inhibitors, there is little doubt that it would be great. It is thus possible that with a suitable choice of inhibitors the I_{50} ratio method could be used to establish, first, whether the whole of the activity towards one substrate is identical with the activity towards another and, secondly, whether any part of it is.

So far, a purified preparation of the DFP-insensitive enzyme has not been obtained, and it is thus impossible to be certain of its exact specificity. It seems unlikely, however, that it can contribute to the ACh hydrolysis of plasma in view of the total inhibition produced in the unpurified preparation by very low DFP concentrations.

SUMMARY

1. Using human plasma as a source of a single non-specific esterase and the cholinesterase inhibitors di-*iso*propyl fluorophosphonate (DFP) and di-(2-chloroethyl)methylamine hydrochloride (DDM), a method is described for characterizing the enzymic activity of the unpurified plasma towards representative choline and non-choline esters. This involves the determination for each substrate of the ratio of the concentrations of each inhibitor which produce 50 % inhibition of hydrolysis (the DDM : DFP I_{50} ratio). The method enables the most probable value of the ratio and the range of probable values to be estimated.

2. The values for the I_{50} ratio for the different esters are closely similar, ranging from 2·28 to $2{\cdot}44 \times 10^5$.

3. The limitations of the method, and its possible wider application, are discussed.

4. Evidence has been obtained for the existence in human plasma of a small amount of a second enzyme, DFP- and DDM-insensitive, which accounts for 5–20 % (depending on the substrate) of the aliphatic esterase activity of the plasma, and which also hydrolyzes triolein.

5. When the I_{50} ratios for tributyrin and triacetin are corrected for the contribution of the DFP-insensitive esterase they become identical with the value ($2{\cdot}28 \times 10^5$) for acetylcholine.

We are particularly indebted to Dr R. B. Fisher for his advice and helpful criticism, especially in connexion with the statistical aspect of our work. We are also grateful to Prof. R. A. Peters and Prof. R. H. S. Thompson for their interest. One of us (D.H.A.) wishes to thank the Department of Scientific and Industrial Research for a grant.

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Studies on Histaminase*

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In a number of papers Zeller and his co-workers (Zeller, 1938*a*, *b*; Zeller, Birkhauser, Mislin & Wenk, 1939; Zeller, Stern & Wenk, 1940) have advanced evidence that histaminase acts on various diamines such as cadaverine, putrescine, agmatine and spermine, and have suggested that this enzyme should be called diaminoxidase. It has, however, been found that the enzyme in placental extracts, and in the serum during pre-eclamptic toxaemia, differs from the enzyme in the serum during pregnancy in having much less effect on histamine compared with cadaverine (Kapeller-Adler, 1944). This suggested that there might after all be two enzymes and the question has, therefore, been reinvestigated by studying the effects of various conditions on a purified preparation of the enzyme.

* Read in part before the Biochemical Society on 19 July 1946 (Kapeller-Adler, 1946).

METHODS

Purification of histaminase. Hog kidneys, as fresh as possible, were obtained from a slaughter house, the medullas were removed and rejected. The cortex of about 24 kidneys was minced to a very fine cream, cooled in the refrigerator and poured into 4 vol. of cooled acetone. The suspension was rapidly filtered with suction, the residue washed several times with cold acetone and dried in air on large sheets of filter paper. The dry acetone powder was then extracted with 10 ml./g. of 1% (w/v) NaCl and heated with stirring to 62°. It was filtered through Buchner funnels, with frequent changes of the filter paper, and the precipitate was rejected. This crude extract was analyzed for protein and tested for histaminase activity. Protein was determined by the method of Robinson & Hodgen (1940) and the enzyme activity was tested manometrically. The activity of histaminase was expressed in arbitrary units adopted by Laskowski (1942), one unit being equivalent to the amount of enzyme which under the experimental conditions (total vol. 3 ml.; 1 mg. histamine hydrochloride as substrate; pH 7·2; 38°; air) utilizes 1 μl. O_2/min. It roughly corresponds to the destruction of 1 mg. of histamine hydrochloride/hr. The degree of purification of the enzyme was expressed as the number of units/mg. of protein.

The crude extract contained 0·05 Laskowski units/mg. of protein. It was then treated with solid $(NH_4)_2SO_4$ using 380 g. of the salt/l. of filtrate to obtain 0·5 saturation. The yellow-brown precipitate was collected and the filtrate discarded. The precipitate was dissolved in 1000 ml. of distilled water, and the solution treated with 380 g. of solid $(NH_4)_2SO_4$ to obtain 0·5 saturation again. The precipitate was redissolved in 300 ml. of distilled water, and the solution again analyzed for protein and histaminase activity. At this stage the average preparation contained 0·3 units/mg. of protein. The enzyme was then adsorbed on $Ca_3(PO_4)_2$ gel, prepared by mixing 700 ml. of 0·4 M-Na_3PO_4 with an equal amount of 0·6 M-$CaCl_2$, adjusting the mixture to pH 7·2, and washing the precipitate formed on a Buchner funnel several times with distilled water. The filtrate did not as a rule show any enzymic activity. The adsorbed enzyme was further purified by elution with 1000 ml. of phosphate buffer pH 7·2. This eluate showed hardly any activity and was discarded. The adsorbate was then eluted with 1000 ml. of phosphate buffer containing 228 g. $(NH_4)_2SO_4$ (0·3 saturation), and this procedure was repeated with another 1000 ml. of the eluting agent. The combined eluates were precipitated with 380 g. $(NH_4)_2SO_4$ to make 0·55 saturation. The precipitate was dissolved in 100 ml. of distilled water, centrifuged and the clear deep-brown solution treated with 45·6 g. $(NH_4)_2SO_4$ (0·6 saturation). The enzyme was redissolved in a small amount of water, analyzed for protein and tested for activity. At this final stage the average degree of purification was 1·3 Laskowski units/mg. of protein. Laskowski's histaminase unit is said to be 5·4 times larger than the unit described by Zeller (1942), about 10 times larger than the unit proposed by Stephenson (1943) and approximately 24 times larger than the 'Torantil' unit of the Winthrop Chemical Company, one Winthrop unit being the amount of histaminase which will destroy 1 mg. of histamine in 24 hr. (Laskowski, 1945). The degree of purification attained compares well with that obtained by Laskowski whose purified preparation contained on the average 1·5 Laskowski units/mg. of protein. Taking into account that the crude extract showed an activity of 0·05 Laskowski units/mg. of protein the average degree of concentration achieved by the described procedure amounts to 26:1. Stephenson (1943) has obtained a preparation 20–33 times more active than the original hog kidney. Swedin's (1943, 1944) purified histaminase preparation showed a 35 times stronger effect than the pig's kidney. By electrophoresis a further purification up to 124 times of the original material was achieved. The purified enzyme preparation consumed, when tested with histamine as substrate, 0·5 mol. O_2/molecule of histamine, which finding is in agreement with Kiese (1940), Zeller (1942), Laskowski (1942), Swedin (1943, 1944) and Stephenson (1943).

RESULTS

Competition between substrates. In confirmation of the results of Zeller (1938*a*) it has been found that, when the enzyme acts simultaneously on histamine and cadaverine or putrescine or agmatine, the oxygen consumption is less than the sum of the oxygen consumptions when it acts on the two substrates separately. It has also been shown by means of pharmacological assays that the addition of cadaverine inhibits the destruction of histamine.

In these experiments an extract of the purified enzyme was incubated with various substrates in Warburg vessels for 30 min. at 37°. The reaction was then stopped by adding an equal amount of 10% trichloroacetic acid (3 ml.). After filtration, this acid was removed with ether, the aqueous solution neutralized with a few drops of 2 N-NaOH and made up with 0·9% NaCl to 250 ml. The histamine in this solution was then estimated in comparison with a known histamine solution by its action on guinea pig's ileum (Barsoum & Gaddum, 1935). In one such experiment the first vessel originally contained 1 mg. of histamine hydrochloride and this appeared to be completely destroyed; the second vessel contained 20 mg. of cadaverine hydrochloride and the extract from this vessel also had no action on the ileum. The third vessel contained 1 mg. of histamine hydrochloride and 20 mg. of cadaverine hydrochloride, and the test indicated that only 50% of the histamine had been inactivated.

Effect of inhibitors. In agreement with Zeller (1942) it was found that the effect of histaminase preparations on histamine, cadaverine or putrescine was more or less strongly inhibited by hydroxylamine, semicarbazide, sodium azide, potassium cyanide and aneurin hydrochloride. Enzyme preparations inhibited by various inactivators were dialyzed in order to find out whether the inhibition was reversible. After dialysis of histaminase preparations inactivated by 0·01 M-hydroxylamine hydrochloride full activity was regained with cadaverine and putrescine as substrates but only 20% of the original activity was restored with histamine as substrate. The activity was furthermore completely restored on dialysis of the enzyme preparation, which had been inhibited by 0·01 M-sodium azide, when tested with cadaverine and putrescine

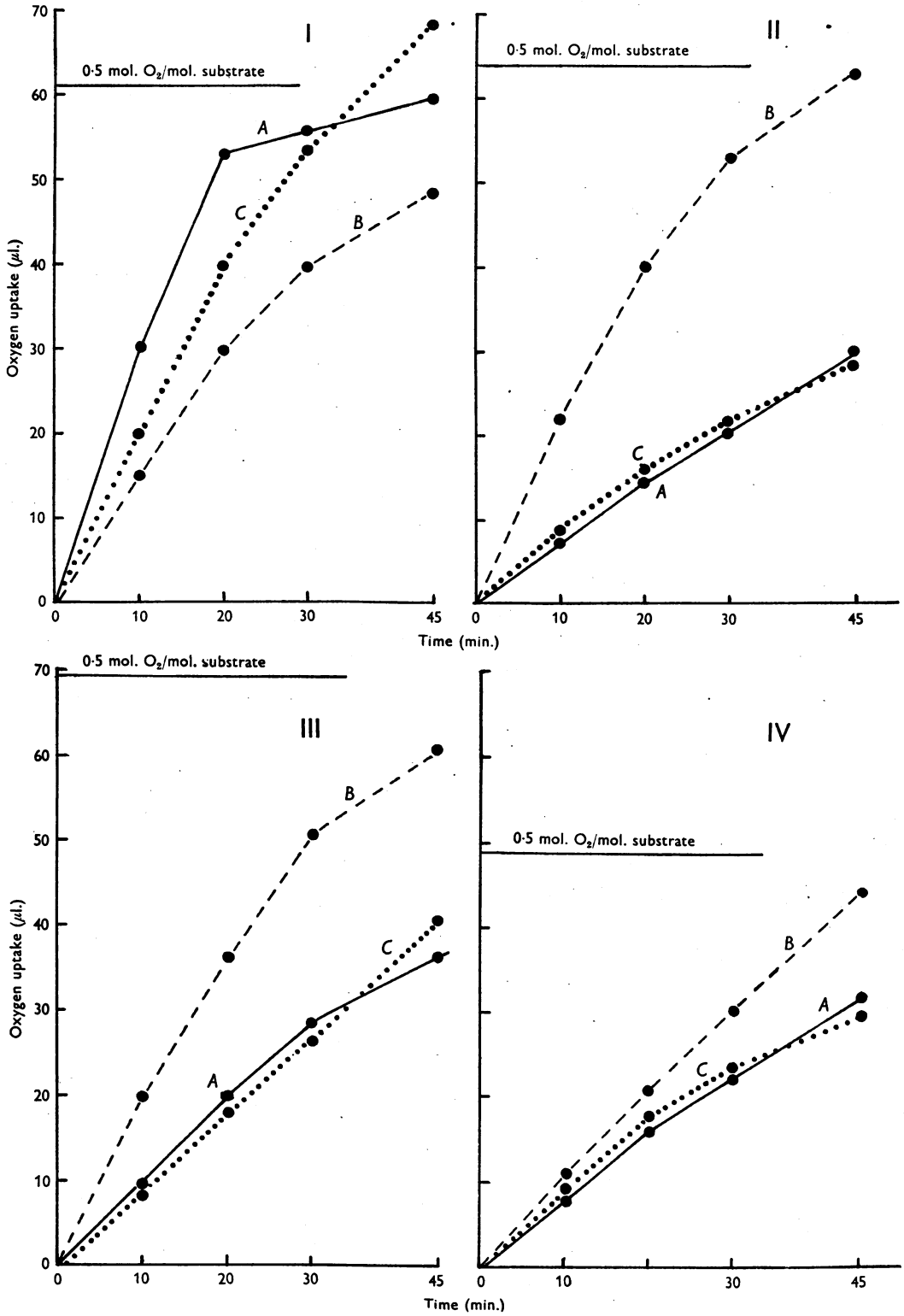

Fig. 1. Activity of histaminase preparation (2·3 mg. protein). *A*, before dialysis; *B*, after dialysis; *C*, after dialysis and addition of yellow extract from histaminase. I, 1 mg. histamine hydrochloride; II, 1 mg. cadaverine hydrochloride; III, 1 mg. putrescine hydrochloride; IV, 1 mg. agmatine sulphate as substrates. pH 7·2; temp. = 38°; air.

as substrates and 80% restored with histamine. Similarly, after inactivation with 0·01 M-cyanide, full enzymic activity was regained by dialysis with cadaverine and putrescine as substrates, but only 40% reactivation was found with histamine. The inactivation of histaminase by semicarbazide was irreversible.

Effects of dialysis. In view of these results it seemed desirable to study the effect of dialysis of purified enzyme solutions on their activity towards histamine and the diamines. Swedin (1944) found that the effect of histaminase on histamine was diminished by dialysis and restored by the addition of a methanol extract of the enzyme. In the present experiments similar results were obtained with histamine, but other substrates have been found to give quite different results. Fig. 1 represents typical results obtained after prolonged dialysis (24–72 hr. against tap water or against distilled water in the cold). It shows that after dialysis the action of the enzyme on histamine is markedly decreased, but that its action on cadaverine, putrescine or agmatine is increased. It was observed that the deep yellow colour of histaminase solutions bleached on dialysis. This suggested that the histaminase might be a chromoprotein and that part of its prosthetic group had been split off during dialysis. Zeller *et al.* (1940) and Swedin (1943, 1944) have in fact suggested that histaminase is a flavoprotein, but the flavin group has not been identified. This problem has been studied by extracting the yellow pigment with acetone.

A purified histaminase preparation was dissolved in water and an equal amount of acetone added. The mixture was heated to 38° for 30 min., the precipitate filtered off and the yellow filtrate concentrated under reduced pressure. The yellow residue was purified with *p*-cresol (Warburg & Christian, 1938) and finally taken up in phosphate buffer pH 7·2. This intensely yellow extract showed greenish fluorescence, and on addition to the dialyzed enzyme reversed more or less completely both the effects of dialysis (Fig. 1). On irradiation in alkaline solution this extract entirely lost its catalytic effects. The yellow colour of the extract was bleached on addition of $Na_2S_2O_4$ and was restored again on shaking the mixture with air. These facts suggest the presence of a flavin group. The active substance in these acetone extracts has been identified as flavin-adenine-dinucleotide (*FAD*) by the following experiments. A preparation of *FAD* was made from yeast (Warburg & Christian, 1938). A purified preparation of D-amino-acid oxidase was obtained and the prosthetic group, which is known to be *FAD*, was split off without denaturing the protein (Warburg & Christian, 1938). This protein had little effect by itself on DL-alanine (Fig. 2), but its activity was greatly increased when the enzyme was reconstituted by adding either the *FAD* from the enzyme itself, or the *FAD* from yeast or the yellow acetone extract from histaminase. This result which was confirmed with a second preparation of D-amino-acid oxidase shows that the purified preparation of histaminase did contain *FAD*. The results in Fig. 3 show that a known preparation of *FAD* obtained from D-amino-acid oxidase reproduced all the observed effects of the acetone extract of histaminase. When it was added to dialyzed histaminase it reversed, either partly or completely, the observed effects of dialysis with all four substrates. It increased the O_2 consumption when histamine was substrate and diminished it when the three other substrates were used. Riboflavin had no action at all in similar experiments.

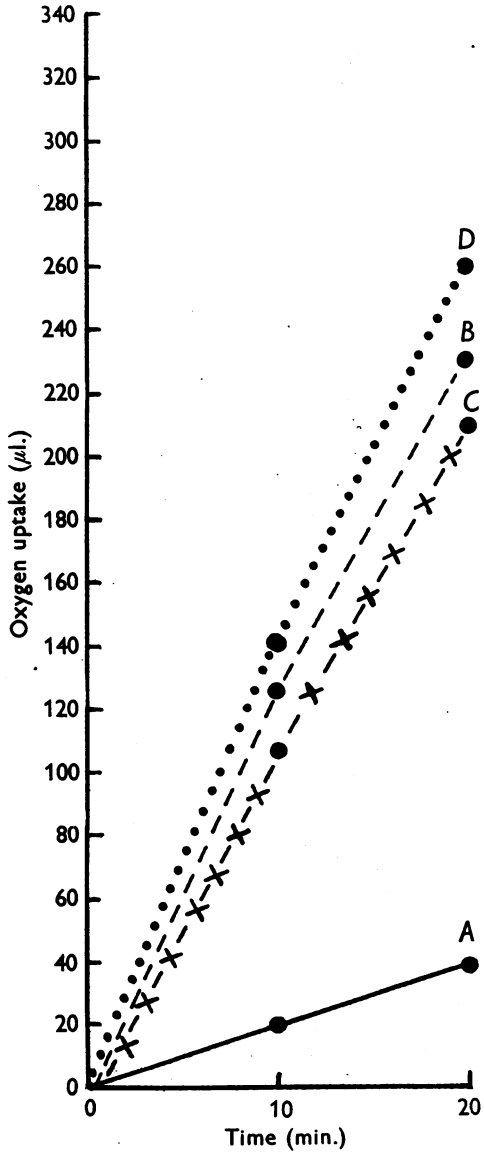

Fig. 2. Reactivation of split D-amino-acid oxidase. *A*, protein part of split preparation (1·6 mg. protein); *B*, +*FAD* from D-amino-acid oxidase; *C*, +*FAD* from yeast; *D*, +yellow extract from histaminase. Substrate: 0·2 ml. 4·5% (w/v) DL-alanine solution. pH 7·2; temp. = 38°; air.

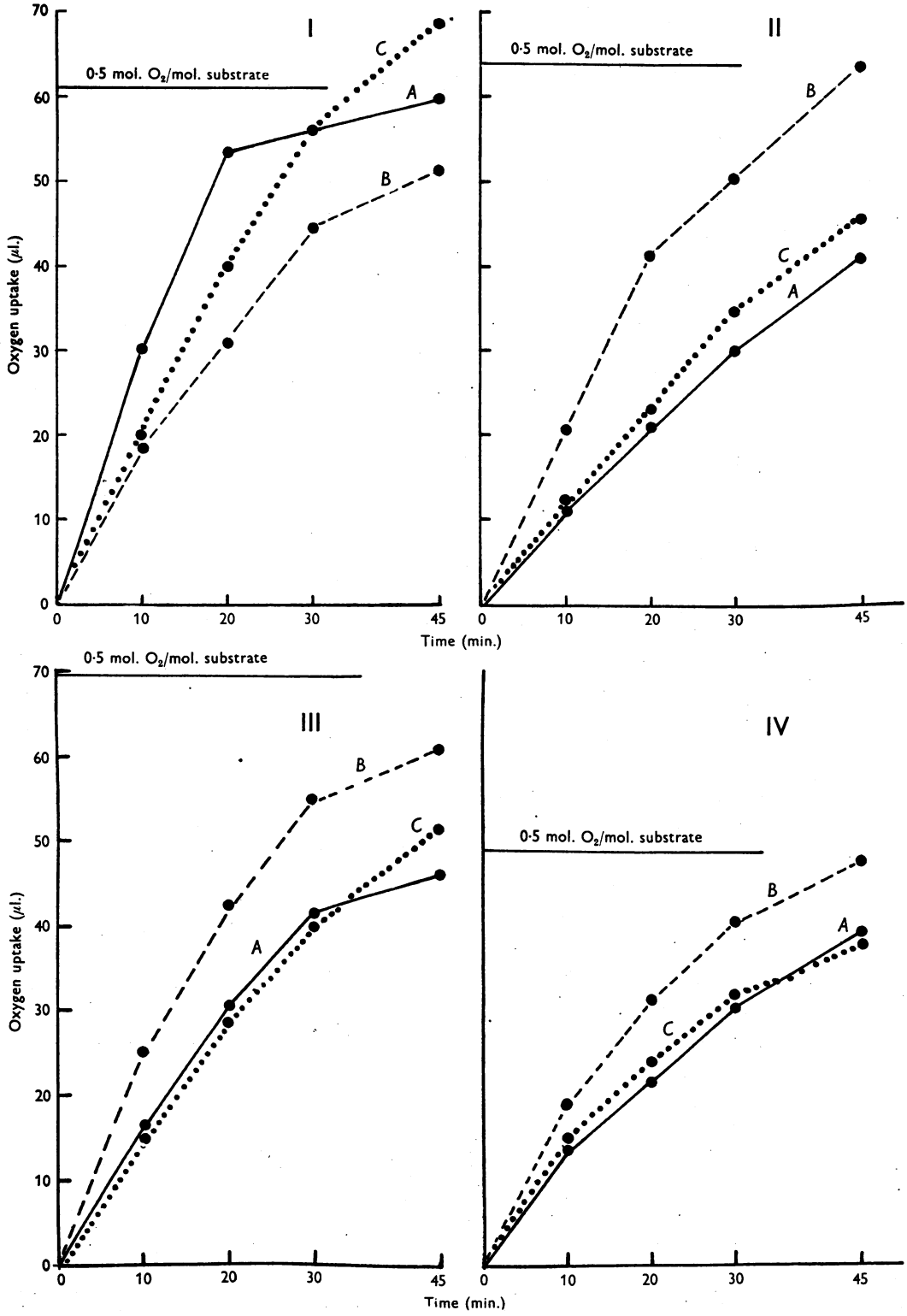

Fig. 3. Activity of histaminase preparation (1·7 mg. protein). *A*, before dialysis; *B*, after dialysis; *C*, after dialysis and addition of *FAD* from D-amino-acid oxidase. I, 1 mg. histamine hydrochloride; II, 1 mg. cadaverine hydrochloride; III, 1 mg. putrescine hydrochloride; IV, 1 mg. agmatine sulphate as substrates. Experimental conditions as in Fig. 1.

Both types of effect are thus presumably due to the restoration of *FAD* which had been removed from the histaminase by dialysis. The experiments with histamine might be explained on the theory that *FAD* is the prosthetic group. It is not, however, clear why this substance should have the opposite effect when the other substrates are used.

An unsatisfactory feature of these experiments is that the effects of dialysis were incomplete. Various attempts were made to split the histaminase preparation completely and reversibly into protein and prosthetic group. The procedures were those of Warburg & Christian (1938), Haas (1938) and Theorell (1935), but in all these experiments the enzyme was irreversibly inactivated. Swedin (1944) had a similar experience and it is evident that the protein is very unstable.

Rupture of the iminazole ring. Whereas Stephenson (1943) and Laskowski (1945) maintain that no rupture of the iminazole ring occurs by the direct action of histaminase on histamine, Best & McHenry (1930) and Swedin (1944) came to the conclusion that the iminazole ring is split in the first stages of this enzymic reaction. To examine further this question experiments were conducted to correlate the amount of O_2 absorbed and the rate of destruction of the iminazole ring.

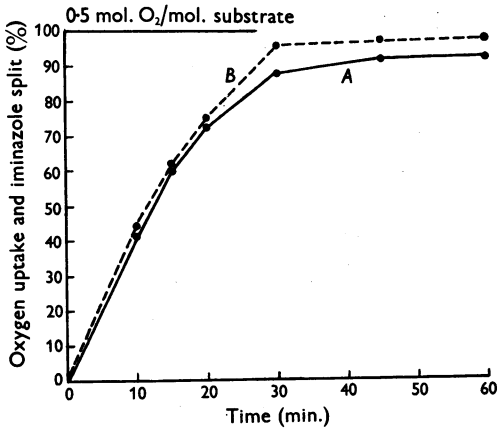

Fig. 4. Correlation of O_2 uptake with the rupture of the iminazole ring in the histaminase-histamine reaction. Histaminase preparation: 2·0 mg. protein; histamine hydrochloride 1 mg. as substrate; pH 7·2; temp. = 38°; air. *A*, iminazole split; *B*, oxygen taken up.

In six manometers the O_2 absorbed in the histaminase-histamine reaction was determined and the manometers withdrawn one at a time after 10, 15, 20, 30, 45 and 60 min. To the contents of the Warburg vessels, transferred into ordinary test tubes, 10 ml. of water and 10 ml. of 4·4% (v/v) $HClO_4$ were added. The protein precipitate was centrifuged off and portions of the clear supernatant fluid were tested for their iminazole content with a slightly modified diazo reaction (Macpherson, 1946). The initial iminazole content was determined in a control, set up in the same way and precipitated at $t = 0$. In Fig. 4 the O_2 uptake of the histaminase-histamine system and the rupture of the iminazole ring are correlated. The results show that one iminazole ring is broken for each atom of oxygen used in the early stages of the histaminase-histamine reaction. Similar results were reported by Swedin (1944).

Finally, the formation of NH_3 in the histaminase-histamine reaction described by Zeller (1938*a*; Zeller *et al.* 1940), Laskowski (1945) and other investigators has been confirmed by Conway's microdiffusion method (Conway & O'Malley, 1942).

Formation of H_2O_2 during the histaminase-histamine reaction. Zeller (1938*a*) suggested that histaminase belongs to the group of dehydrogenases, and that H_2O_2 is formed during the histaminase-substrate reaction. This view has been supported by Stephenson (1943) and by Swedin (1944) but not by Laskowski (1945). An experiment was, therefore, done to test whether coupled oxidation (Keilin & Hartree, 1936, 1945) would occur on addition of ethanol and catalase to the system containing histaminase and histamine. Fig. 5 shows that under these conditions twice the theoretical O_2 uptake was obtained, a fact which can be accepted as proof of the formation of H_2O_2.

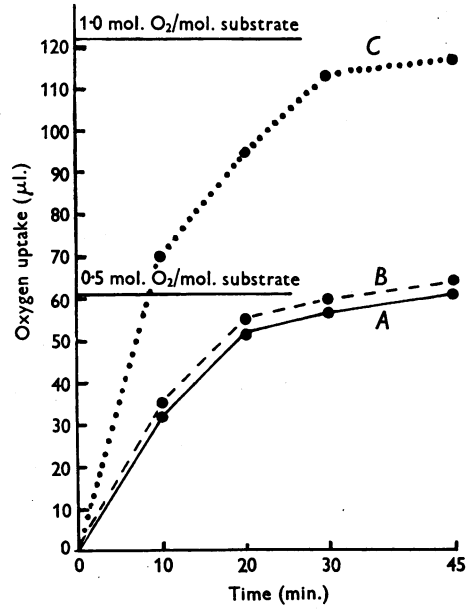

Fig. 5. Coupled oxidation of ethanol by the histaminase system in presence of purified catalase. *A*, histaminase (2·5 mg. protein) + histamine (1 mg. hydrochloride); *B*, as *A* + catalase (0·2 ml. purified, strong); *C*, as *B* + ethanol (15 mg.); pH 7·2; temp. = 38°; air.

Optimal activity and Michaelis constants. The optimal enzymic activity was found to lie between pH 7·2 and 7·7 for all three substrates. The Michaelis

constants were determined manometrically with histamine, putrescine and cadaverine as substrates and were found to be 0·00079, 0·0023 and 0·0030 M respectively. These figures demonstrate that the enzyme has a much higher affinity for histamine than for the diamines tested. The Michaelis constants given by Zeller, Schaer & Staehlin (1939) were 0·00050 for histamine, 0·00056 for cadaverine and 0·0012 for putrescine. Contrary to Zeller's (1938*b*) observation on crude histaminase no enzymic activity was obtained with spermine.

Histaminase and antihistamine drugs. The effect of the following drugs on the activity of histaminase was studied: Benadryl (benzhydryl 2-dimethylaminoethyl ether hydrochloride), Antergan (*N*-phenyl-*N*-benzyl-*N'N'*-dimethyldiaminoethane hydrochloride), Neo-antergan (*N*-2'-pyridyl-*N*-*p*-methoxybenzyl-*N'N'*-dimethyldiaminoethane hydrochloride), Antistin (2-(*N*-phenyl-*N*-benzylaminomethyl)iminazoline), 3277 R.P. (*N*-(dimethylamino*iso*propyl)phenothiazine hydrochloride), 3015 R.P. (*N*-(2-dimethylaminoethyl)phenothiazine hydrochloride). Each of these compounds was tested in two concentrations (2×10^{-3} and 7×10^{-3} M) with a histaminase preparation corresponding to 4·2 Laskowski units. Histamine hydrochloride (1 mg.) was used as substrate. The activity of the enzyme remained unchanged by Benadryl, Antergan, Neo-antergan or R.P. 3015, but was diminished in the presence of Antistin methanedisulphonate or of the drug R.P. 3277. With the Antistin preparation there was an enzyme inhibition of 33 and 45 % with 2×10^{-3} or 7×10^{-3} M solutions respectively, while the drug R.P. 3277 inhibited histaminase activity by 13 and 34 % with 2×10^{-3} and 7×10^{-3} M solutions.

DISCUSSION

These results suggest that histaminase is a flavoprotein with flavin-adenine-dinucleotide as its prosthetic group. Leloir & Green (1946) purified histaminase by electrophoretic separation, and obtained a colourless protein which showed no evidence of a dissociable prosthetic group. From this observation they concluded that histaminase was not a flavoprotein. It is difficult to discuss this work since only a brief account of it has been seen. Swedin (1943), also using electrophoresis for the purification of the enzyme, obtained the purest known preparation, which although almost colourless appeared to contain flavines. Swedin suggested that the enzyme was a flavoprotein.

Histaminase acts not only on histamine but also on cadaverine, putrescine and agmatine. It has a much higher affinity for histamine than for the other substrates. The pH optimum was the same for all the substrates investigated, and similar results were also obtained with these substrates when various narcotics and inhibitors were acting on the enzyme. However, a significant discrepancy in the behaviour of preparations of histaminase towards the substrates mentioned was found on dialysis of the enzyme. After dialysis its action on histamine was decreased, but its action on cadaverine, putrescine or agmatine was increased. The observations on the behaviour of *FAD* towards dialyzed histaminase make it seem probable that *FAD* acts as activator of the histaminase-histamine reaction and as inhibitor of the reaction between histaminase and cadaverine, putrescine or agmatine. The experiments on competition, however, show that there are not two independent enzymes, one responsible for the oxidation of histamine and the other for the oxidation of the diamines. It seems, therefore, probable that histaminase is an enzyme with various affinities and modes of behaviour towards various substrates. In this connexion the dual specificity of xanthine oxidase (Booth, 1938) may be recalled where similar conditions prevail, one enzyme acting on two chemically completely different groups of substrates, purines and true aldehydes; both groups showing different affinities for the enzyme. The reason for the different behaviour of histaminase preparations towards histamine on the one hand, and the diamines on the other, might be found in the presence of the iminazole ring in the histamine molecule which apparently is split in the very first stages of the histaminase-histamine reaction.

Concerning the nomenclature it may be suggested that the enzyme should retain the more specific name of histaminase instead of the term diaminoxidase, introduced by Zeller (1938*a*), since the enzyme has the highest affinity for histamine, and its behaviour on dialysis towards histamine is more characteristic of a flavoprotein than that towards cadaverine, putrescine and agmatine. The observation that the activity of histaminase is not impaired by the presence of most of the antihistamine drugs (Benadryl, Antergan, Neo-antergan, R.P. 3015), may be of importance for the clinical application of these compounds.

SUMMARY

1. A purified preparation of histaminase was made from hog kidney.

2. Dialysis caused a diminution of the action of this preparation on histamine, but an increase of its action on various diamines.

3. These effects were due to the loss of flavin-adenine-dinucleotide, which was shown to be present in the enzyme and to reverse both effects.

4. New evidence is presented that when histaminase acts on histamine, the iminazole ring is split in the early stages of the reaction.

5. Experiments with coupled oxidation showed that hydrogen peroxide is formed when histaminase acts on histamine.

6. The antihistamine drugs Benadryl, Antergan, Neo-antergan and the preparation R.P. 3015 do not inhibit histaminase preparations. Partial inhibition was obtained with Antistin and R.P. 3277.

I wish to thank Prof. J. H. Gaddum, F.R.S., Prof. D. Keilin, F.R.S., and Dr H. Laser for their valuable help and kind interest in this work. My thanks are also due to Dr Malcolm Dixon, F.R.S., for a preparation of split D-amino-acid oxidase and to Dr H. Laser for a sample of D-amino-acid oxidase as well as for a purified preparation of catalase. I am grateful to Miss Mairi A. Mackay for technical assistance, and very much indebted to the Manager of the Fleshing Department, St Cuthbert's Co-operative Society, Edinburgh, for the generous supply of hog's kidneys. The tenure of a whole-time grant from the Medical Research Council is gratefully acknowledged.

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Nicotinamide Metabolism in Mammals

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A number of compounds have been described as metabolites of nicotinic acid or nicotinamide in mammals. These are nicotinic acid (Komori & Sendju, 1926), nicotinamide, nicotinuric acid (Ackermann, 1913), trigonelline (Ackermann, 1913), nicotinamide methochloride (Najjar & Wood, 1940; Huff & Perlzweig, 1943*a*; Ellinger & Coulson, 1943; Coulson & Ellinger, 1943) and, recently, 1-methyl-6-pyridone-3-carbonamide (Knox & Grossmann, 1946, 1947). Dinicotinyl ornithine is a metabolite in the chick (Dann & Huff, 1947) and nicotinamide methochloride is formed by insects and bacteria (Ellinger, Fraenkel & Abdel Kader, 1947). In the earlier papers the metabolites were isolated from urine and identified by chemical analysis. When the physiological importance of nicotinamide was established, large series of analyses were carried out by methods allowing routine assay. Most of them were based on the König (1904) principle. The majority of the investigators were satisfied to estimate 'total nicotinic acid' which generally includes nicotinic acid, nicotinamide, nicotinuric acid and trigonelline. When nicotinamide methochloride was found to be a metabolite, this substance was assayed by special methods (Huff & Perlzweig, 1943*b*; Coulson, Ellinger & Holden, 1944; Najjar, 1944). It soon became evident that most of the metabolite previously described as trigonelline was in fact nicotinamide methochloride (Sarett, 1943; Huff & Perlzweig, 1943*a*). Although single observations have been carried out on man, dog, ferret, rat, mouse, calf, horse, rabbit and guinea pig, apart from the investigations of Johnson, Hamilton & Mitchell (1945) on man, and of Johnson, Wiese, Mitchell & Nevens (1947) on the calf, no distinction was made between

* Some of the results presented in this paper were communicated to the Biochemical Society on 24 January 1948 (Ellinger & Abdel Kader, 1948).