and Schlossmann, 1937; Blaschko and Duthie, 1945) have been shown to be most active towards amines with four or five methylene groups, and no evidence has been presented that different enzymes are responsible for the oxidation of each homologue. Since it would be extremely unusual that two enzymes should be required in the same organ for the oxidation of such close homologues as cadaverine and putrescine, variations in P/C ratios may with some confidence be used as a "control", to indicate the magnitude of the ratio differences to be expected when the same assay method is used to compare activities towards cadaverine and some other presumed substrate, less closely related than is putrescine.

Thus, the H/C ratio, using Method II, is seen to be almost constant after Stage 2 of the purification scheme, the variations from the mean being less than the corresponding variations of the P/C ratio. Therefore if the small variations in H/C are held to imply the presence of separate enzymes for the oxidation of these substrates, a third enzyme, specific for putrescine, must also be postulated.

The author concludes that the differences in P/C ratios in Table 4.3.1 may reasonably be attributed to errors in the individual activity measurements.

Interpreting the P/C ratios as remaining approximately constant throughout purification, it is considered very probable that these diamines are both oxidised by the same enzyme. By an extension of this argument, it is concluded that the approximate constancy of H/C ratios after Stage 2 supports the contention that histamine and the diamines are oxidised by the same enzyme.

Whereas the P/C ratios do not show any progressive changes at any stage, the H/C ratios fall markedly from Stage 1 to Stage 3. Lindahl (1961) reported that imidazole N-methyl transferase, as well as histaminase, is present in placental extracts, and that methylhistamine may be more rapidly oxidised that histamine by placental histaminase. If Stage 1 and 2 preparations also contain the methylating enzyme, the following reactions may both occur when histamine is added to crude placental extracts:

(1) histamine $\xrightarrow{\text{Histaminase}}$ imidazoleacetaldehyde + H_2O_2.

(2) histamine $\xrightarrow[\text{transferase}]{\text{Imidazole N-Me}}$ methylhistamine $\xrightarrow{\text{Histaminase}}$ methylimidazole-acetaldehyde + H_2O_2.

It is conceivable that reactions (2) might result in more rapid H_2O_2 production than reaction (1), and consequently indigometric activities with histamine as substrate would be artificially high. Since the diamines are not attacked by the methylating enzyme, the H/C ratio would be high in early stages of the preparation, and decrease as the methylating enzyme was progressively removed.

This explanation of the high H/C ratios found by Method II does not account for the low H/C ratio of about 0.4 - 0.5 found in early stages of the preparation, using Kapeller-Adler's indigo test (Method I); nor is it in agreement with the results of mixed-substrate experiments quoted by Lindahl (1961), in which the oxidation of histaminase by crude placental extracts was inhibited by methylhistamine.

4.3.4. The use of EDTA.

It was pointed out in the preceding discussion of substrate ratios that the activity of an enzyme towards one of its substrates might be inhibited by impurities in, e.g., the enzyme solution. The inclusion of 0.1 mM EDTA in the assay buffers (Chapter 2) and most of the working buffers used throughout the purification of the enzyme, was introduced in an attempt to eliminate such selective inhibition.

During the preliminary salt fractionation stages of placental preparations carried out early in this project, it was noted that the H/C ratio tended to increase, until virtually no activity towards cadaverine could be discerned at Stage 3. This observation might have led to the conclusion that a true "histamine oxidase" was being separated from a true "diamine oxidase"; but the missing activity towards cadaverine did not appear in any of the rejected fractions. 1 mM EDTA was then included (Kapeller-Adler, 1964) in the assay of Stage 3 enzyme, whereupon the H/C ratio returned to a value similar to that of Stage 1.

Initially EDTA was included in buffers only when it was suspected that activity towards diamines might have been inhibited, but this practice gave rise to confusion. The EDTA-reversible inhibition was not present in all placental preparations, and assay in 1 mM EDTA did not always restore the original H/C ratio. The effect of EDTA on histaminase activity towards histamine and putrescine was rather variable. A consistent policy for the addition of EDTA had to be devised; three alternatives were considered:-

(1) To omit EDTA altogether.

(2) To omit EDTA from the working buffers, so that the bulk of the histaminase preparation retained its "native" distribution of metal ions, but

always to add EDTA to the assay buffer.

(3) To add a rather low concentration (0.1 mM) EDTA to all buffers.

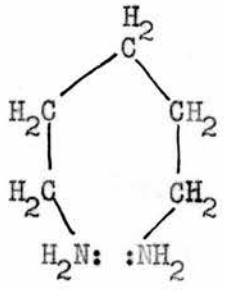
The first alternative was considered too likely to lead to erroneous H/C ratios, especially since contact with metal surfaces, and reagents containing significant amounts of metal ions, could not have been completely avoided. Similarly, the second alternative would have been successful only if the possibility of adding metal ions with the reagents could have been eliminated. An attempt was made to avoid one possible source of metal ions, while omitting EDTA from the early stages, by using only "metal-free" ammonium sulphate. This proved very expensive, and gave no better results than the routine addition of EDTA to the working solutions -- in fact, it was later realised that there may have been a residue of EDTA in the "metal-free" salt. Since contact with metal objects, such as the enamelled iron mincer, could not be avoided, alternative (2) did not provide against the accumulation of fairly high metal ion concentrations in the bulk of the enzyme preparations.

Finally, 0.1 mM EDTA was added routinely to all working and assay buffers. This procedure risked the progressive removal of a metal ion vital to enzymic activity towards one or more of the substrates, but it was considered that spurious substrate ratios were less likely to arise in this way than from the omission of EDTA. In any case, any suspected undesirable effects of EDTA inclusion could be corrected by gel filtration or dialysis of the preparation to remove EDTA, and the controlled addition of selected metal ions to the EDTA-free enzyme. In practice, the consistent addition of a low concentration of EDTA to all buffers did not appear to remove vital metal factors from the enzyme, judging by the high yields of activity obtained at each stage. On the other hand, if EDTA were omitted at any stage, placental preparations

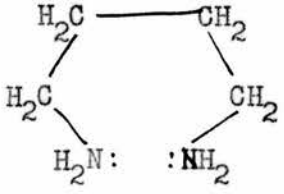
immediately tended to lose activity towards the diamines. For instance, an EDTA-free enzyme solution was concentrated overnight by osmosis against Carbowax, and assayed in buffers containing increasing concentrations of EDTA. It was found that the enzyme had been almost completely inhibited by components diffusing from the Carbowax, and that activity was increasingly restored in the presence of EDTA in concentrations from 10^{-7} to 10^{-5} M; no further increase in activity was gained by higher concentrations of chelating agent. It appeared probable that EDTA acted by chelating trace metals, which might be expected to be present in many reagents at about 10^{-6} M concentration. The worst sources of EDTA-reversible inhibitors were hydroxyl-apatite, cellulosic ion-exchangers, Carbowax, and possibly some batches of Analar ammonium sulphate.

Since the inhibition of histaminase by metal ions, and the reversal of inhibition by EDTA, appears to vary with the substrate used, it seems reasonable to suggest that different metal ions may participate in the enzyme-substrate complex, either as activators or inhibitors.

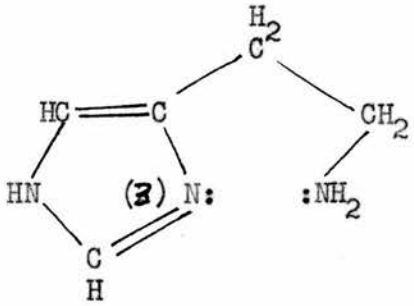
It is conceivable that a different metal ion is required to bring each substrate into the correct spatial and electrical configuration for binding to, or release from, the active centre of histaminase. The projected molecular structures of the commonly-used substrates of histaminase are shown below.



cadaverine



putrescine



histamine

Lone pair electrons of nitrogen atoms, capable of forming co-ordinate (dative) bonds with metal ions, are shown as ":" on the relevant atoms. All bonds joining carbon and nitrogen atoms in the aliphatic diamines are capable of free rotation about the axis of the bond; these diamines may be induced by co-ordinate bonding with a metal ion to form irregular rings consisting of a metal ion, two nitrogen atoms and 4 or 5 carbon atoms. Similarly, nitrogen atom (3) of the imidazole ring of histamine may function in the same way as a free amino-group in the diamines, to co-ordinate with a metal ion and thereby assist the formation of a six-membered ring.

In this way, metal ions of different radius and charge may be required to induce different amines to assume an approximately similar overall configuration, or local configuration — e.g., approximately constant distance between nitrogen atoms — to satisfy the requirements of the enzyme's active centre. Certain ions may not have the appropriate size or charge to fulfil this function, and may act as competitive inhibitors with ions of the correct dimensions. Whatever metal ions may assist histaminase activity towards different substrates, they must be appreciably dissociated from EDTA at pH 6.8, under standard conditions for the assay of histaminase.

Alternatively, it is possible that the formation of co-ordinate bonds with most metal ions is unfavourable to the subsequent reactions of the substrate at the active centre; but that metal ions are more likely to distort the geometry of diamines than of histamine, which has a more rigid molecular structure. No activation by any added metal ion was ever observed, whereas it was suspected that Ca^{++}, Cu^{++} and Fe^{+++}, added incidentally to unrelated experiments, were inhibitory to histaminase activity.

No protracted attempt was made to find additional support for the

explanations outlined above. The study of histaminase inhibition and activation by metal ions would have been a heavy undertaking, involving the analysis of trace metals in the assay components, the application of selective chelating agents, and possibly the preparation of metal-free enzyme. Such a thorough study lay outwith the scope of the present project.

Table 4.3.3.

Ratios of activities obtained by different assay methods.

Stage No.	II_C/I_C	II_C/III_P	II_P/III_P
1	0.09	-	-
2	-	2.1	0.71
3	0.57	2.6	0.86
4	-	2.3	1.10
5	-	2.0	0.78
6	0.81	2.2	1.00

4.3.5. Comparison of Specific Activities using different assay methods.

Methods I and II. In the column headed II/I in Table 4.3.3. are presented the ratios, at several purification stages, of Specific Activities towards cadaverine, as measured by two indigometric methods, Kapeller-Adler's titrimetric test (Method I) and the new spectrophotometric test (Method II). The ratio increases almost ten-fold between Stage 1 and Stage 6. These results may indicate that an inhibitor present in the early stages affects Method II more than Method I; however, since Method II employs much less enzyme solution, and therefore introduces a lower concentration of contaminants into the assay, it might have been predicted that the ratio would decrease after the early removal of inhibitors.

Methods II and III. In the next two columns, the Specific Activities measured by Method II, using either cadaverine or putrescine, are compared with corresponding data measured by Method III, using putrescine as substrate. The ratios are approximately constant, within about the same limits as the P/C ratios for Method II, discussed earlier. The constancy of these ratios is a valuable indication that the "indirect" indigometric assay (Method II) is related to the primary enzymic reaction as closely as the "direct" product-trapping method of Holmstedt and Tham (Method III). Conversely, it is clear that Method I correlates very poorly with the independent Method III.

4.4. Attempts to resolve histaminase from haptoglobin-methaemoglobin in Stage 6 enzyme.

4.4.1. Ion-exchange chromatography.

The early ion-exchange stages of the purification scheme were designed primarily to remove haemoglobin (Hb) and methaemoglobin (MHb), rather than their complexes with haptoglobin (Hp). It was noted during preliminary experiments that the behaviour of histaminase on chromatography was affected by non-specific adsorption to bulk contaminants. It was therefore considered possible that an ion-exchange procedure which had failed to resolve histaminase from haptoglobin-methaemoglobin after Stage 3, for instance, might separate them after Stage 6, when interfering contaminants had been removed. Some of the fractionation attempts recorded in this section are repetitions or variants of experiments reported in Section 4.2.4. In general, however, it was thought unwise to attempt immediately successive separations under exactly the same conditions; any success achieved by such serial separations might be gained more efficiently by increasing column length or decreasing the sample load. Usually, a procedure which had not been effective at Stage 3 was not attempted again until several further separations had radically altered the distribution of contaminants.

While certain other minor contaminants remained with histaminase after Stage 6, the following experiments were aimed at the removal of HpMHb. This haemoprotein obscured the spectral region in which pyridoxal phosphate, one of the suspected cofactors of histaminase, might be detected. The complete removal of HpMHb from histaminase was also sought in order to show conclusively that the activity of the enzyme in the indigo test was independent of the presence of this peroxidase-like contaminant.

(a) Chromatography on cellulose phosphate.

(i) pH 8.6. A 2 x 40 cm. column was packed with the cation exchanger, equilibrated against 5 mM borate buffer, pH 8.6, a small aliquot of Stage 6 enzyme applied, and the column eluted under a shallow linear gradient of sodium chloride concentration. The proteins were strongly negatively charged, and emerged rapidly from the column without appreciable fractionation.

(ii) pH 6.8. During some Stage 5 separations, the extinction of the effluents was measured at 408 mμ, for haemoproteins, as well as at 280 mμ, for total proteins. A slight separation of the 408 mμ pattern from histaminase activity was noted, but it was impossible to tell at this stage, without concentration and electrophoresis of fractions, whether the extinction at 408 mμ was due to MHb, HpMHb, or both. After removal of MHb by gel filtration (Stage 6), however, the brown-coloured effluents contained HpMHb and no other haemoproteins. Many attempts to separate histaminase from HpMHb by cellulose phosphate chromatography of Stage 6 enzyme at pH 6.8 proved ineffective, even when a long column and very shallow salt gradients were used.

(iii) pH 5.5. Chromatography of Stage 6 enzyme at pH 5.5 was complicated by the tendency of both histaminase and HpMHb to become insoluble at low pH. Histaminase survived overnight dialysis in the refrigerator against phosphate or acetate buffer, 10 mM, at pH 5.5, but its stability at room temperature was poor.

Chromatography of Stage 6 enzyme at pH 5.5 was attempted on a 2 x 40cm. column of cellulose phosphate, under a shallow gradient of sodium chloride concentration, from 0 to 1 M over 2 l. No protein was detected in the effluents. The pH and molarity of the eluant were increased in steps, but even 0.34 M

phosphate buffer, pH 6.8, containing 4 M sodium chloride, failed to elute any protein. The highly cationic proteins had either become denatured or irreversibly adsorbed by the strongly attractive electrostatic forces exerted by the adsorbent; the increases in salt concentration of the eluant may have caused precipitation of the proteins on the column.

CM-Sephadex was tried under similar conditions, in case it possessed characteristics less likely to denature the sample, e.g. lower exchange capacity, electrostatic charge, or non-specific adsorption capacity. Again, a variety of pH and molarity changes failed to elute the tightly adsorbed sample.

(b) Chromatography on DEAE-cellulose.

(i) pH 8.6. Many attempts were made (Section 4.2.4) to fractionate Stage 3 enzyme at pH 8.6, using shallow salt gradients to achieve the highest degree of resolution. At that stage it was impossible to tell by simple spectrophotometric examination of the effluents whether histaminase had been separated from HpMHb, as well as from free haemoproteins. Fine resolution was abandoned in the interests of simplicity and high yield, when the compromise Stage 4 was routinely adopted. An attempt was therefore made to fractionate Stage 6 enzyme under optimal conditions at pH 8.6. The sample was applied to a 2 x 40 cm. column of DEAE-cellulose in 5 mM borate buffer, pH 8.6, and eluted under a very shallow salt gradient, 0 to 0.4 M over 2 l. The peaks of histaminase and HpMHb in the effluents coincided almost exactly. The resulting Specific Activity was rather lower than that of the sample, due possibly to long exposure of the highly purified enzyme at the large adsorbent surface, and at room temperature; this was a common feature of chromatography of Stage 6 enzyme, even at pH values normally favourable to the stability of solutions of histaminase.

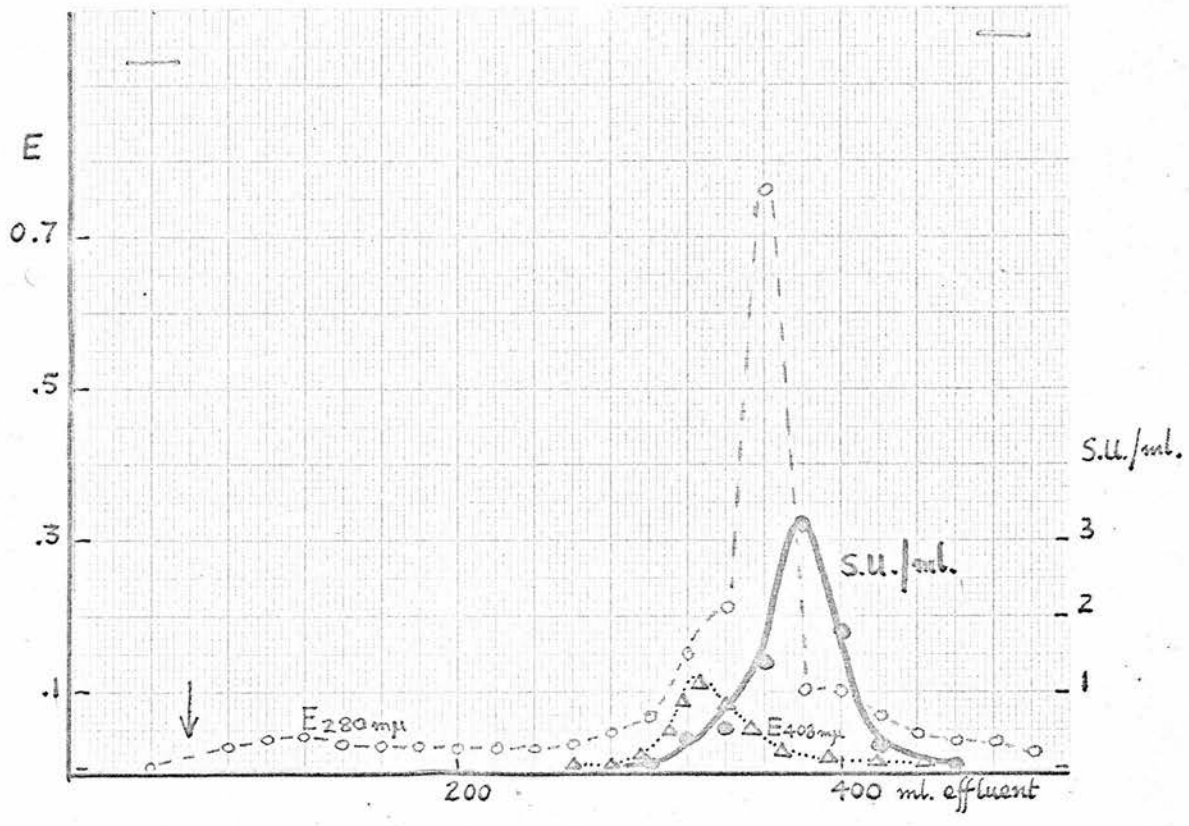


Fig. 4.4.1. Chromatography of Stage 6 enzyme on DEAE-cellulose at pH 5.5.

The sample was 6 mg. Stage 6 enzyme in 2.5 ml. of the starting buffer, 0.01 M phosphate buffer, pH 5.5. A linear gradient of sodium chloride, from 0 to 2 M over 1 l., was started at ↓ .

o---o Protein, $E_{280\ mu}$

△·····△ HpMHb, $E_{408\ mu}$

●——● Histaminase, SU/ml.

(ii) pH 7.4 and pH 6.4. Preliminary batch experiments had revealed little about the anionic properties of histaminase at pH values near neutrality. 2 x 40 cm. columns of DEAE-cellulose were equilibrated against 20 mM phosphate buffers at pH 6.4 and pH 7.4, Stage 6 enzyme applied to each after dialysis against the appropriate buffer, and the columns eluted under a shallow salt gradient, 0 to 1 M over 2 l. No significant separation of histaminase from HpMHb was achieved.

(iii) pH 5.5. Although histaminase is rather unstable at pH 5.5, except in free solution at low temperature, it was predicted that histaminase would be less strongly bound to DEAE-cellulose than to cellulose phosphate at low pH, and that a rapid separation of the enzyme from HpMHb might be effected. Stage 6 enzyme was applied to a 2 x 40 cm. column of DEAE-cellulose in 10 mM phosphate buffer, pH 5.5, and after washing in with 200 ml. of starting buffer, a salt gradient was used to elute the column. All protein emerged, however, before the salt concentration of the effluents had significantly increased. As illustrated in Fig. 4.4.1., considerable separation of histaminase from HpMHb had occurred. Unfortunately, histaminase was almost completely inactivated in the process.

It seemed probable that histaminase inactivation was due to denaturation of the protein at a large adsorbent surface, near the iso-electric point. It was also possible that a vital cofactor had been split from the enzyme at low pH. To test the latter hypothesis, a portion of the most active effluent was brought to pH 6.8 by the addition of phosphate buffer, and this stabilised enzyme was assayed in the presence of several factors which it was thought might include coenzymes of histaminase, by analogy with other forms of DAO. Pyridoxal phosphate, flavin adenine dinucleotide, yeast extract, liver extract,

and a boiled solution of Stage 6 enzyme failed to re-activate the preparation. It was concluded that inactivation of histaminase at pH 5.5 was due to structural denaturation of the protein moiety of the enzyme, rather than to the loss of cofactors.

Sufficient activity remained in the effluents to demonstrate, with the aid of the sensitive indigo test (Method II), that HpMHb had been partially separated from histaminase. The elution pattern shown in Fig. 4.4.1. is direct evidence that HpMHb is not responsible for histaminase activity, or for catalysis of the indigo test, and that, while the two species are usually associated, or have extraordinarily similar physical properties, they have separate identities.

(c) Digestion with neuraminidase.

It was thought that if the HpMHb complex could be dissociated Hp and MHb might be easily separated from histaminase by gel filtration or ion-exchange chromatography. However, a recent review by Laurell and Gronvall (1962) has claimed that Hp binds irreversibly to haemoglobin, and all means of cleaving the complex have so far failed. A more promising approach seemed to be the removal of the potentially anionic neuraminic acid residues of the Hp moiety, by digestion with the specific enzyme, neuraminidase. On chromatography at alkaline pH, the digested HpMHb might possess fewer negative charges than native HpMHb, and be eluted from an anion exchanger more rapidly than histaminase.

There was no evidence in the literature that Hp is a substrate of neuraminidase, and the pH optimum of the enzyme is about pH 5 (Gottschalk, 1960). Digestion of Stage 6 enzyme at this low pH would almost certainly destroy histaminase activity. The sacrifice of a large quantity of laboriously

purified enzyme did not seem justified, but it was considered worthwhile to determine whether incubation of HpMHb with neuraminidase, under conditions not too destructive of histaminase activity, might appreciably alter its mobility on DEAE-cellulose at pH 8.6.

A solution of HpMHb, prepared as described in Appendix 1, was buffered at pH 6.0, with 0.1 M phosphate buffer. 5 mg. crystalline neuraminidase was added to one half of this solution; the remaining half was treated in the same way as the test solution throughout all subsequent operations, and served as a control. Control and test solutions were incubated for 24 hr. at 37° to facilitate digestion, then dialysed overnight at 4° against 5 mM borate buffer, prior to chromatography on 2 x 40 cm. columns of DEAE-cellulose at pH 8.6. The test and control samples were eluted under a shallow salt gradient, 0 to 1 M over 2 l. No significant difference in the elution volumes of digested and undigested HpMHb was observed.

It was not possible to reduce the pH of digestion, since histaminase would not have withstood incubation at a lower pH. The chromatographic system used was considered a valid test of the expected change in mobility of HpMHb. Further pursuit of this means of separating HpMHb and histaminase therefore did not promise to be rewarding.

4.4.2. Recycling gel filtration on Sephadex G-200.

Before recycling equipment became available, Stage 6 enzyme had been concentrated and re-filtered on a 1.5 x 100 cm. column of G-200, and sometimes a very slight separation of the two components had been achieved. Usually, histaminase travelled rather more slowly than the brown band of HpMHb, but occasionally the enzyme peak slightly preceded the brown peak. This was difficult to explain, until it was realised that different preparations, each from ten placentae, were likely to possess different distributions of Hp types, all of which, in combination with MHb, should be eluted together in the first peak at Stage 6. If the relative elution volume of histaminase remained constant while the shape of the composite peak comprising several HpMHb types changed with each preparation, the displacement of histaminase from HpMHb in the elution pattern would be variable.

According to the definition of relative elution volume outlined in Section 3.3., the ratio of the relative elution volume of HpMHb to that of MHb alone is the same as the ratio of their elution volumes, measured on the same column. Using the elution volumes of Fig. 4.2.3.:

$$V_{(HpMHb)} / V_{(MHb)} = 0.58.$$

The data of Killander (1964), who investigated the mobility of free and Hb-bound haptoglobins on G-200, may be compared with this value:

$$V_{(Hp_{2-2}Hb)} / V_{(Hb)} = 0.52.$$

$$V_{(Hp_{2-1}Hb)} / V_{(Hb)} = 0.55.$$

$$V_{(Hp_{1-1}Hb)} / V_{(Hb)} = 0.62.$$

It may be concluded that all three HpMHb types were present in the fast-moving brown component in Fig. 4.2.3. Starch-gel electrophoresis of Stage 6

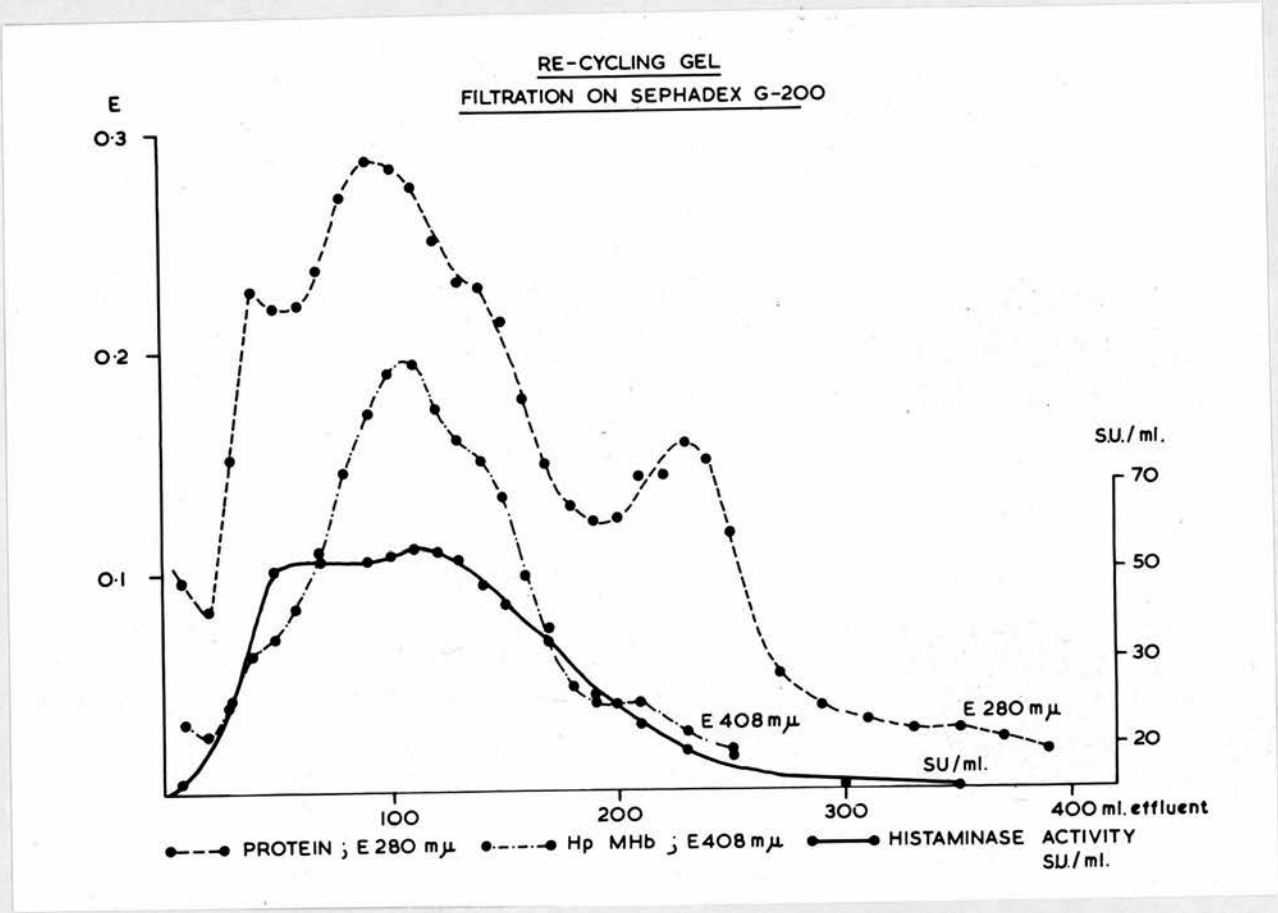


Fig. 4.4.2. Re-cycling gel filtration on Sephadex G-200.

enzyme, followed by detection of HpMHb with the H_2O_2/dianisidine stain, were also consistent with this conclusion. The elution volume of histaminase in Fig. 4.2.3. was almost indistinguishable from that of the HpMHb composite peak. It remained possible that recycling gel filtration of a small sample of Stage 6 enzyme might separate the enzyme from some or all of the HpMHb types.

Stage 6 enzyme was concentrated to small volume, and 3 ml., containing 55 mg. protein, was cycled twice through a 3 x 100 cm. column of G-200, according to the procedure outlined in Section 3.3. The selector valve was closed to start recycling when the brown band of HpMHb had traversed two-thirds of the column on its first cycle, and opened again to collect fractions when the band had traversed two-thirds of the column on its second cycle. Zone spreading precluded a third cycle, in the absence of a continuous record of protein effluent, which would have permitted unambiguous selection of fractions for recycling.

Fig. 4.4.2. shows the elution pattern after two cycles of concentrated Stage 6 enzyme from placental preparation No. 13.

The main peak, collected between 40 and 400 ml. effluent, consisted of histaminase and HpMHb, both components being eluted in unsymmetrical peaks. A slower, colourless protein followed the main peak, and it is possible that a little β-lipoprotein emerged just before the main peak — the first effluents appeared faintly milky, but there was insufficient protein in the first fractions to permit staining of protein bands after electrophoresis. β-lipoprotein travels very slowly towards the anode on starch gel electrophoresis at pH 8.5, and might easily be obscured by HpMHb bands.

The main peak, called Stage 7 enzyme, was concentrated to very small

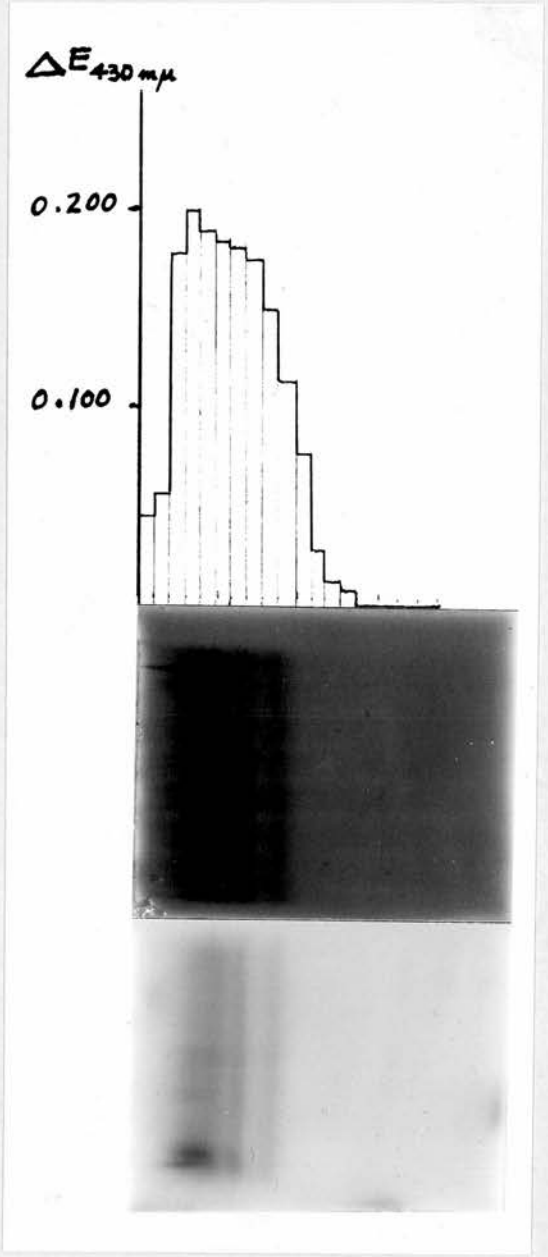


Fig. 4.4.3. Starch-gel electrophoresis of Stage 7 enzyme. Electrophoresis (according to Smithies, 1955) at pH 8.5 for 20 hr. at 4°, and with constant current of 15 mA. Three sections of same gel stained and compared as described in Section 3.5.

volume and subjected to starch gel electrophoresis, as described in Section 3.5. The sample was applied on a long strip of filter paper and, after running for 20 hr. in the refrigerator, four corresponding sections of the gel were used to detect different components, (Section 3.5).

(1) Total protein — nigrosin stain, in gel.

(2) HpMHb — H_2O_2/dianisidine stain, in gel.

(3) Histaminase — putrescine/o-aminobenzaldehyde stain, in gel.

(4) Histaminase — eluted gel strips, assayed in vitro.

The in-gel stain for histaminase, (3), showed a single diffuse yellow band which could not be photographed adequately. The position and intensities of the diffuse band appeared similar to the pattern of activity revealed by the in vitro test, (4). The results of tests (1), (2) and (4) are shown in parallel in Fig. 4.4.3.

Stage 7 enzyme appeared to be free from the MHb complex of the low-M.W. Hp component which is present in type 1-1 and 2-1 sera, and which migrates most rapidly on starch-gel electrophoresis at pH 8.6. Killander (1964) has separated $Hp_{1-1}Hb$ from all other serum components by recycling gel filtration on G-200.

Gel (1) shows no distinct protein band which cannot be attributed to the HpMHb bands in gel (2) — although there may be an additional diffuse band near the origin in gel (1). No single ~~bed~~ band attributable to histaminase is visible in the gels. Comparing gels (1) and (2) with (4), it seems rather that histaminase activity migrates in association with all the high M.W. HpMHb bands. The association of histaminase with the HpMHb species would also explain the almost coincident, but broad and unsymmetrical peaks of both these components in Fig. 4.4.2. Either histaminase is specifically bound to HpMHb, or more

probably, it is present in such small amounts that it tends to become adsorbed to HpMHb and migrate with this contaminant under the conditions of gel filtration and starch gel electrophoresis.

Chapter 5. Some properties of placental histaminase

5.1 The substrate specificity of placental histaminase.

5.2 Some kinetic constants.

5.3 The inhibition of placental histaminase.

5.4 Cofactors of placental histaminase.

5.5 The M.W. of placental histaminase.

5.6 The stability of placental histaminase.

Table 5.1.1

Symbols

H	histamine
C	cadaverine
P	putrescine
B	benzylamine
Sp	spermine
Ag	agmatine

e.g. $P > H$ putrescine oxidised more rapidly than histamine.
$H = C$ histamine and cadaverine oxidised by same enzyme.
$H \neq C$ histamine and cadaverine not oxidised by same enzyme

Purity

C.E.	crude extract
P.P.	partly purified
P.	pure

These indications of the purity of the enzyme preparation used are only very approximate. The original author's estimate of the purity of the enzyme preparation has been accepted for the present purpose.

Enzyme	Reference	Purity	Basis of Assay	Amines Oxidised	Amines not Oxidised	Substrate Competition	No Substrate Competition	Conclusion
Pea seedling DAO	Kenten and Mann (1952)	C.E.	O_2 uptake	C>P>H, Ag	-	-	-	(H = C)
	Werle et al. (1961)	C.E.	O_2 uptake; NH_3	C>Sp>H	-	-	-	(H ≠ C)
	Hill and Mann (1964)	P	O_2 uptake	P=C>Ag>H>B	Sp	-	-	(H = C)
Clover seedling DAO	Werle and Hartung (1956)	C.E.	NH_3	C>H	-	-	-	H ≠ C
Bovine serum spermine oxidase	Tabor et al. (1954)	P.P.	O_2 uptake	B, Sp	H, C, P	-	-	-
Hog serum benzylamine oxidase	Buffoni and Blaschko (1964)	P	O_2 uptake	B>H	-	-	-	H = B
Human serum benzyl-amine oxidase	McEwen (1965)	P.P.	NH_3	B>H>P	-	-	-	(H = B)
Hog kidney DAO	Zeller (1938 a,b)	C.E.	NH_3	H>C>P	-	H with C	-	H = C
	Kapeller-Adler (1949)	P.P.	O_2 uptake	H>P>C>Ag	-	H with C, P, Ag	-	H = C
	Tabor (1951)	P	O_2 uptake	P>H	-	-	-	-
	Kapeller-Adler (1956)	P.P.	Indigo oxidn.	H>C>P	-	-	H with C, P, Ag	H ≠ C
	Davidson (1956)	P.P.	O_2 uptake	H>C	-	-	-	H = C
	Waton (1956)	C.E.	Biological (H)	H	~~C, P~~	H with C, P, Ag	-	(H = C)
	Kapeller-Adler and MacFarlane (1963)	P	Indigo oxidn.	H	C, P	-	-	H ≠ C
	Mondovi et al. (1964)	P	O_2 uptake	C>H	~~C~~	-	-	H = C
	Uozomi et al. (1964)	P	O_2 uptake	H	C	-	-	(H ≠ C)
Human placental histaminase	Zeller et al. (1939)	C.E.	O_2 uptake; NH_3	C>P>H	-	-	-	H = C
	Kapeller-Adler (1956)	P.P.	Indigo oxidn.	C>P>H	-	-	H with C, P	H ≠ C
	Lindahl (1961)	C.E.	O_2 uptake	C>H	-	H with C	-	H = C

5.1. Specificity of placental histaminase.

5.1.1. Introduction.

Table 5.1.1 summarises the major conclusions reached by different authors on the specificity of some amine oxidases, with particular reference to the substrates histamine, cadaverine and putrescine. The table cannot completely assess each author's views, and only the most relevant data have been selected from each publication. Conclusions in parentheses were not specifically stated by the author, but seemed able to be drawn from the published experimental results and discussion. It is difficult to discern much agreement between the work of all authors, even with regard to the specificity of the enzyme from a single source.

Before assessing the criteria used by different workers in deciding whether histamine and the diamines are oxidised by one or more enzymes from the same source, the related problem of methodology must first be discussed.

5.1.2. Assay methods.

Investigation of the range of substrates oxidised by a single DAO preparation requires an assay method measuring one of the reactants, oxygen, ammonia or hydrogen peroxide, common to the action of the enzyme on all substrates (cf. Section 2.1). The difficulties involved in applying existing methods may be illustrated by reference to the assay of DAO activity towards histamine and cadaverine.

Manometric estimation of oxygen consumption requires the presence of a second enzyme, catalase, which may introduce further enzymically active impurities into the reaction system; the relatively high enzyme concentrations required for manometric assays also increase the probability of a minor contaminant contributing to oxygen consumption in the presence of one of the substrates; and it has been shown by Born (1953) that the presence of Cu^{++} may lead to spuriously high oxygen consumption in the presence of histamine, but not of cadaverine.

Zeller (1963) has objected to the comparative assay of activity towards histamine and cadaverine in Conway units, on the grounds that the product of cadaverine oxidation is diffusible, and interferes with the Nessler reaction.

More importantly, for the present project, Zeller (1965) has claimed that the indigometric assay of hydrogen peroxide produced in the DAO reaction is not equally satisfactory when histamine and cadaverine are used as substrates. He has shown that, under certain experimental conditions (not including the control of metal ions) histamine and certain derivatives and related substances exert a catalytic effect in the oxidation of indigo by synthetic hydrogen peroxide, which otherwise proceeds very slowly; cadaverine does not have this catalytic effect.

Zeller's theory that cadaverine is not an appropriate substrate for indigometric assays is incompatible with indigometric data presented by Kapeller-Adler (1956 b); although hog kidney extracts oxidised histamine faster than cadaverine, the reverse order obtained for placental extracts. Also, the H/C ratio obtained by Lindahl (1961) for placental histaminase, using manometry, agreed well with the indigometric measurements of Kapeller-Adler (1956 b).

Kapeller-Adler and MacFarlane (1963) found that during the purification of hog kidney DAO, the specific activity of the preparation towards histamine increased, while the Specific Activity towards cadaverine decreased to zero. Zeller (1965) has attempted to explain this by proposing that in the early stages of purification, the oxidation of indigo by hydrogen peroxide is catalysed chiefly by the histidine residues of proteins in the assay mixture, whereas indigo oxidation in the assay of highly purified enzyme solutions, containing little protein, is catalysed chiefly by the substrate; the failure of cadaverine to catalyse the second stage of the indigo test therefore results in failure to detect enzymic activity towards this substrate in indigometric assays of purified DAO.

It was found in the present work that, using the indigometric Method II, the H/C ratio of placental histaminase did not tend to increase during purification, although the assays of the purest preparations contained less than 0.1 μg. protein/ml.

It was concluded that Zeller's (1963, 1965) criticisms of indigo tests, based on model experiments on the oxidation of indigo by synthetic hydrogen peroxide, were not applicable to the enzymic tests used by Kapeller-Adler (1951, 1956b) nor to Methods I and II used in the present work.

5.1.3. Criteria of purity.

It is a common practice to attempt to isolate an enzyme completely, and to define the specificity of the enzyme as that of the purest preparation obtained. With comparatively recent advances in protein fractionation, particularly chromatographic techniques, this definition has become more satisfactory, but it must remain only approximate and provisional. The substrate specificity of the purest preparation obtainable can be regarded as the specificity of a single enzyme, until such time as it is resolved into two or more separate entities, each being active towards only some of the substrates metabolised by the previous purest preparation — loss of activity towards one group of substrates does not constitute resolution.

No single analytical method can conclusively demonstrate the homogeneity of an enzyme preparation. Most methods, e.g. electrophoresis, crystallisation to constant Specific Activity or ultracentrifugation, might fail to detect a minor contaminant representing less than 10% of the total protein. Furthermore, an apparently homogeneous and enzymically active band, obtained on starch gel electrophoresis, might consist almost entirely of a contaminant protein having the same electrophoretic mobility as the enzyme. As an increasing number of different analytical methods are applied, the possibility that a single band represents more than one species may be considered to diminish, but this is a valid conclusion only if the analytical methods exploit many different properties of the proteins; most rely on molecular weight or charge differences only.

These difficulties are illustrated by the failure to separate histaminase from HpMHb by a combination of salt precipitation, ion-exchange over virtually the whole pH range in which histaminase is stable, gel

filtration, and starch gel electrophoresis. The only protein bands observed after electrophoresis were attributable to HpMHb, yet these bands also contained histaminase activity. The apparently homogeneous HpMHb bands might have contained other enzymes besides histaminase, and it was impossible to tell from the electrophoretic pattern whether the purest preparations were contaminated with other amine oxidases of similar physical properties. This will be further discussed in Sections 5.3 and 6.1.

One difficulty in approaching specificity problems in this way is the assessment of the purity of preparations obtained by other workers, often using different assay methods, and applying different criteria in support of the purity of their enzyme preparations. Of those authors who have recently published purification schemes for amine oxidases, Buffoni and Blaschko (1964) have laid most emphasis on the probable purity of their pig serum benzylamine oxidase; they demonstrated homogeneity of their enzyme under starch gel electrophoresis and ultracentrifugation, after salt precipitation, ion-exchange chromatography, and several re-crystallisations. On the other hand, Mondovi et al. (1964) and Uozomi et al. (1964) offered no evidence for the purity of their final hog kidney DAO preparations. Kapeller-Adler and MacFarlane (1963) relied solely on cellulose acetate electrophoresis as the criterion of purity of their hog kidney preparation, obtained after salt precipitation, heating, and simple elution from DEAE-cellulose columns. The low resolving power of these techniques has been noted in Sections 3.5 and 4.2.4. No separation by molecular weight was attempted. Hill and Mann (1964) showed that their pea seedling DAO was not completely homogeneous in the ultracentrifuge, and McEwen (1965 a) emphasised that his human serum benzylamine oxidase had not been purified completely.

Before concluding that an enzyme has been purified, vigorous attempts to demonstrate heterogeneity of the purest preparation should be made, in addition to reporting a purification factor, and care must be taken not to rely on criteria of poor sensitivity (e.g. cellulose acetate electrophoresis); nor on methods which test only one physical parameter.

Criteria of homogeneity should include (1) tests depending almost solely on charge properties, such as ion-exchange chromatography or column electrophoresis, and (2) tests depending almost solely on molecular weight properties. With the proviso that the size of the enzyme should permit its partial retardation on one of the Sephadex grades, gel filtration should be comparable in sensitivity to ultracentrifugation as a test for homogeneity by molecular weight, and recycling gel filtration may be even better. Electrophoresis on starch or poly-acrylamide gels, which separate by both charge and molecular weight, can sometimes be used as an additional test, preferably over a wide range of pH, and followed by specific enzyme stains; protein staining alone carries little conviction.

The enzyme preparation (Stage 7) used in the present determination of the specificity of placental histaminase was not completely pure, but extensive purification produced evidence of only one contaminant, HpMHb, which is not active as an amine oxidase (Section 2.3.6). Nevertheless, it was considered advisable to supplement specificity measurements with mixed-substrate experiments.

5.1.4. Mixed-substrate experiments.

Dixon and Webb (1964, p.201) have discussed the application of mixed-substrate assays in determining whether the oxidation of two substrates is effected by one or more enzymes in the same preparation. They emphasised that the demonstration of competition between two substrates may not be conclusive proof that a single enzyme is responsible for both oxidations: each substrate may be a competitive inhibitor of an enzyme specific for the other substrate. Equally, failure to demonstrate competition by an assay method measuring the formation of a product common to both substrates may not prove that both substrates are oxidised by different enzymes, since the same enzyme may have the same affinity for both substrates, and oxidise them at the same rate. Mixed-substrate experiments are, therefore, best accompanied by a comparison of the rates of oxidation of the individual substrates. The significance of competition experiments may be defined in two sections, according to the type of assay method employed.

(a) Method measuring a common product of both substrates.

If a single assay method is capable of measuring a product common to enzymic activity towards all substrates (e.g. hydrogen peroxide, measured by Method II), and if

x is the rate of reaction in the presence of substrate X

r is the rate of reaction in the presence of a reference substrate R

m is the rate of reaction in the presence of an equimolar mixture of X and R,

then if m were equal to r + x, this would indicate that two enzymes in the same preparation were acting independently on R and on X, each reaction providing part of the measured product. On the other hand, if m lay between r and x, this

would be strong evidence that both substrates were competing at the active centre of the same enzyme, and the proximity of m to r and x would be an approximate index of the comparative affinities of the enzyme for R and X.

(b) Methods applicable to only one substrate.

The specific measurement of the product formed from the oxidation of a single reference substrate R (e.g. putrescine, Method III) may be inhibited by the addition of a substrate X which can compete with R at the active centre of the same enzyme. Independent tests may have to be applied to ensure that X alone is oxidised by the enzyme preparation, and that it is not a specific inhibitor of the enzyme.

5.1.5. Experimental results.

(a) Comparative rates of oxidation of different substrates.

In these experiments, the rate of oxidation of each substrate was measured as the rate of decrease of indigo extinction when Stage 7 enzyme was incubated with the substrate under the assay conditions of Method II.

The extinction of each test, and that of a control containing substrate but no enzyme, was measured at 3 hr. intervals, and the oxidation rate calculated over the period during which indigo oxidation proceeded linearly, after a brief "lag" period. Increased amounts of enzyme were used in the presence of the more slowly oxidised substrates.

Table 5.1.2.

Indigo: 0.287 mM.

Enzyme: Stage 7.

Substrates: 2.28 mM.

Incubation at 37°, pH 6.8, 0.067 M phosphate buffer, 0.1 mM EDTA.

Rate of indigo oxidation: 1000 x $\Delta E_{610\ m\mu}$/hr.

Substrate	Rate of oxidn.	Enz.soln. ml.	Rate/ml. enzyme	Comparative Rate (C = 100)
Diaminoethane	2.1	1.0	2.1	5
Diaminopropane	4.6	0.5	9.2	23
Diaminobutane (P)	4.4	0.2	22	54
Diaminopentane (C)	8.0	0.2	40	100
Diaminohexane	8.0	0.5	16	40
Histamine	1.8	0.2	9.0	23
Spermine	1.5	1.0	1.5	4
Agmatine	3.4	0.5	6.8	17
Benzylamine	2.0	1.0	2.0	5

(b) Mixed-substrate experiments, using Method II.

Semi-quantitative experiments were carried out to determine whether some of the amines oxidised only very slowly by the purified histaminase solution were really substrates of that enzyme, or of some contaminating oxidase present in only small amounts. Single measurements of extinction changes after 24 hr. were adequate for this purpose. Activity was measured, according to the standard Method II, in the presence of each substrate alone, and with an equimolar admixture of putrescine.

Table 5.1.3.

Indigo: 0.287 mM.

Substrates: 2.28 mM.

Enzyme: Stage 7. Same volume of enzyme solution used in each test.

Incubation at 37°, pH 6.8, 0.067 M phosphate buffer, 0.1 mM EDTA.

Rate of oxidation: (S.U.) = $\Delta E_{610\ m\mu}$ after 24 hr. incubation.

Substrate (S)	Rate of oxidation (S.U.)	
	S alone	S + putrescine
Diaminobutane (P)	0.096	
Diaminoethane	0.013	0.078
Histamine	0.038	0.050
Spermine	0.015	0.109
Agmatine	0.029	0.039
Benzylamine	0.015	0.328

The remarkable amount of indigo oxidation obtained in the presence of benzylamine+putrescine (more than the added values in the presence of each substrate alone) was reproducible.

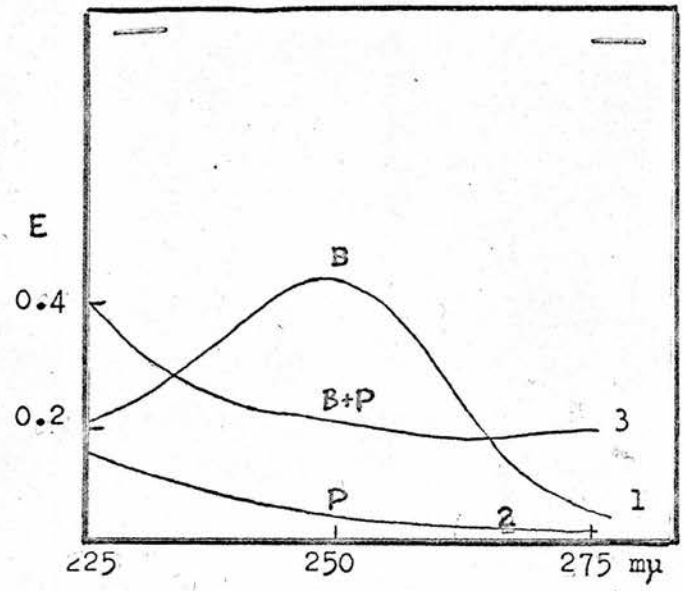


Fig. 5.1.1. Spectra of completed assays of histaminase activity towards benzylamine and putrescine.

Assay conditions were as described in Section 5.1.5.(c)

Substrates: 1 Benzylamine.
2 Putrescine.
3 Benzylamine + putrescine.

(c) Competition between benzylamine and putrescine.

After the unsatisfactory result obtained in (b) above, it was considered necessary to check the possibility of competition between these substrates using an independent assay method. The oxidative deamination of benzylamine may be followed by the appearance of benzaldehyde, which has an extinction maximum at 250 mμ (Tabor, Tabor and Rosenthal, 1954). The oxidation of benzylamine and putrescine, separately and in equimolar mixture, was examined spectrophotometrically. Tests were incubated with substrate and enzyme; substrate was added to control assays, incubated without substrate but with enzyme, immediately before extinction measurements.

Table 5.1.4.

Substrates: 2.28 mM.

Enzyme: Stage 7. Same volume of enzyme solution added to each test.

Incubation at 37°, pH 6.8, 0.067 M phosphate buffer, 0.1 mM EDTA.

Extinction differences measured after 24 hr. incubation.

Test No.	Substrate	$\Delta E_{250\ m\mu}$
1	Benzylamine	0.425
2	Putrescine	0.055
3	Benzylamine + putrescine	0.255

Spectra of the completed tests (Fig. 5.1.1), recorded with the appropriate control solution in the reference cuvette, showed that Test 1 had a U.V. extinction maximum at about 250 mμ, whereas Tests 2 and 3 showed no clear maximum in this region.

(d) Mixed-substrate experiments, using assay Method III.

The method of Holmstedt and Tham (Method III) is specific for a restricted range of aliphatic diamines, whose corresponding monoaldehydes can cyclise and condense with o-aminobenzaldehyde to form yellow compounds. Putrescine was used as the typical DAO substrate in experiments designed to test whether other amines, thought to be substrates of placental histaminase, might competitively inhibit the action of the enzyme towards diamines. These experiments served as a check on results obtained by the more complex indigo test (Method II), which may be susceptible to anomalous effects due to the catalytic action of some substrates or oxidative products on the oxidation of indigo by hydrogen peroxide formed in the primary enzyme-substrate reaction. The assay conditions in these experiments were essentially those of Method III (Section 2.4.1.). Controls contained substrate, but no enzyme.

Table 5.1.5.

Substrates: 1.14 mM.

o-aminobenzaldehyde; 5 mM.

Enzyme: Stage 7. Same volume of enzyme solution added to each test.

Incubation at 37°, pH 6.8, 0.067 M phosphate buffer, 0.1 mM EDTA.

Extinction measured at 430 mμ after 3 hr.

Substrate (S)	Activity $\Delta E_{430\ m\mu}$	
	S alone	S+putrescine
Diaminoethane	0.003	0.244
Diaminopropane	.004	.340
Diaminobutane (P)	.416	-
Diaminohexane	.253	.372
Histamine	.004	.034
Spermine	.002	.347
Agmatine	.026	.067
Benzylamine	.004	.365

5.1.6. Conclusions

On the basis of results presented in Section 5.1.5(a), it appears that the purest preparation of placental histaminase oxidises the C_4-C_6 diamines more rapidly than the other known substrates. Aliphatic diamines of shorter chain length are less rapidly attacked. Histamine is also oxidised by this placental preparation, but spermine, agmatine and benzylamine are attacked only very slowly.

The results of mixed-substrate experiments reported in Section 5.1.5(b) confirm that histamine and the short-chain aliphatic diamines, although rather slowly attacked by comparison with putrescine, are in fact oxidised by the same enzyme. That the weak oxidation of agmatine by purified enzyme is attributable to histaminase is supported by the extent to which it competes in the indigo test with putrescine; however, since agmatine (1-amino, 4-guanidinobutane) may be regarded as substituted putrescine, the objection that agmatine may be a competitive inhibitor of specific DAO activity may acquire particular significance. It is doubtful whether the mixed-substrate oxidation of spermine + putrescine is sufficiently different from that of putrescine alone to justify the conclusion that two enzymes are acting separately on these substrates. The confidence with which these results can be interpreted is reduced when the rate of oxidation of spermine approaches the error involved in the assay of the activity of the same amount of enzyme, with putrescine as substrate. At the least, it might be concluded that spermine is a very poor substrate of placental histaminase.

The mixed-substrate experiment with benzylamine and putrescine was difficult to interpret and may have been due to anomalous catalysis of indigo oxidation by a product of the reaction. Since the excessive indigo oxidation

required a combination of enzyme and both substrates, it was considered that any interference with the normal course of indigo oxidation must have occurred as a result of a reaction between putrescine and benzylamine, or one of these with the aldehyde of the other, or the aldehydes of both. The phenomenon was not further pursued, and the possibility of substrate competition between putrescine and benzylamine was tested in other ways. The mixed-substrate experiment with putrescine and benzylamine described in 5.1.5.(c) is a much more convincing demonstration (i) that benzylamine is specifically oxidised by a purified preparation placental histaminase and (ii) that the same enzyme oxidises both benzylamine and putrescine.

The mixed-substrate experiments using Method III, reported in Section 5.1.5.(d) indicate that all the amines suspected from indigometric data to be substrates of histaminase were able to compete with putrescine in this independent test. However, the affinity of the enzyme for spermine and benzylamine, as judged by the relatively small inhibition of putrescine oxidation, appeared to be low. It was noted that, with the exception of these two unusual cases, the percentage inhibition of activity towards putrescine was of a similar order, measured by either Method II or III. Using Method III, no increased oxidation of putrescine was observed in the presence of benzylamine. Conversely, Section 5.1.5.(c) showed that putrescine did not stimulate increased enzymic oxidation of benzylamine. It was concluded that the increased indigo oxidation observed, using Method II, when putrescine was mixed with benzylamine, was due to a disturbance of indigo oxidation, rather than of the primary enzyme-substrate reaction.

While, as stated in the introductory sections 5.1.1 - 3, it is possible to ascribe activity towards several classes of substrate to one enzyme only

with certain reservations, the data presented here with the aid of highly purified enzyme considerably reduce previous uncertainty about the specificity of placental histaminase. In particular, the evidence that the enzyme is a DAO, which also attacks histamine, will be assembled in Chapter 6.

5.2. Some kinetic constants of placental histaminase.

5.2.1. Introduction.

The inhomogeneity of the purest preparations of placental histaminase was an obstacle to the investigation of the physical properties of the enzyme, but its catalytic properties were not expected to be affected by the presence of contaminating HpMHb. The most highly purified enzyme was used in these experiments to diminish the risk of interference by unidentified contaminants or inhibitors in the enzyme solution. The aim was to determine the Michaelis constants (K_m) and the pH optima for the activity of placental histaminase towards histamine and the aliphatic diamines using the new spectrophotometric indigo test (Method II) and the method of Holmstedt and Tham (Method III).

Method II. All assay methods should measure the initial velocity of the enzyme-substrate reaction. It was shown in Section 2.3 that, after an initial lag period, oxidation of indigo proceeded linearly with time, under the standard conditions of Method II. It was concluded that single measurements of extinction changes after 24 hr. were valid for the quantitative comparison of the histaminase content of similar solutions, under standard conditions. There could be no assurance, however, that any departure from standard conditions might not disturb the linear progress of indigo oxidation, and therefore invalidate comparisons of activity based only on single extinction measurements after 24 hr. In the determination of K_m, low substrate concentrations might limit the rate of enzymic reaction before this period had elapsed, and the slight variation of lag period with substrate concentration (Section 2.3.7) might further increase the error of single extinction measurements.

In this series of kinetic experiments, where quantitative comparisons of histaminase activity had to be carried out under non-standard conditions,

initial velocity was defined as the rate of decrease of extinction of indigo at 610 mμ, during the linear period of indigo oxidation following the initial lag period. Progress curves were constructed for each test by making extinction measurements at regular intervals, as in the lag-period experiments described in Section 2.3.7.

The determination of the pH optimum was carried out in a similar way, maintaining the standard substrate concentrations, but varying the pH of the M/15 Sorensen buffers in the assay. Although comparisons of activity did not have to be numerically precise to indicate optimal pH values, it was considered advisable to measure "initial" rates of linear indigo oxidation, in case the usual course of oxidation was disturbed by pH changes, or the enzyme was rapidly inactivated at extreme pH. Model experiments were also carried out using synthetic hydrogen peroxide in place of the enzyme, to check that the observed pH optima were not due to artificial limitation of the overall reaction by slow oxidation of indigo in the secondary reaction.

Method III. As stated in Section 2.4.1., Method III does not produce a linear extinction increase over a convenient incubation period. Deviations from linearity were found to be greatest at low substrate concentrations, indicating that exhaustion of substrate is one of the limiting factors in the overall reaction.

For the determination of K_m, the extinction increase after 30 min. incubation gave an approximate measure of initial velocity. This procedure may have resulted in under-estimates of initial velocities at low substrate concentrations, but measurable extinction changes could not be obtained over a shorter interval.

For the determination of the pH optimum, carried out at the relatively high standard concentration of putrescine, it was sufficient to measure the extinction changes after a longer interval. Precise initial velocities were not required for this limited investigation, and colour development appeared to proceed continuously at all pH values, i.e. there was no evidence of extraordinarily rapid, but short-lived, activity at extreme pH.

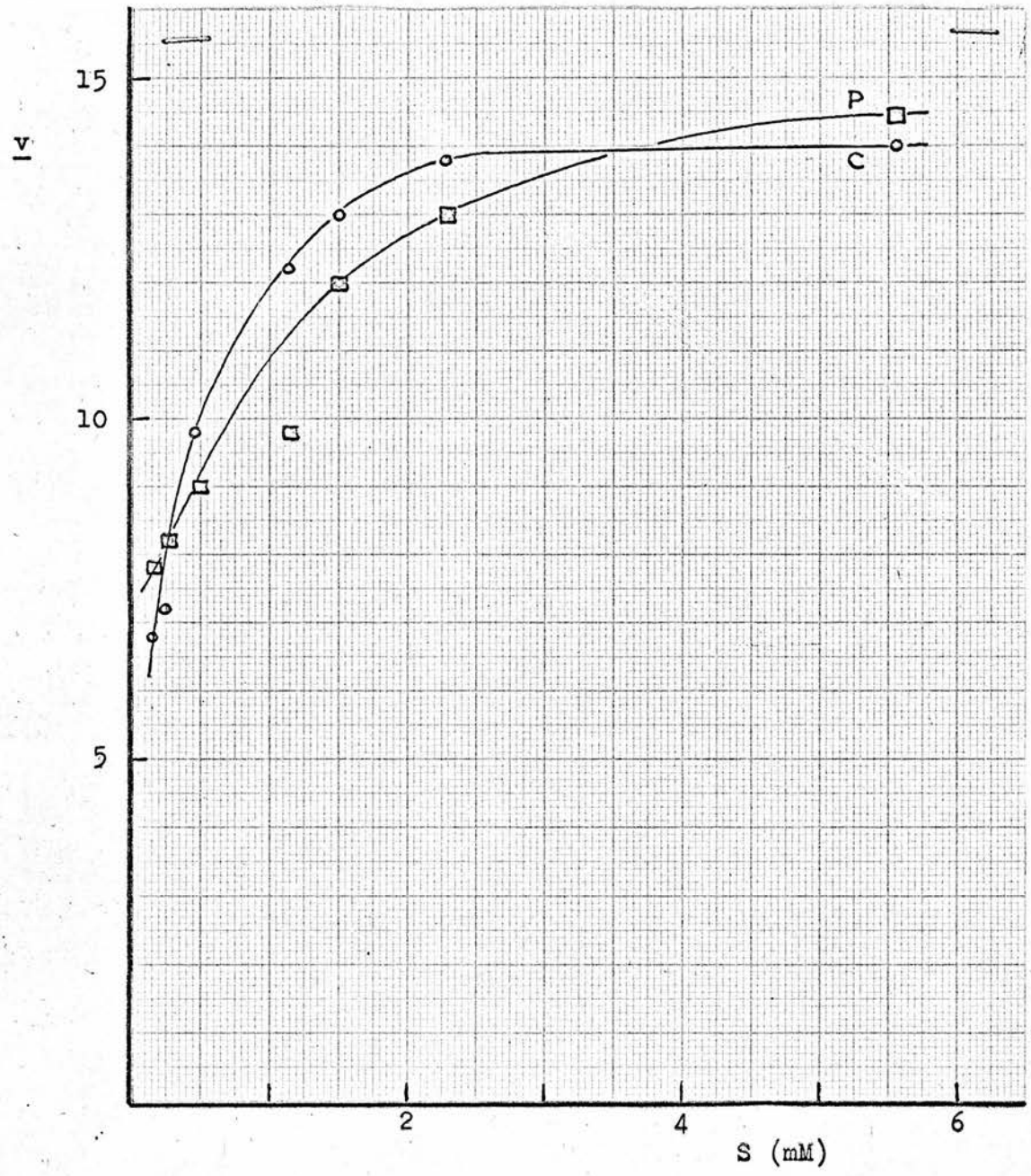


Fig. 5.2.1. Variation of histaminase activity (Method II) with concentration of cadaverine and putrescine.

S is expressed in mM units, v is expressed as described in Section 5.2.2.

o—o Cadaverine
□—□ Putrescine

5.2.2. Determination of K_m.

Method II. The concentration of substrate was varied in assay solutions containing the following components:

Indigo: 0.287 mM.

Enzyme solution: Stage 7. The same amount of enzyme was used at all concentrations of the same substrate; but, to allow for different rates of oxidation, the enzyme concentration was varied for different substrates. Controls contained no enzyme solution.

Phosphate buffer, 0.067 M, pH 6.8, 0.1 mM EDTA; to 4 ml. final volume.

Assays were incubated at 37°, and extinction of tests and controls read at 610 mμ at 3 hr. intervals. Initial velocities enzymic activity, v, were expressed as 1000 x $\Delta E_{610m\mu}$/hr., during the period of linear indigo oxidation.

(a) Cadaverine and putrescine.

The curves shown in Fig. 5.2.1 are hyperbolic in shape, consistent with the Michaelis-Menten theory. No inhibition at higher substrate concentrations was noted, in the range of concentrations used.

The Michaelis-Menten equation may be written:-

$$v = \frac{V(S)}{K_m + (S)} \qquad \text{Eq. 1.}$$

where S is the initial substrate concentration, v the enzymic activity at that concentration, V the maximum velocity at optimal substrate concentration and K_m the Michaelis constant, an index of the "affinity" of the enzyme for the substrate.

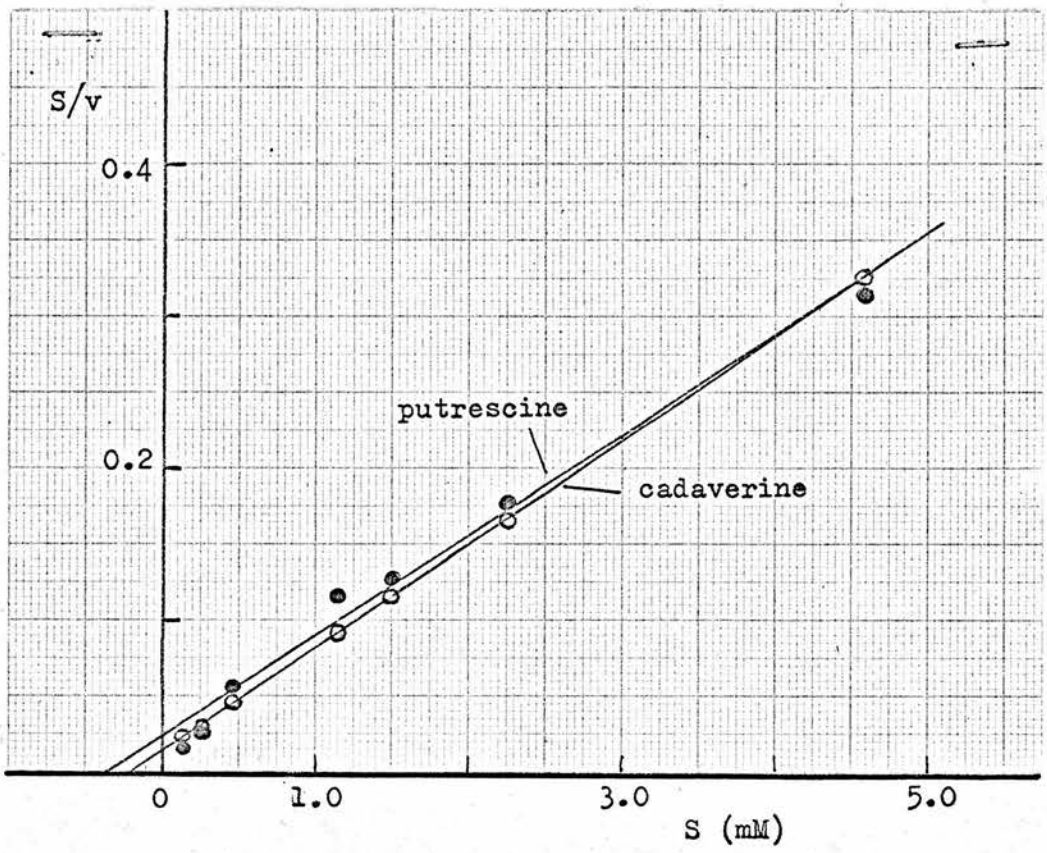


Fig. 5.2.2. Determination of K_m(cadaverine) and K_m(putrescine) by Method II.

S is expressed in mM units, v is expressed as described in Section 5.2.2. Data are plotted according to the method of Hanes (1932).

●—● Putrescine
○—○ Cadaverine

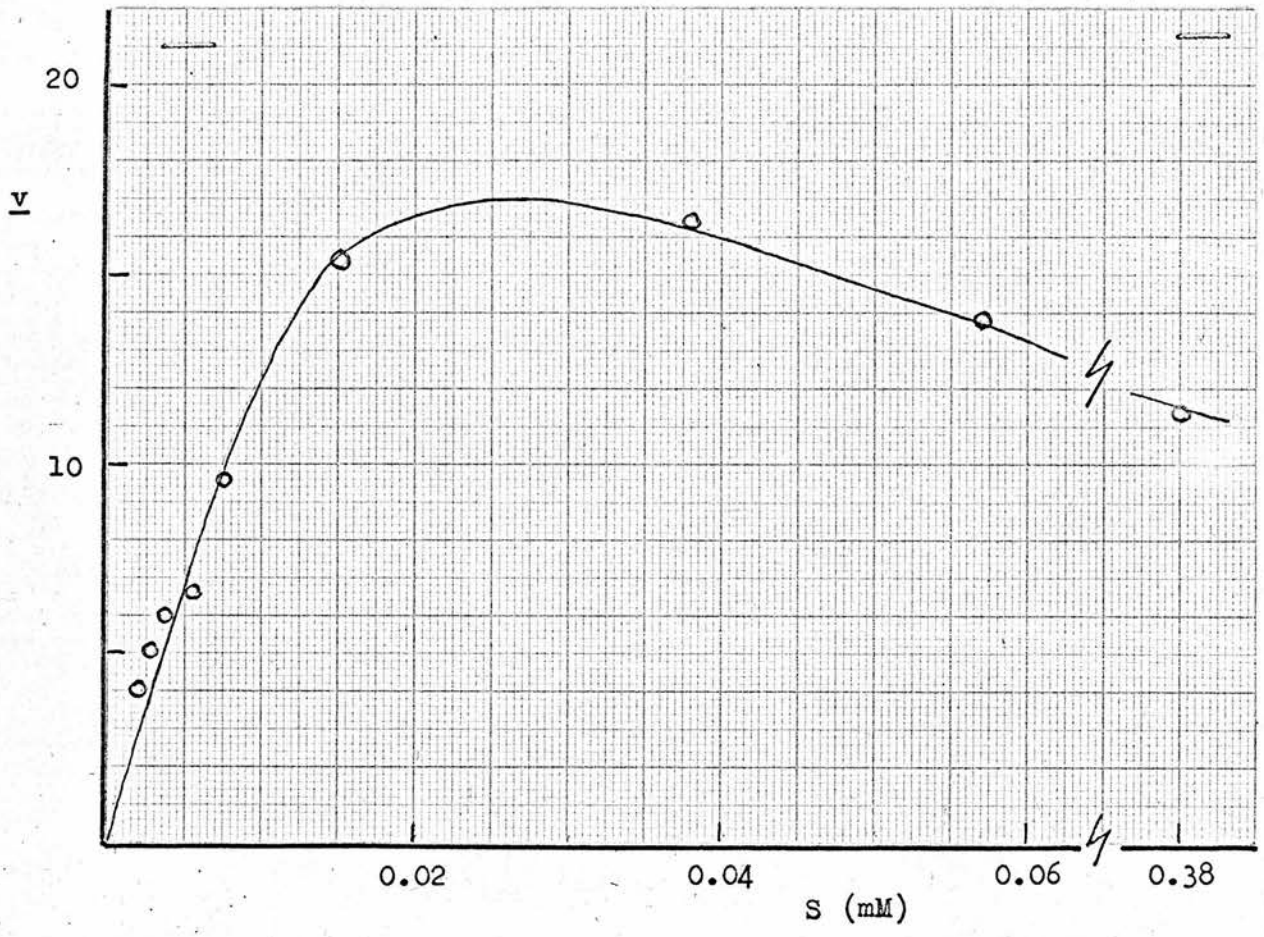


Fig. 5.2.3. Variation of histaminase activity (Method II) with concentration of histamine.

v is expressed as described in Section 5.2.2.

Multiplying Eq. 1 by (S) the following equation may be derived (Hanes, 1932):

$$\frac{(S)}{v} = \frac{K_m}{V} + \frac{1}{V}(S) \qquad \text{Eq. 2.}$$

If a straight line is fitted to experimental values of (S)/v plotted against (S) the intercept of that line on the ordinate will be K_m/V, and the gradient 1/V. This method of plotting does not tend to give undue weight to activities at low substrate concentrations, where measurements are often least reliable.

The data for cadaverine (Fig. 5.2.2.) fitted a straight line almost perfectly, and the following Michaelis constant was calculated by the method of least squares: K_m cadaverine = 0.21 mM.

Results for putrescine were not readily fitted by a straight line. Although it was considered that this was due to random errors in the individual reaction velocity measurements, it did not seem valid to quote, as a characteristic of placental histaminase, a K_m constant derived by assuming a conformity with orthodox Michaelis-Menten kinetics which was not apparent from the experimental data.

(b) Histamine.

Fig. 5.2.3. shows that there is an optimal concentration for histamine in the indigo test, beyond which histaminase was inhibited by excess substrate. The standard concentration of histamine in Method II was 0.034 mM, and lay within the optimal range, about 0.02 - 0.04 mM.

Hanes's (1932) method of plotting was not used for histamine, since it obscured the existence of a substrate concentration optimum, and did not emphasise activities at low concentrations of substrate; the latter were chosen to suit the reciprocal plot of Lineweaver and Burk (1934):

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{(S)} + \frac{1}{V} \qquad \text{Eq. 3.}$$

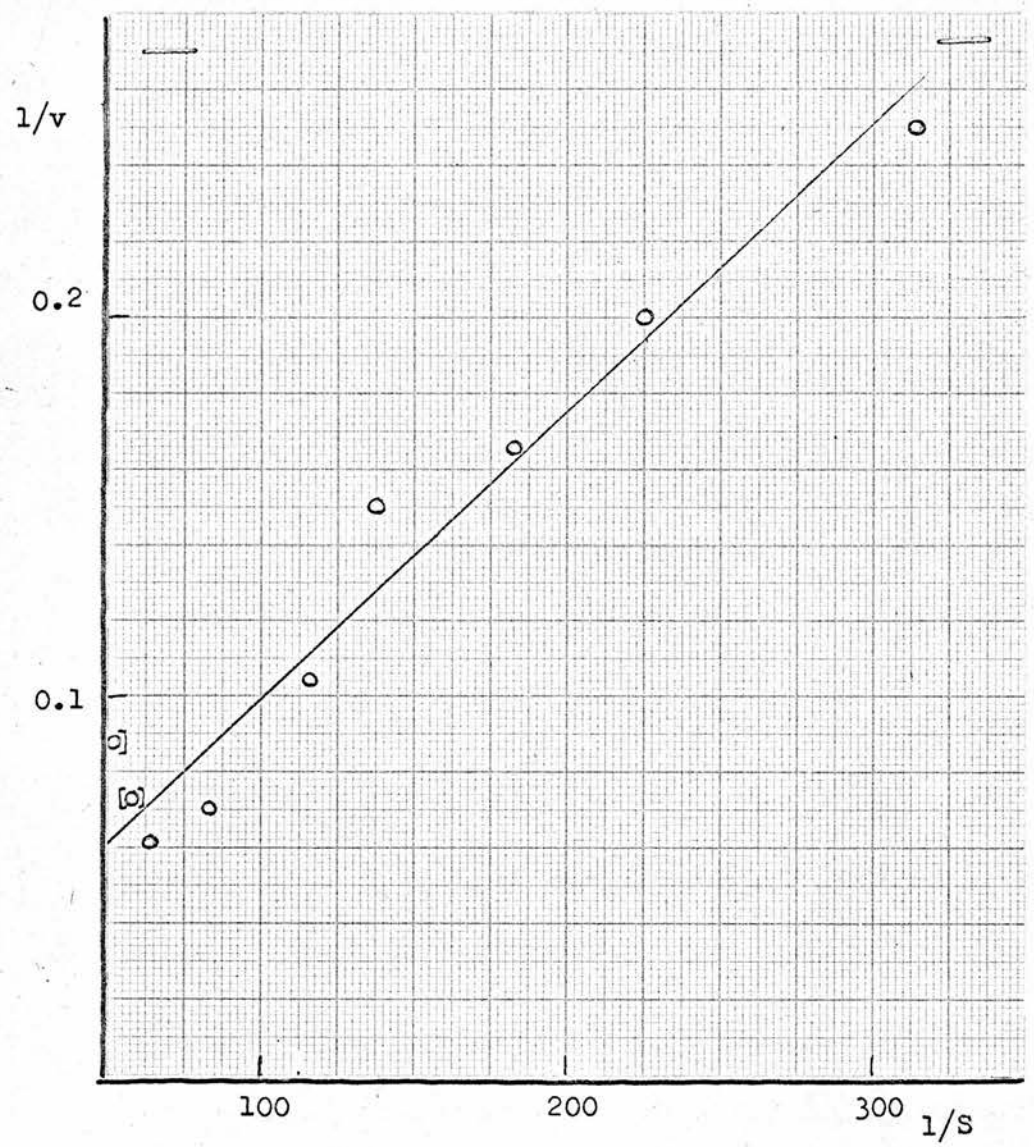


Fig. 5.2.4. Determination of K_m (histamine) by Method II.

S is expressed in mM units, $\underline{v}$ is expressed as described in Section 5.2.2. Data are plotted according to the method of Lineweaver and Burk (1934). The bracketed points were omitted from the calculation of K_m.

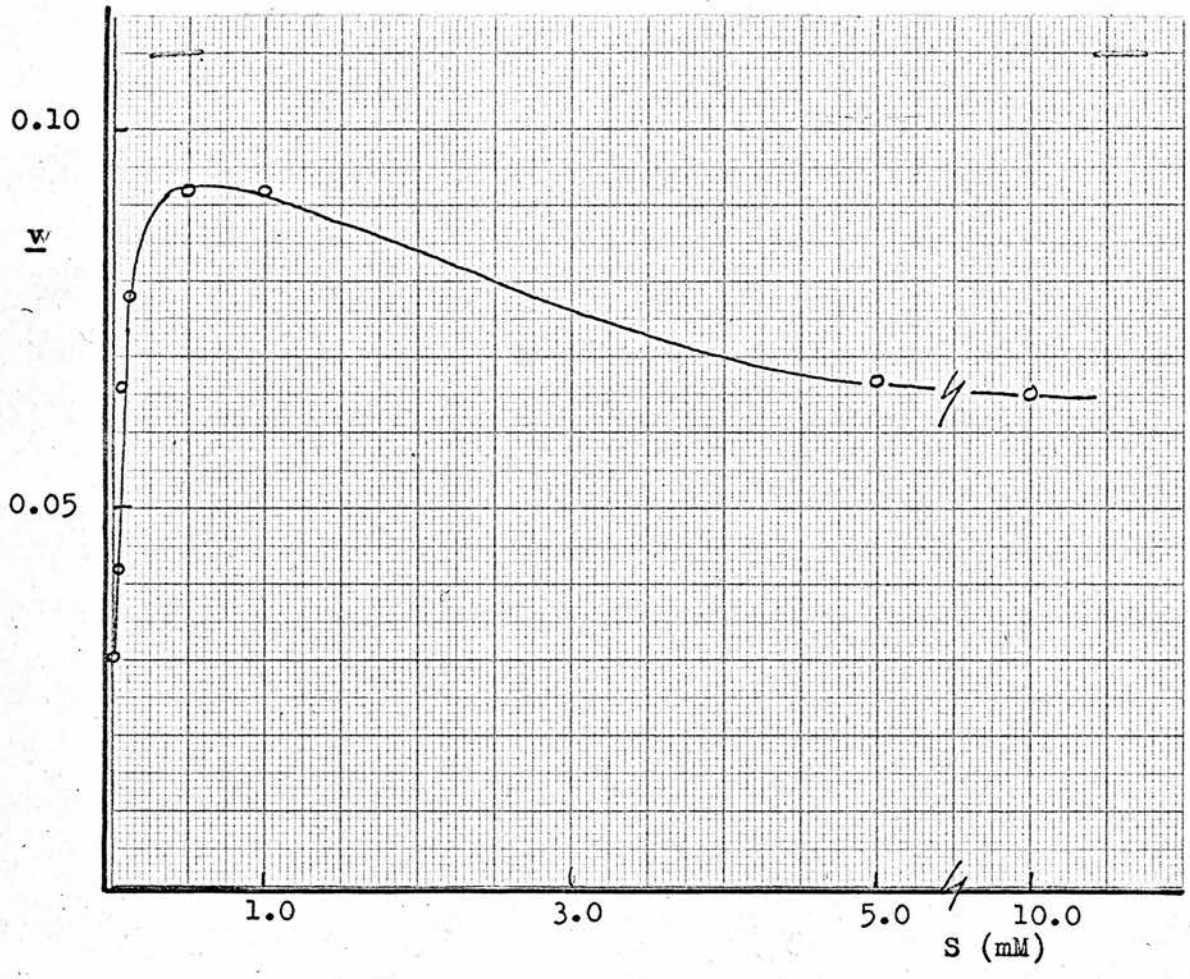


Fig. 5.2.5. Variation of histaminase activity (Method III) with concentration of putrescine.

v is expressed as described in Section 5.2.2.

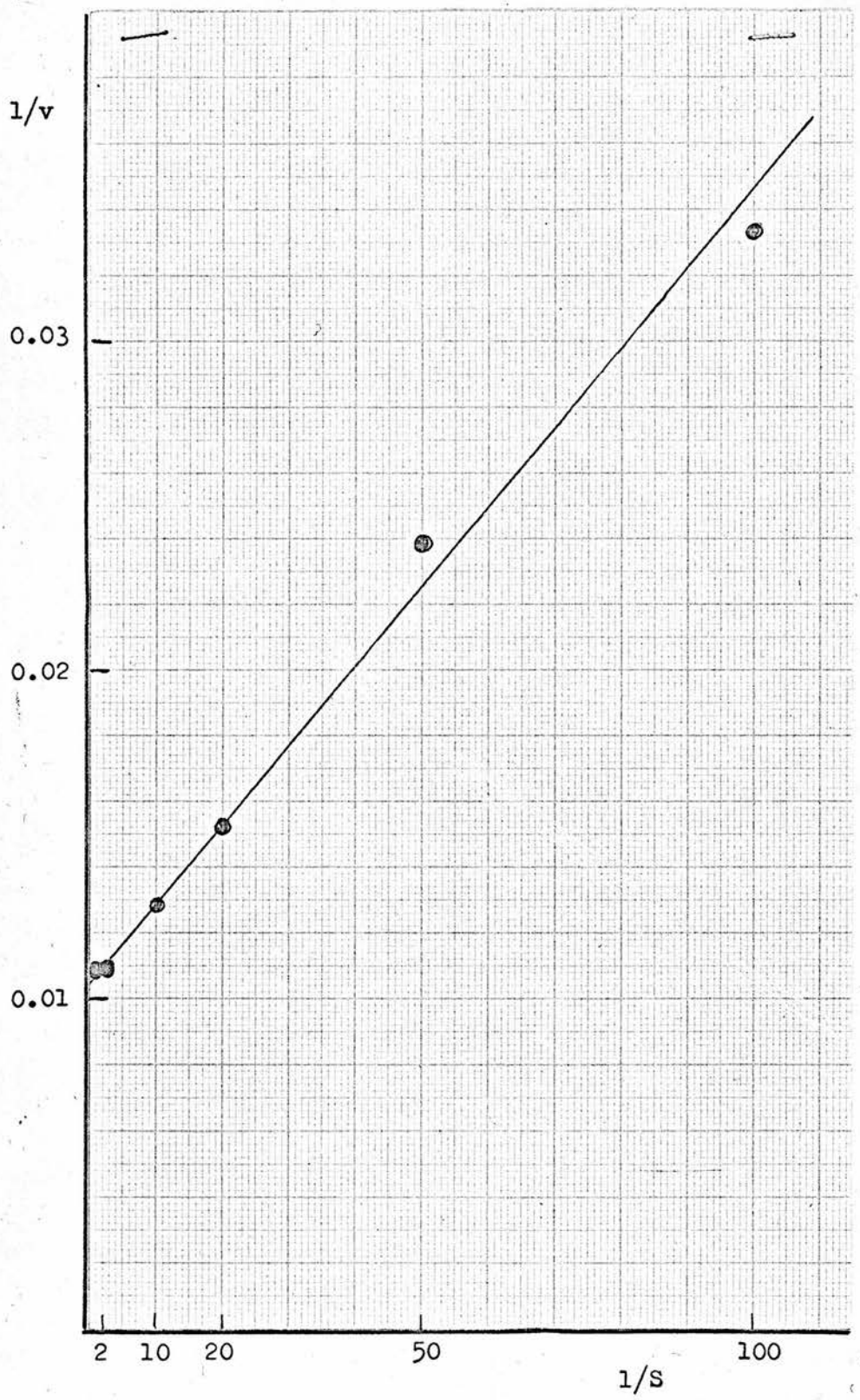


Fig. 5.2.6. Determination of K_m (putrescine) by Method III.

S is expressed in mM units, v is expressed as described in Section 5.2.2. Data are plotted according to the method of Lineweaver and Burk (1934).

Where the graph of 1/v against 1/S has an intercept on the ordinate axis equal to I/V, and the gradient is K_m/V.

When data obtained at high substrate concentrations were omitted, the experimental points fitted closely to a straight line derived by the method of least squares. The constant calculated in this way was:

$$K_m \text{histamine} = 6.25\ \mu M.$$

Method III; putrescine.

In these experiments, the concentration of putrescine was varied in assays of the following composition:

O-aminobenzaldehyde, 5 mM ; 2.0 ml.

Enzyme solution, Stage 7 ; 0.1 ml.

Phosphate buffer, 0.067 M, pH 6.8, 0.1 mM EDTA ; to 4.0 ml. final volume.

Test assays and controls without enzyme were incubated at 37°, and extinctions read after 30 min. and at 1 hr. intervals thereafter. The rate of product formation did not remain constant (cf. Fig. 2.4.1). The extinction increase after 30 min. incubation was used as an approximate measure of initial velocity, v, in Figs. 5.2.5 and 5.2.6.

Fig. 5.2.5. shows a substrate concentration optimum at about 1.0 mM. Consequently, the reciprocal plot of Lineweaver and Burk was chosen to present the data for determination of K_m by the method of least squares. Data obtained at high concentrations of putrescine were omitted from the reciprocal plot in Fig. 5.2.6. The calculated constant was; K_m putrescine = 0.023 mM.

Discussion

There have been no previous attempts to measure the Michaelis constants for the activity of placental histaminase towards any of these substrates. The present determinations were carried out primarily in order to characterise the enzyme by several constants largely independent of the purity of the preparation. The assay methods available at present are not well suited to a broader survey of the Michaelis constants for the activity of several DAOs towards many different substrates.

Methods II and III did not give the same results when applied over a similar range of putrescine concentrations. The discrepancies in substrate optimum and K_m values for putrescine measured by Methods II and III may arise from the catalytic effect of substrate, or oxidised substrate, on the second stage of the indigo test; inhibition of the enzyme-substrate reaction at high substrate concentrations might be concealed by an increased rate of indigo oxidation, but the demonstration of an optimum histamine concentration does not support this theory. The apparent consistency of the experimental data for cadaverine with Michaelis-Menten kinetics may be an argument in support of the validity of indigometric measurements, but, in principle, assay Method III is much more direct, and there was no evidence from the reciprocal plot (Fig. 5.2.6.) that this method of measuring initial velocities gave spuriously low activities at low substrate concentrations.

Using an assay method very similar to Method III, McEwen (1964) found an optimum concentration of putrescine at about 1.0 mM for pregnancy serum histaminase, originating in the placenta; this is in agreement with the putrescine concentration optimum found in the present work. Holmstedt and Tham (1959) found an optimum concentration of putrescine, about 10 mM for hog

kidney DAO which compared well with the value, 8 mM, found by Zeller et al. (1959) using manometry.

The inhibition of DAOs by excess histamine has been attributed by Kapeller-Adler and MacFarlane (1963) to irreversible compound formation between histamine and one of the postulated cofactors of histaminase, pyridoxal phosphate (Schott and Clark, 1952), but such a mechanism would not account for the oxidation of histamine at lower concentrations. It might be necessary to propose that undissociable compound formation involves functional groups different from those which take part in the reaction at the active centre of the enzyme, i.e. that histamine may approach the enzyme-pyridoxal phosphate centre in two ways, one favourable to the enzymic oxidation of histamine, and the other leading to inhibitory and irreversible compound formation between substrate and cofactor. It would be interesting to incubate the enzyme with a high concentration of histamine, remove excess histamine by dialysis, and then test the activity of the enzyme at low concentrations of histamine. If the cofactor of histaminase forms an undissociable compound at high concentrations of histamine, subsequent assays at low concentrations of histamine should be inhibited.

The simpler mechanism of histaminase inhibition by high concentrations of histamine, proposed by Zeller (1951), also takes into account a similar inhibition by high concentrations of agmatine, which does not form undissociable compounds with pyridoxal phosphate. Zeller suggested that unsymmetrical diamines, in which one basic group had a much greater affinity for the enzyme's active centre than the other, might at high concentrations become bound via the high-affinity group to only one acidic group at the active centre, whereas binding of both basic groups might be necessary for enzymic

breakdown of the substrate. This theory is similar in some respects to that proposed for the inhibition of urease by high concentrations of urea. It required fewer assumptions than the theory of Kapeller-Adler and MacFarlane (1963), but does not explain the inhibition of DAO by high concentrations of pyridoxal phosphate found by the latter authors. Possibly both mechanisms operate.

5.2.3. Determination of pH optima.

Method II. Initial velocities of indigo oxidation were measured, essentially as described in Section 5.2.2. The substrates were dissolved in distilled water and used at standard concentrations giving optimal activity at pH 6.8. The assay buffers were made by mixing M/15 solutions of potassium dihydrogen phosphate and disodium hydrogen phosphate in the proportions given by Sorensen (1909), and adding 0.1 mM EDTA. The pH of the buffers was estimated to within 0.05 units at a glass electrode. The other components of the assay were present in such low concentrations that they had no detectable effect on the pH of the assay buffers. The concentration of enzyme in the assays was adjusted for each substrate used.

In a parallel experiment, enzyme solution was replaced by 0.05 μ mole synthetic hydrogen peroxide, incubated with standard concentrations of indigo and cadaverine in the buffers of different pH, to act as a non-enzymic model for the second stage of the indigo test.

Method III. Assays were set up essentially as described in Section 5.2.2., except that the concentration of putrescine was maintained at 1.0 mM, and the pH of the buffer was varied. An approximation to initial velocity was made by measuring the extinction changes after $3\frac{1}{2}$ hr. incubation.

Fig. 5.3.1. illustrates the results of these experiments, with curves drawn through the experimental points. The upper curve was obtained by Method III, with putrescine as substrate. The lower curves represent pH optima for the action of histaminase on histamine, cadaverine and putrescine, as measured by Method II, and the pH optimum for the oxidation of indigo by synthetic hydrogen peroxide, under similar conditions.

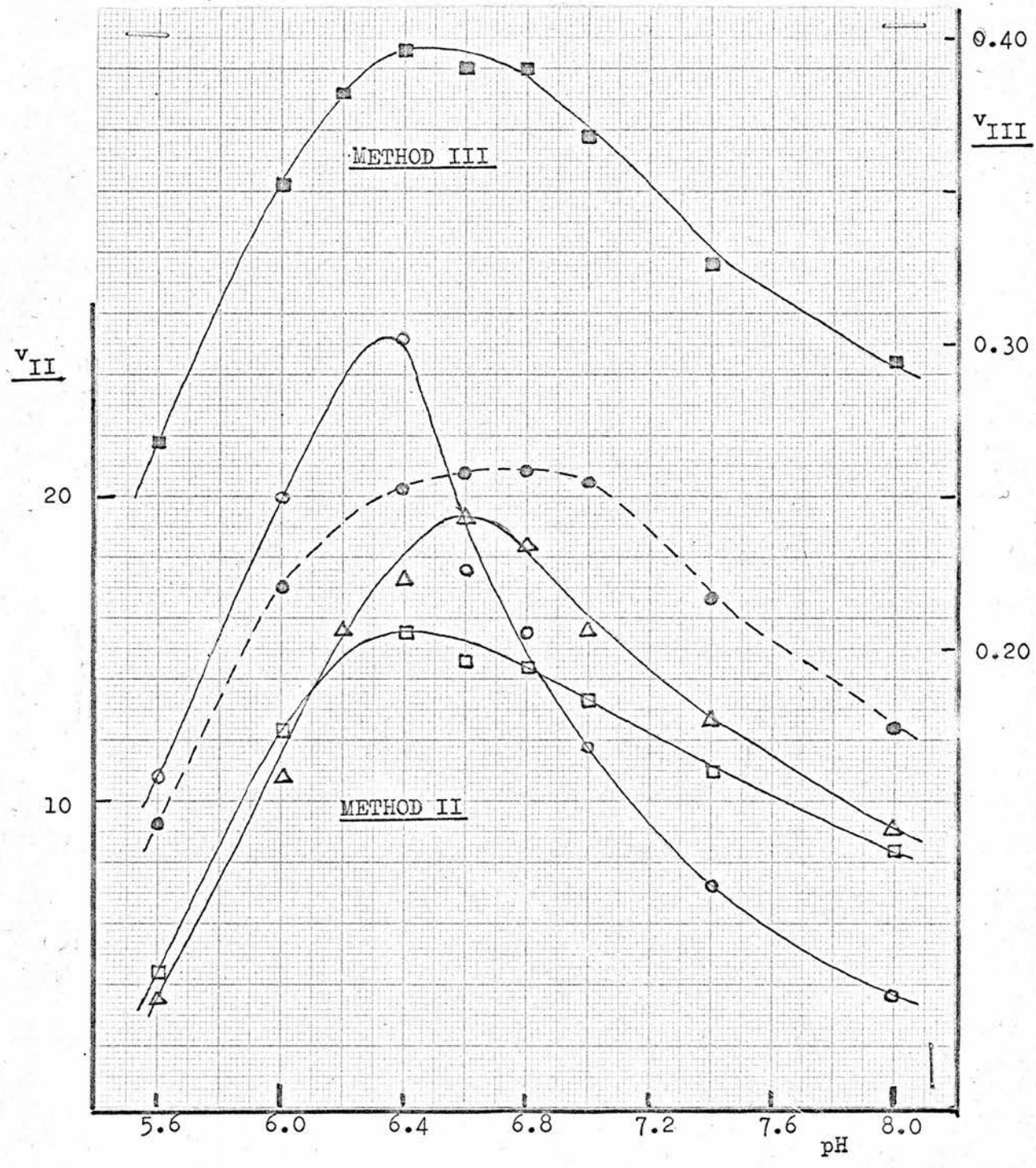


Fig. 5.3.1. pH optima of histaminase activity.

Assays were carried out in phosphate buffers of different pH, and velocities of reaction expressed as described in Section 5.2.3.

●- - ● Oxidation of indigo by H_2O_2 in presence of cadaverine.

○—○ Histaminase activity towards cadaverine (Method II)

△—△ " " " histamine "

□—□ " " " putrescine "

■—■ Histaminase activity towards putrescine (Method III)

Results obtained by Method II indicate different pH optima for histaminase activity towards histamine and the diamines. This may be due to the different dissociation constants of these substrates, and the requirement of the enzyme's active centre for amino-groups in a specific ionisation state. If metal ions activate or inhibit the reaction at the active centre, the ionisation state of EDTA in the reaction mixture might also influence the pH curve. The different shapes of pH curve for the closely homologous cadaverine and putrescine suggests that interpretation of these curves may require a precise knowledge of the geometry of the enzyme's active centre, and the conformation in which the diamines approach it.

The different pH curves cannot be explained on the grounds of dissociation constants alone, since cadaverine and putrescine have almost identical titration curves (Kenten and Mann, 1952). These authors found widely disparate pH curves for the activity of pea seedling DAO on the two diamines, and pointed out that below pH 8 both were dicationic. The reactions of histamine at the active centre are likely to be even more difficult to elucidate, since the primary amino-group on the side-chain and the imino-group in the imidazole ring have different dissociation constants, and probably different affinities for the acidic groups at the active centre. The observed pH optima are reported here as characteristic constants which may aid recognition of the enzyme in future work.

Changes in the pH of the assay medium may not only alter the velocity of the primary enzyme-substrate reaction, but may also interfere with the secondary reaction used to measure the product of the primary reaction. Since the oxidation of indigo, used to measure hydrogen peroxide production in Method II, involves several complex reactions, the validity of Method II for the determination of pH optima merits discussion.

The pH optimum found by Holmstedt and Tham (1959) for the action of hog kidney DAO was similar to that found by Best and McHenry (1930), and since formation of the yellow complex in Method III proceeds spontaneously even at pH 5(Schöpf and Oeschler, 1930), it seems unlikely that Method III is subject to limitation by the secondary reaction, at least at low pH. If Method III is assumed to be valid at all pH values, the results presented here may be used to support the validity of Method II. Synthetic hydrogen peroxide does not react in exactly the same was as "enzymic" hydrogen peroxide in the oxidation of indigo (Section 2.3.6) but it is the only model substance which can be used at present to check that the second stage of Method II is not rate-limiting under non-standard conditions of pH. The pH optimum for the oxidation of indigo by synthetic hydrogen peroxide was found to be about pH 6.8. If the second stage of Method II had limited the rate of the overall reaction, the pH optimum found by this method would have been shifted to a higher pH than the optimum found by Method III. These experiments showed that the pH optimum for putrescine found by Methods II and III were identical, but distinct from the pH optimum for the second stage of Method II. This observation is consistent with the view that the rate-limiting reaction in Method II is the primary enzyme-substrate reaction, and that initial rates of indigo oxidation accurately reflect the variation in the rate of the primary reaction with changes in pH. This view is also supported by the fact that the pH curve for the activity of histaminase towards cadaverine, obtained by Method II, is markedly different from the pH curve for the secondary reaction of Method II in the presence of cadaverine.

Summary. The following kinetic constants derived from the present study of highly purified placental histaminase, under defined assay conditions, are believed to be characteristic of a single enzyme:

Assay Method	Substrate	K_m	(S) optimum	pH optimum
Method II	Histamine	6.3 μM	0.03 mM	6.6
	Cadaverine	0.21 mM	None	6.4
	Putrescine	*	None	6.4
Method III	Putrescine	0.023 mM	1.0 mM	6.4

* See Section 5.2.2.(a).

Table 5.3.1

Method II. Standard assay components (Section 2.3.2.)

Controls contained no enzyme, but contained inhibitor and substrate.

Incubation for 24 hr. at 37°, pH 6.8.

Results expressed as percentage inhibition of activity of parallel assay without addition of any inhibitor.

Method III. Standard assay components (Section 2.4.1).

Controls contained no enzyme, but contained inhibitor and substrate.

Incubation for 24 hr. at 37°, pH 6.8.

Results expressed as in Method II.

Inhibitor	10^{-3} M		10^{-5} M		10^{-7} M	
	Method		Method		Method	
	II	III	II	III	II	III
Sodium azide	0	0	2	0	2	0
Ammonium chloride	100	0	43	0	6	0
Sodium cyanide	33	98	0	0	6	0
Aminoguanidine	100	100	100	100	63	62
Isoniazid	*44	78	24	53	0	7
Diethyldithiocarbamate (DDC)	*100	66	44	12	13	0
Semicarbazide	*100	100	56	61	2	14
Hydroxylamine	* -	100	100	100	42	40

* Controls severely affected by inhibitor.

5.3. The inhibition of placental histaminase.

The susceptibility of histaminase to some commonly used inhibitors of amine oxidases was studied to provide evidence for the classification of the enzyme, and to distinguish it from other oxidases which might have been present in the original placental extract. Zeller (1963) has recognised four main types of DAO inhibitors.

Amines (e.g. aminoguanidine)

Carbonyl reagents (e.g. cyanide, semicarbazide, hydroxylamine)

Hydrazines (e.g. isoniazid)

Chelating agents (e.g. EDTA, diethyldithiocarbamate [DDC]).

Inhibition of placental histaminase activity towards putrescine, a typical DAO substrate, was determined using assay Methods II and III. It was recognised that Method II might be subject to interference by some of the inhibitors, which might reduce indigo or hydrogen peroxide; but it was considered unlikely that the same reducing agents would be capable of interfering with the spontaneous cyclisation and condensation of Δ^1-pyrroline in Method III.

Experiments were carried out on the purest enzyme preparation obtained, Stage 7, under the standard conditions for assay Methods II and III defined in Chapter 2, except for the addition of inhibitors immediately prior to starting the reaction. Activity measurements were made over a fixed incubation period without a continual record of the progress of extinction changes. For classifying the enzyme, these semi-quantitative comparisons of the efficiency of different inhibitors ~~was~~ were considered sufficient. The results are presented in Table 5.3.1.

Using Method II, the extinction of the controls and tests was frequently reduced by reaction of indigo with the inhibitor. Where this effect was severe, the assay is marked with an asterisk. Data for hydroxylamine at

10^{-2} M are not quoted, because of the severity of indigo reduction.

Amines. The most efficient inhibition was caused by aminoguanidine, which is commonly used as a specific inhibitor of DAOs, *in vivo* and *in vitro*. Both assay methods are in good agreement, showing considerable inhibition down to 10^{-7} M. It was found several times during the present project that incomplete dialysis of histaminase solutions after ammonium sulphate precipitation resulted in severe inhibition of activity in the indigo test (Method I or II). This inhibition now appears to be due to interference by ammonium ions with indigo oxidation rather than with the enzyme-substrate reaction, which remains unaffected when measured by Method III. This is rather unexpected, since ammonia catalyses the oxidation of dianisidine by hydrogen peroxide and peroxidase (Fridovich, 1963).

Carbonyl reagents. All the carbonyl reagents used in this experiment severely inhibited histaminase activity, hydroxylamine being the most effective. Agreement between the two assay methods is excellent for semicarbazide and hydroxylamine, but cyanide appears to inhibit histaminase activity in Method III more than in Method II. It is possible that cyanide reacts more rapidly than the other carbonyl reagents with γ-amino butyraldehyde before the latter cyclises and condenses with o-aminobenzaldehyde in Method III; Method II does not depend on the estimation of aldehyde production.

Hydrazines. Isoniazid (isonicotinyl hydrazide) inhibited histaminase, but not at such low concentrations as aminoguanidine or hydroxylamine. No explanation could be offered for the greater effectiveness of the inhibitor in Method II than in Method III.

Metal-chelating agents. Histaminase activity was unaffected by sodium azide, suggesting that the enzyme does not require a heavy-metal cofactor, and that

Table 5.3.2.

Enzyme	Amino-guanidine	Carbonyl reagents	Hydra-zines	DDC.	Refs.
Histaminase (human placenta)	+	+	+	+	
DAO (hog kidney)	+	+	+	+	1.
DAO (pea seedling)	+	+	+	+	1.
MAO (mitochondria)	-	-	+	+	1.
Spermine oxidase (bovine plasma)	+	+	+	+	1, 2.
Benzylamine oxidase (human plasma)		+	+	-	3.
Benzylamine oxidase (pig plasma)	+	+	+		4.

References: 1. in Zeller, 1963.

2. Tabor, Tabor, and Rosenthal, 1954.

3. McEwen, 1965 (b).

4. Blaschko, 1962.

inhibition by cyanide is due to the reaction of the latter with a carbonyl group of the enzyme, rather than to its chelating effect. On the other hand, histaminase was inhibited by DDC down to 10^{-5} M, suggesting that copper might be involved in histaminase activity. The possible role of copper will be discussed in Section 5.4. Method II was more severely affected by DDC than was Method III, perhaps because of interference of the inhibitor, also a reducing agent, with the second stage of the indigo test.

The inhibitor specificity of some amine oxidases are compared in Table 5.3.2. with that found for placental histaminase.

Of the other amine oxidases which might have been present in the crude extract of human placentae, serum benzylamine oxidase is distinguishable from histaminase by its insensitivity to DDC, and MAO by its insensitivity to aminoguanidine and carbonyl reagents. Table 5.3.2 shows a close similarity in behaviour towards inhibitors between placental histaminase and the typical DAOs, from hog kidney and pea seedlings.

5.4. Cofactors of placental histaminase

5.4.1. Introduction.

The substances most frequently studied as possible cofactors of DAOs are pyridoxal phosphate, FAD and metals, particularly cupric ions.

Werle and Pechmann (1949) suggested that pea seedling DAO required both FAD and pyridoxal phosphate, and presented a reaction mechanism involving these co-enzymes. Werle, Trautschold and Aures (1961) later suggested, chiefly on the evidence of enzyme inhibition by chelating agents, that copper was a cofactor of plant DAO. The theory of DAO activation by cupric ions has been refined by Hill and Mann (1964); they have shown that cupric ions may catalyse the breakdown of the enzyme-substrate complex, which involves Schiff-base formation between the substrate and a carbonyl group at the active centre, possibly pyridoxal phosphate. This work implies a similarity between pea seedling DAO and the bovine serum spermine oxidase studied by Yamada and Yasunobu (1962, a, b). These latter authors found that cupric ions were required for the activity of the oxidase, but did not undergo any valency change during the reaction. Only when the oxidase was freed from copper by treatment with diethyldithiocarbamate (DDC) did it show an absorption spectrum typical of enzyme-bound pyridoxal phosphate.

The cofactor requirements of hog kidney DAO have been investigated chiefly by reactivation experiments, following attempts to split off cofactors by exhaustive dialysis or acidification (Swedin, 1943; Laskowski, Lemley and Keith, 1945; Kapeller-Adler, 1949; Kapeller-Adler and MacFarlane, 1963; Uozomi et al., 1964). Most workers have found that FAD and pyridoxal phosphate added in controlled amounts, may reactivate hog kidney DAO after such treatment. In addition, Kapeller-Adler and MacFarlane[a] presented spectral evidence for the

presence of both FAD and pyridoxal phosphate in their purified hog kidney preparation. Hill and Mann (1964), however, have pointed out that the disproportionately small amounts of FAD detected by Kapeller-Adler and MacFarlane were probably due to impurities in their preparation.

One of the original aims of this project was the identification of the cofactors of histaminase. This problem could best be approached through spectral examination of completely purified enzyme. Apart from the well-defined spectra possessed by FAD and pyridoxal phosphate in free or enzyme-bound form, the presence and absence of cupric ions may result in spectral changes which may be useful in following the kinetics of the enzyme-cofactor-substrate reaction (Hill and Mann, 1964; Yamada and Yasunobu, 1962, b). Spectral changes following the addition of substrate offer clearer evidence of the specific involvement of cofactors in enzymic activity than does the simple detection of the cofactor by spectral or chemical means, in an enzyme solution of uncertain homogeneity.

The present work failed to purify placental histaminase completely and, while it was thought probable that the only major contaminant remaining was HpMHb, the latter obscured the absorption spectrum of pyridoxal phosphate, and it was impossible to determine the proportions of histaminase and HpMHb in the final preparation. Starch gel electrophoresis did not exclude the possibility that histaminase was a minor component of even the purest preparation, and that the preparation might contain small amounts of other enzymes. It therefore seemed premature to initiate any detailed study of the cofactors of placental histaminase.

However, during the project several interesting observations were made, which might be investigated more closely when the enzyme is finally isolated.

5.4.2. Experimental observations.

The absorption spectrum of the enzyme solution was recorded between 240 and 700 mμ at all stages of the preparation. From Stages 1 to 3, the spectrum was qualitatively indistinguishable from that of haemoglobin, and from Stage 4 to Stage 7 the spectrum was dominated by peaks in the region of 280 mμ and 408 mμ, due to HpMHb.

Section 4.4.1. included a description of the separation of histaminase, in a partially inactivated form, from HpMHb by chromatography on DEAE-cellulose at pH 5.5. The fraction having highest histaminase activity showed negligible absorption at 408 mμ, and only a single broad absorption band from 240 to 400 mμ, with a maximum at about 255 mμ. This may have represented the spectrum of pure histaminase, another contaminant protein, or degraded protein.

"Copper-free" placental histaminase was prepared from Stage 6 enzyme by adding 1.0 mM DDC to the enzyme solution and removing the chelating agent, after 24 hr. at 4°, by gel filtration through Sephadex G-25. The "copper-free" enzyme solution has no detectable histaminase activity, but the only change in the absorption spectrum was due to the reduction of HpMHb to HpHb by DDC. In particular, no new absorption maximum appeared at 380 mμ, as found by Yamada and Yasunobu (1962, b) for spermine oxidase. If cupric ions are a cofactor of placental histaminase, they are therefore probably not bound to the enzyme in association with pyridoxal phosphate, as in spermine oxidase and pea seedling DAO.

The absorption spectra of histaminase solutions showed no maxima in the region of 380 mμ (suggesting pyridoxal phosphate) or of 450 mμ (suggesting FAD). Any absorption in the visible region due to histaminase must have been obscured by the spectrum of HpMHb. It is possible that histaminase was present

in such small amounts, even in the purest preparation, that its spectrum was not detectable, but even fluorimetric investigations, following the procedure used by Kapeller-Adler and MacFarlane (1963) for hog kidney DAO, failed to reveal absorption bands, other than those of HpMHb.

Inhibition by DDC and by carbonyl reagents (Section 5.3) might suggest that copper and pyridoxal phosphate were cofactors of placental histaminase, but these reagents are not specific for the proposed cofactors -- most of the inhibitors might conceivably act by reducing an important group at the enzyme's active centre. EDTA did not inhibit the enzyme. The most sensitive chemical test for copper, a colour reaction with diphenylcarbazone (Mikac-Devic, 1962) failed to detect copper in the enzyme solution, even after the removal of EDTA. Any cofactors of histaminase must be extremely tightly bound to the apoenzyme. Histaminase activity was never lost by lengthy dialysis between pH 8.6 and pH 6.8; inactivation at lower pH did not appear to be caused by the loss of specific cofactors (Section 4.4.1).

5.4.3. Conclusions

Since histaminase was not completely purified, and since even the proportion of histaminase to HpMHb in the purest preparation was unknown, it was not possible to determine whether pyridoxal phosphate and FAD were absent from histaminase preparations, masked by other components, or present in concentrations below the limits of detection by spectrophotometry. The only evidence suggesting the presence of copper or pyridoxal phosphate was the inhibition of histaminase activity by DDC and carbonyl reagents.

5.5. The molecular weight of placental histaminase.

Some information about the molecular weight of histaminase was gained from gel filtration on Sephadex G-200 (e.g. Section 4.2.6 and Section 4.4.2); histaminase and the high-M.W. HpMHb species could not be separated on this material. It is possible that histaminase was specifically bound to HpMHb, but the close agreement between the relative elution volumes found here for HpMHb, and by Killander (1964) for HpHb does not suggest that the two species were eluted as a single compound molecule. Neither gel filtration nor ultracentrifugation would separate these proteins if they were chemically bound.

If it is assumed that histaminase and HpMHb travel independently under the conditions of gel filtration, it may be concluded that the enzyme has a similar M.W. to that of HpMHb, or that the molecular size of both species lies outside the exclusion limit of the gel (about M.W. 200,000). Herman-Boussier, Moretti and Jayle (1960) calculated an average M.W. of 400,000 for Hp_{2-2} from ultracentrifugal data, and Smithies (1959) found that Hp_{2-1} had an average M.W. of 200,000. Combination with MHb would increase these M.W.s by at least 68,000, and such large molecules would probably not be fractionated on Sephadex G-200.

It was therefore considered probable that histaminase had a M.W. of at least 200,000, and that the determination of its exact M.W. must await further purification, and ultracentrifugal analysis. It is unlikely that the Sephadex matrix could be made more porous, to permit fractionation of larger molecules, without the gel becoming unmanageable in columns, but a more porous Sephadex could be used in thin layers for rapid M.W. estimations, and would permit detection of the position of the enzyme after applying a sample containing contaminants; the recovery of separated layers from the ultracentrifuge cell for identification is more difficult.

Table 5.6.1.

Treatment of enzyme	Purification Stage.	Recovery %.		
		pH 8.6	pH 6.8	pH 5.5
Dialysed 24 hr. at 4°.	4	100	100	86
	6	100	95	32
Stored 24 hr. at room temperature	4	97 (91)*	95 (80)	58 (54)
	6	98 (85)	91 (76)	33 (13)
Frozen 24 hr. at -15°.	4	100	90	76
	6	92	83	47

* Results in brackets are for solutions diluted 100-fold before storage. All other solutions contained about 1 mg. protein/ml., and were diluted immediately before assay of enzyme activity.

5.6. The stability of histaminase.

The best way of storing crude histaminase was as a suspension in 65% saturated ammonium sulphate at 4°. These suspensions lost no activity over at least six months. Exploratory tests (Section 3.4) suggested that salt precipitation was not a good method of stabilising dilute, purified solutions of the enzyme.

Crude extracts could be protected from bacterial action by the addition of chloroform, or a layer of toluene on the surface of the solution in a conical flask. Again, these preservatives were less suitable for purified solutions, since they caused some precipitation of protein. Sodium azide (0.01 mM) was preferred for the protection of purified histaminase, during prolonged operations at room temperature. Histaminase solutions in 5 mM borate buffer at pH 8.6 showed a remarkable resistance to inactivation and bacterial action. One highly purified solution containing less than 0.1 mg. protein/ml. remained perfectly clear and lost no activity after storage for more than a year in this buffer, at 4°, without aseptic precautions.

Table 5.6.1. shows the results of storing histaminase solutions at different stages of purity under different conditions of temperature and pH. The buffers used were borate/HCl, 5 mM, pH 8.6; phosphate, 20 mM, pH 6.8; phosphate, 10 mM, pH 5.5. The stability of the enzyme decreased as

(a) the pH of storage was reduced from pH 8.6.

(b) the purity of the preparation increased.

(c) the dilution increased.

At pH 6.8 or 8.6, the stability of histaminase was not greatly affected by temperature but, as found in Section 4.4.1, the enzyme deteriorated rapidly at pH 5.5 at room temperature, especially if highly purified and dilute. Recoveries

after dialysis of Stage 6 enzyme at pH 5.5 and 4° varied from about 30% to 95%; the reason for this variation was not discovered.

In contrast to hog kidney DAO which can be considerably purified by heating at 60° (Kapeller-Adler and MacFarlane, 1963; Mondovi et al., 1964), placental histaminase was rather thermolabile. When Stage 3 enzyme was heated at 60° for 20 min., 65% of its activity was lost.

Chapter 6. General discussion, and suggestions for future work.

6.1 Classification of placental histaminase as a DAO.

6.2 Comparison of placental histaminase with other DAOs.

6.3 The isolation of placental histaminase.

6.4 Other suggestions for future work.

SUMMARY.

6.1. Classification of placental histaminase as a DAO.

Table 4.1.1 showed that the H/C ratio remained virtually constant after the earliest stages of the purification of histaminase, using assay Method II throughout. Moreover, the pattern of histaminase activity in all column chromatograms was the same, using either histamine or cadaverine as substrate. Although in some early experiments activity in column effluents towards cadaverine was sometimes masked by EDTA-reversible inhibitors, no separation of a "histamine oxidase" from a "cadaverine oxidase" peak was ever achieved by any of the high-resolution chromatographic experiments described in Chapter 4.

The purest preparation of histaminase (Stage 7) oxidised both histamine and cadaverine. The enzyme preparation also metabolised histamine under the conditions of the fluorimetric assay of Shore et al. (Section 2.4.3), which shows that activity towards histamine was not an artifact of the indigo test. The indigometric assay method normally employed ensured that the metabolism of histamine was due to oxidative deamination; imidazole N-methyl transferase activity would not have been detected by this method.

No other known histamine metabolising enzyme was likely to be present in the Stage 7 preparation. Although the mitochondrial MAO of some species slowly oxidises histamine, no particulate enzyme would be expected to have survived the lengthy chromatographic purification scheme. It is possible that serum benzylamine oxidase was present in the early preparations, but even if this enzyme had survived the purification scheme, the data of Table 5.1.2 do not support the view that benzylamine oxidase was responsible for the oxidation of histamine by Stage 7 enzyme. Purified histaminase oxidised histamine much more rapidly than benzylamine, whereas McEwen (1965, b) observed that human

serum benzylamine oxidase oxidised benzylamine fifty times more rapidly than histamine. Therefore, even if the low activity of Stage 7 enzyme towards benzylamine is attributed to serum benzylamine oxidase, it must be concluded that the activity towards histamine was due to a distinct enzyme.

Finally, mixed-substrate experiments using Stage 7 enzyme and independent assay methods were consistent with the interpretation that histamine and putrescine were oxidised at the active centre of the same enzyme. It was concluded, in satisfaction of one of the major aims of this project, that histamine and the aliphatic diamines are oxidised by the same enzyme from human placenta.

The results of Section 5.1 also indicate that aliphatic diamines of longer and shorter chain length than cadaverine and putrescine are oxidised by the same enzyme, although the rate of oxidation of diaminoethane is very low. Agmatine and benzylamine are oxidised by purified histaminase at an appreciable rate, and there is considerable evidence that they compete with putrescine, a typical DAO substrate, in mixed-substrate experiments. Whether significant spermine oxidation occurs is less certain.

As it becomes possible to examine the specificities of purer enzymes with the aid of more sensitive assay methods it may become more common to report as specific substrates substances whose oxidation might earlier have been ascribed to a contaminant enzyme. Lists of substances "oxidised" and "not oxidised" by a certain enzyme are now often superseded by comparative oxidation rates, leaving the reader to assess at which lower limit a substance can no longer properly be termed a substrate. Thus, Hill and Mann (1964) regarded L-adrenaline as a substrate of pea seedling DAO, although it was oxidised 100 times more slowly than the aliphatic diamines, cadaverine and putrescine.

While the assay method used in the present work was not appropriate for the distinction of activities less than 10% of the measurable maximum, it was not considered that the slowly-oxidised substrates were of no physiological importance. On the contrary, it is conceivable that amines oxidised only slowly *in vitro* might be the substrates against which histaminase is directed *in vivo*. Determination of the specificity of placental histaminase gave no grounds for perpetuating the assumption that histaminase is exclusively related to the metabolism of histamine. General acceptance of the name "placental DAO" to replace "placental histaminase" might contribute to changing the emphasis of research on this enzyme, and would render the classification of at least one section of the amine oxidases a little less confusing.

Since the discovery of differences in the insulins produced by different mammals, it has become generally accepted that proteins performing similar hormonal and enzymic functions in different species may vary in their structural and catalytic properties. It is therefore unwise to draw firm conclusions about hog kidney histaminase solely by analogy with placental histaminase. It may, however, be relevant to suggest here a possible explanation for the discrepancies in the specificity of hog kidney DAO, as reported by different workers. Whereas the preparation of Tabor (1951) and of Mondovi et al., (1964) maintained activity towards both histamine and the diamines throughout purification, the preparations of Kapeller-Adler and MacFarlane (1963) and of Uozomi et al., (1964) progressively lost activity towards cadaverine. Neither of the latter groups of workers recovered the "lost" activity towards cadaverine in rejected fractions. A similar position was arrived at in the present work with placental histaminase, until the addition of EDTA to the buffers restored activity towards the diamines. It is possible that the hog kidney preparations of Kapeller-Adler and MacFarlane and of Uozomi et al. may have become contaminated with metallic inhibitors

not present in the preparations of Tabor and of Mondovi et al., and that the true DAO activity of the former might be restored by treatment with selective chelating agents. Tabor (1951) was perhaps the first to point out that an inhibitor might have different effects on the activity of a single enzyme towards different substrates. The discussion of EDTA-reversible inhibition of placental histaminase presented in Section 4.3.4 offers a practical example of this phenomenon and a partial explanation of its mechanism.

6.2. Comparison of placental histaminase with other diamine oxidases.

The specificity of placental histaminase for substrates and inhibitors qualitatively resembles that of hog kidney or pea seedling DAO. The placental enzyme differs considerably from the human serum benzylamine oxidase (McEwen, 1965, b) in its sensitivity to DDC and in the rates of oxidation of benzylamine and histamine, and it differs from mitochondrial MAO in its sensitivity to carbonyl reagents.

Placental histaminase is more thermolabile than hog kidney DAO, and its C/H ratio was much higher (cf. Kapeller-Adler and MacFarlane, 1963), provided that inhibitors were removed with EDTA. The pH optimum using histamine as substrate was similar to that found by Kapeller-Adler and MacFarlane for hog kidney DAO. However, the optimal concentration of histamine in the assay of placental histaminase by Method I was about 0.2 mM, and using Method II about 0.03 mM, whereas Kapeller-Adler and MacFarlane found an optimum histamine concentration of about 0.3 to 0.6 mM for hog kidney DAO, using an assay method very similar to Method I. Zeller et al., (1939) reported that the optimum histamine concentration for the manometric estimation of hog kidney histaminase was about 1.0 mM. The placental enzyme appeared to be more readily inhibited by the products of the o-aminobenzaldehyde test (Holmstedt and Tham, 1959) and the o-dianisidine test (Aarsen and Kemp, 1964), reducing the validity of these assay methods for the activity of placental histaminase. There can be little doubt that placental histaminase and the DAOs from hog kidney, etc., are different entities, although they catalyse the oxidation of similar substrates.

One difficulty in comparing the placental enzyme with other DAOs was the failure to demonstrate the involvement of any cofactors, the only evidence for the presence of copper or pyridoxal phosphate being the inhibition of the

enzyme by DDC and carbonyl reagents. Inhibition by such reagents, or the reactivation of the enzyme by an added cofactor, cannot constitute proof that a cofactor is specifically involved in the enzyme-substrate reaction. It has been shown that cupric ions and pyridoxal phosphate are involved in the reactions of pea seedling DAO, bovine serum spermine oxidase, and pig serum benzylamine oxidase, and theories of activation have been suggested by Yamada and Yasunobu (1962, b) and by Hill and Mann (1964). Determination of the identity and mode of action of the cofactors of placental histaminase might help to explain the role of metals in the activation and inhibition of the enzyme in vitro, and whether this might have any physiological significance.

6.3. The isolation of placental histaminase.

Apart from difficulties with assay methods, the purification of placental histaminase presented many interesting technical problems. Since histaminase is present in only small quantities in placenta, many organs had to be prepared together, and the chromatographic separations carried out on a very large scale. The successful operation of large columns demanded much greater care and more preliminary trials than the columns usually used for the resolution of small quantities of an enzyme. The foundations were laid for the preparation of pure histaminase in amounts sufficient for the study of its cofactors and physical structure, as well as its catalytic properties. Large-scale preparation of pure histaminase might be required in immunochemical studies, perhaps as an adjunct to the comparison of DAOs from different animal sources. Purified histaminase might also find application in the laboratory for the enzymic assay of diamines.

Stages 4 to 6 of the purification scheme, and the preliminary experiments leading to them, might be of value to other workers wishing to eliminate haemoproteins from tissue extracts. The ion-exchange techniques are particularly suitable for the removal of massive amounts of haemoproteins, whereas gel filtration is the simplest way of dealing with small amounts of these contaminants. Conversely, any future publication concerning the ion-exchange behaviour of HpMHb, or its selective degradation under mild conditions, might be applied to the still unsolved problem of separating HpMHb from histaminase.

Sections 4.2.4 and 4.4.1 demonstrated the remarkable similarity in the electrical properties of histaminase and HpMHb, as judged by ion-exchange chromatography, except at very low pH values. Sections 4.2.6 and 4.4.2 have shown that these proteins also have a very similar molecular size, as judged

by gel filtration. It is therefore unlikely that electrophoretic methods, which often combine separations by charge and molecular size, will be successful in resolving these two proteins. It might be fruitful in future work to consider the following possibilities.

(1) Elimination of HpMHb before extraction.

It is unlikely that, in vivo, any binding or non-specific adsorption of intracellular histaminase to serum Hp occurs, and association of these proteins must occur after extraction of the placentae. In principle, therefore, much would be gained by the complete removal of blood proteins prior to extraction. In the author's experience, even very lengthy desanguination of the placenta by the gravity perfusion method described in Section 4.2.1 never freed the organ completely from haemoglobin; by inference, Hp may also have been present in the most thoroughly perfused tissue. An improved blanching procedure which did not destroy or wash out intracellular histaminase might be devised. It is doubtful, however, whether the perfusion of small segments of the placenta would be more efficient than utilising the intact vascular bed.

Since Hp has a very high affinity for Hb and MHb, it is unlikely that these haemoproteins could be specifically removed from the extract at an early stage, before they could combine with Hp. New means of splitting HpMHb might be sought, but standard methods of promoting dissociation, such as heating or treatment with urea, would probably denature histaminase.

(2) Selection of placentae by Hp type.

The high M.W. fractions of Hp types 2-2 and 2-1 are numerous, and their MHb complexes may have a broad range of molecular weights, overlapping that of histaminase. However, the enzyme appears to have a higher M.W. than

the MHb complex of the smallest, 1-1, Hp, and it should be possible to separate this single HpMHb species by gel filtration.

Since only about 20% of the population possess type 1-1 Hp (Riding, 1965) the selection of placentae from women with this Hp type would be a difficult task. Hp typing could not await delivery of the placenta, which must be blanched immediately, but would have to be carried out beforehand, preferably on all women attending the ante-natal clinic of a large maternity hospital. Pre-typing, and collection of placentae from suitable subjects immediately after delivery, would demand considerable organisation, and might yield about two placentae per 24 hr.

(3) Ion-exchange chromatography at low pH.

Of the ion-exchange procedures described in Section 4.2.1., the only method resulting in appreciable resolution of histaminase from HpMHb was chromatography on DEAE-cellulose at pH 5.5. This method failed as a preparative technique because of the low capacity of the adsorbent for both proteins, and, more important, extensive and irreversible denaturation of the enzyme at low pH and room temperature. Clearly, if histaminase could be kept stable at pH 5.5, chromatography on DEAE-cellulose would offer the best means of finally purifying the enzyme. Unfortunately, substances which might be thought to increase the stability of histaminase might also be expected to interfere with chromatography. The addition of ammonium sulphate would result in an increase in ionic strength which might further decrease the adsorption of proteins, or even cause precipitation on the column. Non-electrolytes, e.g. sucrose, also appear to interfere with chromatographic separations.

Chromatography at a low temperature might be more successful in preserving activity, since histaminase survived dialysis at pH 5.5 in the

refrigerator. Cold-room facilities, and a large column of adsorbent to counterbalance reduced exchange efficiency, would be required. There remains the risk of enzyme denaturation at the large solid-liquid interface provided by the adsorbent.

Stability of histaminase might be improved by carrying out the pH 5.5 chromatography at an early stage of the purification. The benefits of protection by other proteins would possibly be cancelled by poor resolution, due to the low protein capacity of the anion exchanger at low pH, and to non-specific adsorption of histaminase to bulk contaminants.

6.4. Other suggestions for future work.

Chapter 1 described in some detail the failure of many attempts to show that histaminase has a necessary role in the metabolism of histamine. Administration of small doses of a histaminase inhibitor, aminoguanidine, (Bjüro et al., 1964) did not appear to harm pregnant women, but the design of this experiment did not justify the conclusion that placental histaminase is dispensable. If repetition of such inhibition experiments could be justified, it would be interesting to assess the effect of injected aminoguanidine, in increased doses, on the serum concentration of histamine and the diamines. The production of aliphatic diamines by the foetus and by pregnant women might be compared with that of normal subjects; histaminase is the only known enzyme capable of metabolising the C_3 - C_6 aliphatic diamines at a considerable rate, whereas the mother is adequately protected from foetal histamine by imidazole N-methyl transferase.

Although there is considerable evidence that histamine metabolism in pregnancy differs considerably between species, little is known about the functions of the diamines. Since serum histaminase is elevated only in human pregnancy, it is unlikely that experiments with other mammalian DAOs will throw much light on the functions of the enzyme and of diamines in pregnancy. The study of the DAO of human kidney and intestine might serve as a useful link between animal and human experiments, and between experiments on pregnant and non-pregnant subjects. It is rather surprising that so little is known about this other human DAO, particularly whether it is catalytically and structurally identical with placental histaminase. Current interest in isoenzymes might stimulate comparative study of these DAOs, particularly if the role of the placental enzyme and its substrates in toxaemias of pregnancy was clarified.

The chief obstacle to further progress in determining the mode of action and comparative properties of the DAOs, apart from the difficulty of purifying the enzymes, is the inadequacy of existing assay methods applicable to all substrates. The need for a better assay method may become apparent only as higher purity is achieved, and the enzyme becomes more susceptible to inhibition by products of the assay reaction. One lesson learned from the present project was that, when the problems of following enzyme purification and the simultaneous need for a better assay method threaten to become interdependent, the best solution is to make do temporarily with an unsatisfactory assay until enzyme preparations of a wide range of purity are available, then to assess the value of new assay methods at all stages of the purification. It is possible, however, that an assay method may cease to function altogether when a vital contaminant is removed by chromatography, or an unsuspected inhibitor is added with the reagents. A somewhat similar problem in this work was that alternative assay methods (those of Holmstedt and Tham (1959) and of Aarsen and Kemp (1964)) developed for hog kidney DAO were not completely valid for placental histaminase. Now that the latter has been added to the list of amine oxidases available in a highly purified state, more progress may be made towards the development of a new assay method for all these enzymes, measuring a common product of oxidative deamination — ammonia or hydrogen peroxide.

When dealing with placental preparations, hydrogen peroxide is not the best metabolite to measure, for reasons discussed in Section 2.1.5. Manometric assays also depend on the quantitative liberation of oxygen from hydrogen peroxide, and are subject to the same objections, besides the problems of secondary oxygen uptake. It is now clear that the complex mechanism of

indigo oxidation in the indigo test makes it unsuitable for further development. The same difficulties might arise from the use of other dyes to detect hydrogen peroxide, but the extreme sensitivity of the hydrogen peroxide assay of Keston and Brandt (1965) would justify serious attempts to apply it to the assay of oxidative deamination.

The proposal of Zeller (1963) to use α-oxo-glutaric acid, glutamate dehydrogenase and NADPH to detect ammonia production should find wide application in the comparative study of amine oxidases from different sources, using many different substrates. It may prove difficult, however, to study the effects of inhibitors or pH variation in this way, since the changes in the assay medium may affect not only the amine oxidases, but also the secondary enzymic detection system. Determination of reaction velocities at different pH values and in the presence of inhibitors requires a direct method of measuring changes in the concentration of a substrate or product. McEwen (1965b) has carried out very interesting kinetic studies on serum benzylamine oxidase, using U.V. spectrophotometry to measure benzaldehyde formation. Of the available methods of measuring histaminase activity, the method of Holmstedt and Tham approaches this ideal most closely; if the difficulty of maintaining initial velocity cannot be overcome by using other substrates or by protecting the enzyme in some way, the early linear extinction increases might be magnified with the aid of a recorder external to the spectrophotometer. Since benzylamine is slowly oxidised by placental histaminase, it might be possible to find some related aromatic amine, perhaps with a second aliphatic amine substituent, which is more rapidly oxidised than benzylamine, but whose aldehyde has a molar extinction coefficient comparable with that of benzaldehyde; unfortunately, the kinetics of oxidation of such a substrate might be different from that of the simple aliphatic diamines and histamine.

The development most likely to facilitate research on diamine oxidases would be a simple and sensitive test for ammonia, unaffected by pH changes and by common inhibitors, and preferably suitable for continuous recording of the progress of the primary enzyme reaction. In the continued absence of an adequate assay method for kinetic work, it is likely that reaction of the DAOs with their substrates and inhibitors will be poorly understood, and the significance of these enzymes in human physiology may remain unexplained.

SUMMARY

Saline extracts were made from desanguinated human placentae, and histaminase was purified from these extracts by salt fractionation, ion-exchange chromatography on cellulosic adsorbents, and by gel filtration on Sephadex G-200. The Specific Activity of the preparation increased approximately 800-fold from Stage 1 to Stage 6, part of this increase being due to the removal of enzyme inhibitors. Further attempts to resolve Stage 6 enzyme by ion-exchange chromatography and re-cycling gel filtration removed some of the remaining contaminants, but did not result in further increases in Specific Activity. Starch gel electrophoresis of the purest preparation revealed that the enzyme had not been separated from high-molecular weight haptoglobin-methaemoglobin species.

A new spectrophotometric test for histaminase was developed from Kapeller-Adler's (1951) indigo test, which proved unsuitable for the present work. The new test was about 50 times more sensitive than any previous method measuring a common product of oxidative deamination of all substrates. Extinction changes after 24 hr. incubation were linearly related to enzyme concentration. Zeller's criticisms of indigo tests (1956, 1965) could not be substantiated. The new indigo test gave very similar results to the method of Holmstedt and Tham (1959) in almost all applications.

Although the oxidation of substrate and the production of hydrogen peroxide proceeded immediately, indigometric assays were subject to an initial delay in indigo oxidation, lasting several hours. The oxidation of indigo by synthetic hydrogen peroxide was not subject to any delay; the mechanisms of indigo oxidation by synthetic hydrogen peroxide and by hydrogen peroxide produced in the enzyme-substrate reaction were not the same. More than the

stoichiometric amount of indigo was oxidised in tests using synthetic or enzymic hydrogen peroxide, due to oxidation by atmospheric oxygen, catalysed by substrates and products of the indigo test. No way was found to eliminate, or correct results for, hyper-stoichiometric indigo oxidation, and therefore rates of oxidation of indigo could not be translated into conventional expressions of enzymic activity.

The specificity of the purest preparations of placental histaminase was found to resemble that of hog kidney and pea seedling DAO. With supporting evidence from mixed-substrate experiments, it was concluded that histaminase oxidised not only the C_3 - C_6 aliphatic diamines, but also agmatine, benzylamine and histamine. Activity towards histamine could not have been due to any known contaminant.

The Michaelis constants for the oxidation of several substrates by placental histaminase were determined. K_m for putrescine, and the appearance of an optimal substrate concentration, varied with the assay method employed. The histamine concentration giving optimal activity was lower than that for hog kidney DAO. The pH optima for the activity of placental histaminase towards several substrates were determined.

The inhibitor specificity of placental histaminase resembled that of hog kidney and pea seedling DAO, but was distinct from that of benzylamine oxidase or monoamine oxidase (MAO).

Activity towards the aliphatic diamines, but not towards histamine, tended to diminish as the enzyme was purified, unless EDTA was added to the working buffers. The possible significance of metal ions in the activation and inhibition of histaminase was discussed, and it was suggested that discrepancies in earlier reports on the specificity of hog kidney DAO might be due to contamination of enzyme solutions by metal ions.

Contamination of the purest preparation by other proteins precluded detailed study of the cofactors of placental histaminase, but the possible involvement of copper and pyridoxal phosphate was discussed. The molecular weight of histaminase was probably at least 200,000. The enzyme was much more thermolabile than hog kidney DAO, but it was extremely stable at low temperatures in dilute borate buffer, pH 8.6.

Priorities for future work were considered to be:

(a) Development of a satisfactory assay method for oxidative deamination.

(b) Separation of histaminase from haptoglobin-methaemoglobin.

(c) Study of the metabolism of aliphatic diamines in normal and pregnant human subjects.

Appendix 1. Materials.

Unless otherwise stated, inorganic reagents used in the present work were of Analar quality. Organic reagents were the purest commercially available. The sources of some important reagents are listed below.

Reagent	Grade	Supplier
Indigo disulphonate	Analar	BDH
Histamine 2 HCl	Biochemical	BDH
Agmatine sulphate	80% pure	Sigma
Benzylamine. 2 HCl	Laboratory	BDH
Spermine. 4 HCl	Unspecified	Sigma
Diaminoethane. 2 HCl	Laboratory	BDH
Diaminopropane. 2 HCl	Chromatographically pure	Calbiochem.
Diaminobutane. 2 HCl (putrescine)	Crystalline	Sigma or Roche
Diaminopentane. 2 HCl (cadaverine)	Crystalline	Sigma or Roche
Diaminohexane. 2 HCl	Laboratory	Eastman-Kodak
Isatin	Analar	BDH
Aminoguanidine. $2H_2SO_4$	Laboratory	BDH
Semicarbazide. HCl	Analar	BDH
Hydroxylamine. HCl	Analar	BDH
Isoniazid	Unspecified	Mann
Phenylhydrazine. HCl	Analar	BDH
o,o'-dianisidine. 2 HCl	Specially purified	BDH
o-aminobenzaldehyde	Unspecified	Sigma
o-phthaldialdehyde	Unspecified	Sigma

Neuraminidase	Crystalline	Sigma
FAD	90 - 99% pure	Sigma
Pyridoxal phosphate	98 -100% pure	Sigma
Liver concentrate	—	Sigma
Yeast concentrate	—	Sigma

Catalase-free Hb and HpMHb

Hb and HpMHb, free from erythrocyte catalase, had to be prepared for experiments with the indigo test, reported in Chapter 2. About 1 ml. of sedimented erythrocytes was mixed with distilled water and frozen, to cause cell lysis. The thawed extract was centrifuged, and the supernatant diluted five-fold, and Hb was freed from catalase by gel filtration through Sephadex G-200. Catalase (M.W. 200,000) emerged in the first peak, and Hb in the third. Effluents containing the highest Hb concentration were used as crude, catalase-free Hb.

A little of this preparation was added to 5 ml. of non-haemolysed serum, and 50 mg. potassium ferricyanide and the mixture subjected to gel filtration on G-200. The first effluent peak contained HpMHb and the other high-M.W. serum proteins. Simple gel filtration of haemolysed serum would have resulted in contamination of the HpMHb peak with catalase, which has a similar M.W.

Glassware, etc.

Pyrex glassware was used wherever possible, and contact of histaminase solutions with materials other than glass or plastic vessels was avoided. It was necessary to use rubber bungs in assay tubes; these were rinsed thoroughly and boiled before use, and cleaned occasionally in detergent solution. To safeguard against erratic results, special attention had to be paid to the cleanliness of test-tubes used in Assay Method II (sensitive spectrophotometric indigo test). After each use, these tubes were soaked in Pyroneg detergent, rinsed twice in warm water, immersed overnight in chromic acid cleaning solution, and washed five times in tap water and twice in distilled water. Other glassware was washed in detergent or chromic acid solution, rinsed in tap and distilled water, and dried in an air oven at 90°. Volumetric ware, including pipettes, was dried with the aid of acetone.

Sephadex and anion exchange adsorbents, which cling stubbornly to glass, had to be removed from the walls of chromatographic columns by filling the latter with chromic acid solution. Plastic vessels and tubing which could not safely be treated with acid were washed in many changes of tap and distilled water, before being rinsed with the buffer solution to be used next.

Visking dialysis tubing was soaked in 5 mM borate buffer, pH 8.6, containing 0.1 mM EDTA, to remove enzyme inhibitors. Before use, the tubing was rinsed in several changes of distilled water, and buffer.

Glass-distilled water, in which all reagents were prepared, was obtained from a Scorah still, or a Loughborough glass still with a chromium-plated element.

Appendix 2. Key to purification stages and assay methods.

Stage No.	Description
1	Crude saline extract.
2	1st ammonium sulphate ppt^{n}.
3	2nd ammonium sulphate ppt^{n}.
4	After DEAE-cellulose chrom.
5	After cellulose-phosphate chrom.
6	After gel filtration on G-200.
7	After re-cycling gel filtration.

Assay method	Description	Std. conditions
I	Kapeller-Adler's (1951) indigo test.	2.2.2
II	New spectrophotometric indigo test.	2.3.2
III	Holmstedt and Tham's test	2.4.1

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