They cannot be explained as being solely due to a dilution effect, since addition of octanoate results in an equal decrease in the formation of both products, whereas addition of palmitate results in an inhibition of acetoacetate synthesis with relatively little effect on $^{14}CO_2$ production. The results obtained with ethylthioacetate are similar to those obtained with palmitate but ethylthioacetate does not form acetyl-CoA, so that a dilution effect with this substance may be eliminated. Further, it has been shown by Tabor, Mehler & Stadtman (1953) that synthetic palmityl-CoA and CoA itself both inhibit the acetylation of *p*-nitroaniline by synthetic acetyl-CoA. It seems likely, therefore, that the presence of palmityl-CoA affects the rate of formation of acetoacetate in animal tissues.

SUMMARY

1. The addition of fatty acids, such as butyric and octanoic, produces an inhibition of acetoacetate synthesis in extracts which are capable of forming acyl-CoA derivatives from fatty acids. Similar effects on sulphanilamide acetylation may also be obtained.

2. The effects of acyl-CoA compounds on the acetylation of sulphanilamide are reversed by acetyl-CoA prepared *in situ* from acetyl phosphate in the presence of *Esch. coli* extracts.

3. Studies on the oxidation of carboxyl-labelled butyrate by respiring rat-liver mitochondria have confirmed the fact that other fatty acids may compete for the butyrate activating enzyme system and that, on oxidation of these fatty acids, isotopic dilution occurs.

4. The addition of compounds such as benzoate, ethylthioacetate and palmitate to rat-liver mitochondria in the presence of [*carboxy*-^{14}C]butyrate results in a considerable decrease in the amount of radioactive acetoacetate formed but in little effect on the amount of radioactive CO_2 produced.

5. It is suggested that these results are consistent with the hypothesis that various reactions of acetyl-CoA (and possibly of acetoacetyl-CoA) are specifically inhibited by acyl-CoA compounds and that these inhibitory effects influence the kinetics of fatty acid oxidation to acetoacetate and carbon dioxide.

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Hypoglycin A and B, two Biologically Active Polypeptides from *Blighia sapida*

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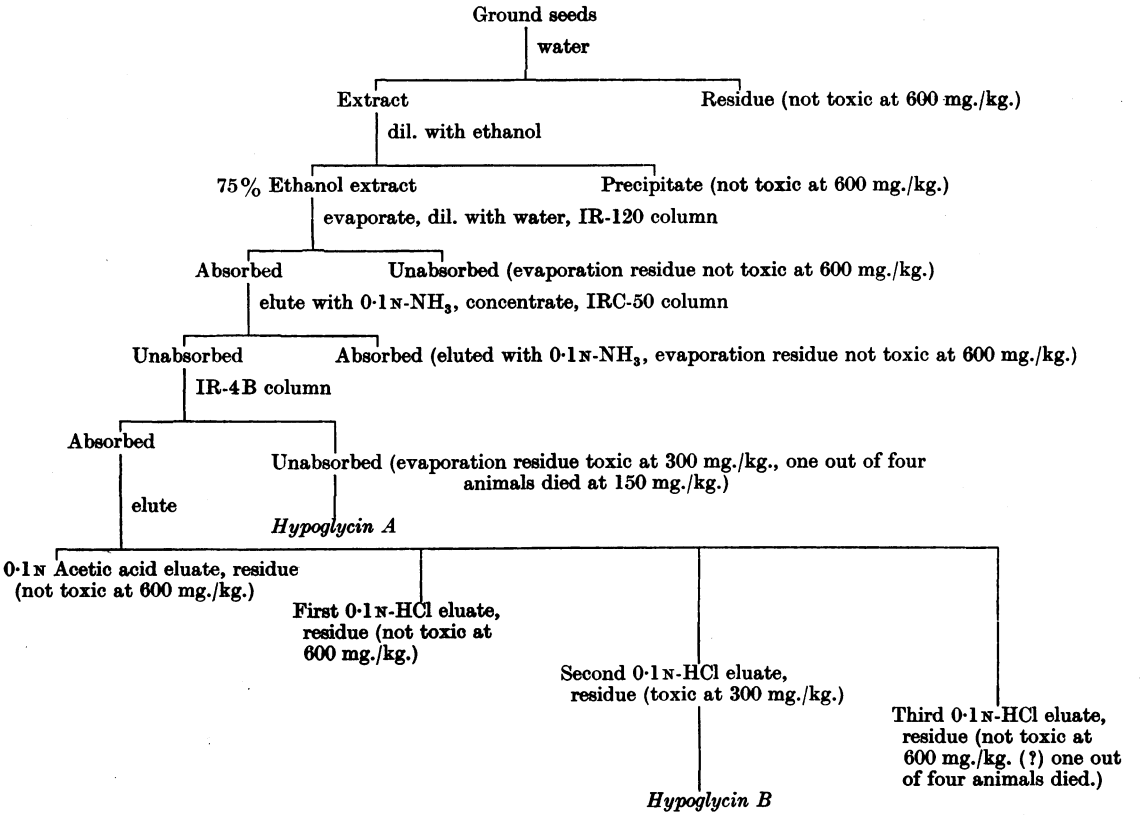
Several studies involving qualitative tests on animals (Bowrey, 1887; Connal & Ralston, 1918; Lynch, Larson & Doughty, 1951; Evans & Arnold, 1938; Jordan & Burrows, 1937) have indicated the presence of water-soluble toxic material in sections of the fruit of *Blighia sapida*, a common article of diet in Jamaica. Particular interest attaches to these observations in view of the suggestions by Scott (1916) that these fruit are responsible for 'vomiting sickness', an important local disease of undefined etiology (Hill, 1952; Jelliffe & Stuart, 1954). Although this view has not been generally accepted (Williams, 1952), it is supported by a considerable weight of circumstantial evidence and appeared worthy of closer examination.

Table 1. *Isolation of hypoglycin A and B from seeds*



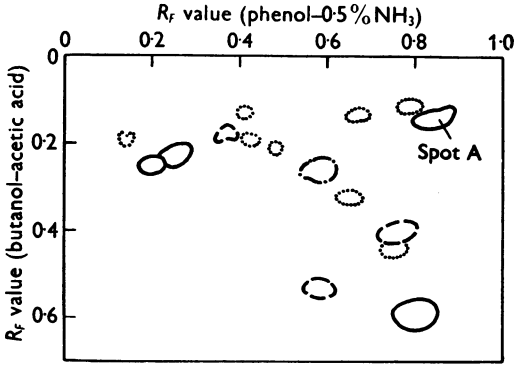


Fig. 1. Paper chromatography of mixture absorbed on Amberlite IR-120. Intensities of spots decrease in the order I–IV. I, ——; II, —·—; III, — —; IV, ·····.

We have attempted the isolation of the toxic constituents of the fruit so that their biological properties might be defined and compared with the characteristics of the vomiting sickness syndrome. Two toxic polypeptides, hypoglycin A and hypoglycin B, have been isolated according to the scheme summarized in Table 1. The separation was followed by toxicity tests and by paper chromatography.

The toxic compounds and amino acids in the aqueous extract of the seeds were separated from associated carbohydrates, pigments and plant acids by absorption on the ion-exchange resin Amberlite IR-120. The mixture which was eluted by ammonia from the ion-exchange resin contained at least sixteen ninhydrin-positive compounds (Fig. 1).

The basic amino acids in the mixture (Fig. 1, R_F 0·84 in phenol–NH_3; 0·14 in butanol–acetic acid; spot A) were removed when the ammonium salts eluted from the IR-120 resin were passed through a column containing the hydrogen form of the carboxylic ion-exchange resin, Amberlite IRC-50. It has been shown that there is 25% leakage when each of the basic amino acids arginine, histidine and lysine are passed through IRC-50 columns in this form (Winters & Kunin, 1949). No doubt, the fact that ammonium salts were involved, accounted for the almost quantitative absorption in this case. This step also had the advantage of removing ammonium ions, the presence of which

would interfere with the use of the basic ion-exchange resin in the next stage of the separation (Cleaver & Cassidy, 1950).

The acidic constituentswere absorbed on the resin Amberlite IR-4B. The mixture of unabsorbed substances contains, as the major component, a crystalline compound, m.p. 280–284°, $[\alpha]_D^{30} + 9{\cdot}2 \pm 2°$, which has been named hypoglycin A. As far as can be judged by comparison of chromatograms before and after hydrolysis with 10N hydrochloric acid, the constituents which accompany hypoglycin A in the IR-4B 'unabsorbed fraction' are amino acids.

When the acidic components absorbed on Amberlite IR-4B were eluted with dilute acetic and hydrochloric acids in turn, four distinct fractions were collected. Only one of these proved to be definitely toxic. This yielded a crystalline polypeptide, double m.p. 194–195°, 200–206°; $[\alpha]_D^{32} + 9{\cdot}6 \pm 2°$. This acidic compound has been named hypoglycin B.

The arilli of the fruit have been submitted to a similar scheme of separation to that used for the seeds. Hypoglycin A has been isolated from both ripe and unripe arilli but there was no evidence of hypoglycin B. The concentration of hypoglycin A in the arillus was estimated by a paper chromatography procedure similar to that developed for amino acids by Moore & Stein (1948) and by Boissonnas (1950). Values of $0{\cdot}008 \pm 0{\cdot}001$% and $0{\cdot}111 \pm 0{\cdot}005$% were obtained for the concentration in ripe and unripe arilli collected at the same time from the same tree.

The method of separation which has been developed followed from the observation, at the outset of this study, that the toxic constituents were present in a mixture of ninhydrin-positive compounds in which amino acids predominated. Animal experiments indicated that the bulk if not all of the toxicity was due to the polypeptides hypoglycin A and B.

The occurrence of a variety of amino acids in the aqueous extract of the fruit calls for little comment. Several studies using various plant species (for example, Allsopp, 1948; Dent, Stepka & Steward, 1947; Hulme & Arthington, 1950) have indicated that this is a normal result. However, there is very little information on the occurrence of peptides in higher plants. 'Bound' amino acids have been reported. Synge (1951) has obtained evidence of diffusible peptides in rye grass extracts. Vickery, Pucher, Wakeman & Leavenworth (1937) found that fresh tobacco leaves contain 0·05–0·26 g./kg. of 'peptide N'. Viscotoxin is the only peptide from a higher plant that has been studied in any detail. This cardiotoxic compound, isolated from the mistletoe, *Viscum album*, includes in its molecular structure a phosphate radical, a peptide chain consisting of one cysteine, one serine and two arginine units, a glucuronic acid radical and a substituted, hydrogenated naphthalene ring (Winterfeld & Rink, 1949).

Some biological properties of hypoglycin A and B have been defined (Hassall, Reyle & Feng, 1954). An estimation of the LD_{50} using adult rats gave a value of approximately 90 mg./kg. for hypoglycin A. The toxicity was more than doubled when fasted animals were used. Hypoglycin B was approximately half as toxic as hypoglycin A. The most striking pharmacological property is hypoglycaemic activity. Blood sugar invariably fell to less than 20 mg. % before death. Histological evidence indicated a marked reduction in the concentration of liver glycogen.

The hypoglycaemic activity of these compounds assumed special significance in view of the general agreement that the clinical evidence on vomiting sickness suggests that the condition is due to the effect of an unknown poison upon subjects, especially children, who are usually poorly nourished. Furthermore, low blood sugar and liver glycogen values (Jelliffe & Stuart, 1954; Patrick, 1954) are a regular characteristic of the vomiting sickness syndrome. The estimation of the concentration of hypoglycin A in the arillus provides evidence in support of the common belief that the unripe arillus is poisonous and corrects the general assumption that the ripe arillus, the portion of the fruit which is normally eaten, contains no toxic material (Scott, 1916). These observations provide further strong circumstantial evidence of a connexion between vomiting sickness and the toxic constituents of the fruit of *Blighia sapida*.

EXPERIMENTAL

Melting points were determined on a Kofler hot-stage.

Toxicity tests. The toxicity tests which were employed to follow the separation made use of kittens (288–852 g.), guinea pigs (185–420 g.) and adult albino rats (127–385 g.). The animals had been kept on a normal diet. There was no selection with respect to strain or sex. In the case of the results which are summarized in Table 1, four rats were used for each of the dose levels 50, 150 and 300 mg./kg. for the toxic and 300 and 600 mg./kg. for the non-toxic fractions. The test material was injected subcutaneously. A dose was regarded as toxic when all four animals died within 72 hr.

Paper chromatography. Paper chromatography experiments employed Whatman no. 1 filter paper in a stainless-steel tank (Woiwod, 1949) at $27 \pm 2°$. 5 µl. of 5% aqueous solutions of samples were applied to the paper with a micropipette to give a spot of about 3 mm. diameter. In two-dimensional runs 25% (w/v) phenol–water (Draper & Pollard, 1949) containing 0·04% 8-hydroxyquinoline (Block, 1950) was run in the presence of 0·5% NH_3 in the longer dimension of the paper. Butanol–acetic acid–water (3:1:6, by vol.) was used for the second dimension. The spots were developed with 0·2% ninhydrin in acetone using the

dipping technique (Smith, 1953). The intensity of spots is given as I–V, in decreasing intensity. Spots of intensity V are just visible.

Toxic constituents of the seeds

Amberlite IR-120 *fraction.* The ground seeds (4·57 kg.) of normal, unripe fruit were boiled under reflux for 1 hr. with water (5·5 l.). The mixture was cooled, strained through cheesecloth and diluted with sufficient ethanol to bring the alcohol concentration to 75%. The ppt. which formed was allowed to settle overnight at 0° and separated by filtration. Purification at this point by means of neutral and dibasic lead acetate (Jordan & Burrows, 1937) was avoided, since considerable loss of toxic material occurred in the lead salt precipitates. The filtrate, which was freed from ethanol by distillation at 50° *in vacuo* was introduced at the rate of 1 l./hr. on a column (38 × 6 cm.) of the cation-exchange resin Amberlite IR-120 (650 g., 80–100 mesh) which had been purified by alternate washing with 10% HCl and 10% NaCl solution and finally 10% HCl and water. When all the filtrate had been applied, the column was treated with distilled water until the washings were neutral. The washings and unabsorbed filtrate were discarded.

The column was eluted with 0·1 N-NH_3 (1 l./hr.). An acidic eluate (12·9 l.) was collected until the brown ammonia band visible in u.v. light reached the bottom of the column. It was concentrated *in vacuo* to 5 l. Elution was continued until the effluent no longer contained ninhydrin-positive material. The second eluate (15 l.) was evaporated to dryness *in vacuo* to remove NH_3 and then dissolved in the concentrated first eluate. When this fraction was subjected to paper chromatography there was evidence of basic, acidic and neutral amino acids in the mixture.

Separation of the ninhydrin-positive basic, acidic and neutral fractions. The separation made use of the Amberlite ion-exchange resins IRC-50 and IR-4B. The resins were ground to 80–100 mesh and purified according to the procedure outlined by the manufacturers (Rohm and Haas bulletins M-2-48, M-3-47). In the case of IR-4B, it was necessary to remove all coloured and ninhydrin-positive impurities by prolonged washing with 0·1 N-HCl. The resin was buffered by washing with 0·25 N sodium acetate solution. Solutions were allowed to flow through columns at the rate of 1 l./hr.

The basic constituents were removed when the aqueous solution (5 l.) of the mixture of ammonium salts from the IR-120 column was passed through a column of Amberlite IRC-50 (35 × 6 cm., 408 g.). After the acidic effluent (pH 4·0) had been collected, the column was washed with water until the eluate was no longer ninhydrin-positive. The absorbed material was eluted with 0·1 N-NH_3. The eluate was evaporated *in vacuo* to give a yellow syrup (2·10 g.) which was non-toxic.

The acidic constituents were removed from the combined effluent and washings from the IRC-50 column by passing the solution (8 l.) through a column of Amberlite IR-4B (33 × 5 cm., 252 g.). The effluent and ninhydrin-positive washings (10 l.) were evaporated at low pressure (35°) to give a yellow crystalline residue (18·7 g.). This material was toxic.

The acidic fraction was eluted from the IR-4B column by treatment with 0·1 N acetic acid followed by 0·1 N-HCl. The acetic acid (15 l.) eluted a crystalline fraction (10·1 g.). This was non-toxic and consisted largely of glutamic acid (m.p. and mixed m.p. with authentic L-glutamic acid, 204–205°). When 0·1 N-HCl was added a distinct band moved down the column. The solution which was collected just before the band reached the bottom of the column (7·5 l.) yielded on evaporation a small residue (0·8 g.) of non-toxic, yellow, crystalline material. The band was eluted from the column until chloride ion appeared. This solution (1 l.) yielded 8·0 g. of yellow amorphous residue which was toxic. Further elution with HCl (1 l.) removed all ninhydrin-positive material. Evaporation of this solution *in vacuo* gave a yellow-brown syrup (5·00 g.). This was slightly toxic, owing no doubt to the small amount of hypoglycin B which was shown to be present by paper chromatography experiments.

Purification of the toxic compounds

Hypoglycin A. The yellow crystalline residue (18·7 g.) obtained from the solution of material which was not absorbed on Amberlite IR-4B was recrystallized 4 times from methanol–water to yield 7·0 g. of colourless plates, m.p. 277–282° (decomp.). Paper chromatography using butanol–acetic acid–water gave the following spots with ninhydrin (R_F values and intensity of spots): 0·28 (V), 0·42 (V), 0·48 (V), 0·61 (I), 0·69 (V). Final purification involved chromatography on powdered cellulose. Whatman no. 1 powdered cellulose or Brown's Solka-floc (B.W. grade, 200 mesh) were purified by washing with N-HCl, distilled water, 0·2% $NaHCO_3$ solution, distilled water and ethanol in turn. The powder was dried at 90° overnight, washed with benzene until no further impurity was removed and dried again at 90°. In a typical experiment a column (67 × 1·5 cm.) of purified cellulose (35 g.) was prepared from an acetone slurry. The acetone was displaced with butanol–acetic acid–water, freshly prepared at 2° below room temp. The crystalline material (50 mg.) in 2 ml. of the butanol–acetic acid mixture was allowed to drain into the cellulose slowly. The column was developed by allowing solvent to pass at the rate of 3 ml./hr. After 54·5 ml. of eluate had been collected the solution showed a ninhydrin reaction. After this, 65 × 1 ml. fractions were collected separately. Fractions 1–39 and 61–65 contained traces only of ninhydrin-positive material and were discarded. Fractions 40–60 were evaporated to dryness, and 100 μg. of each were used in one-dimensional paper chromatograms with butanol–acetic acid–water. The results obtained are summarized in Table 2. A similar result was obtained using a column 63 × 5 cm. (363 g.) for the purification of 530 mg. of material.

Fractions 45–50 were crystallized from methanol–water to give hypoglycin A as colourless, irregular plates, m.p. 280–284°. Crystallization from water or ethanol–water gave hexagonal plates which decomposed at 230° without melting; $[\alpha]_D^{30} + 9·2 \pm 2°$ in water (*c*, 1·00). [Found (material dried at 80°/0·2 mm.): C, 59·4; H, 8·1; N, 11·2%.] A copper salt was precipitated from aqueous solutions by copper acetate. Hypoglycin A was hydrolysed in 48 hr. by 10 N-HCl to a mixture of new ninhydrin-positive compounds. Paper chromatography using *n*-butanol–acetic acid–water, phenol–water–NH_3 and benzyl alcohol–*n*-butanol–water (1:1:1, by vol.) gave single spots with R_F values 0·60, 0·83 and 0·29 respectively. The compound sublimed unchanged at 180°/5 mm.

Attempts to purify crude hypoglycin A by the use of the ion-exchange resin IR-120 or the copper salt were not successful. Hypoglycin A was not modified by the process but no separation from associated impurities was achieved.

Table 2. *Purification of hypoglycin A by chromatography on powdered cellulose*

100 μg. of each fraction were chromatographed on paper using butanol–acetic acid–water as described in the text. Decreasing intensities of spots are denoted by I–V, V being just visible.

Fraction no.	Weight (mg.)	R_F values and intensities of spots					
40	2	0·20 (IV)	0·37 (II)	0·47 (V)	0·67 (I)	0·74 (IV)	0·80 (V)
41–44	14		0·36 (IV)		0·66 (I)	0·74 (V)	
45–50	23				0·65 (I)		
51–55	5	0·23 (V)	0·37 (V)	0·59 (V)	0·66 (I)		
56–60	1	0·22 (V)		0·59 (V)	0·66 (I)		

Hypoglycin B. The toxic, yellow, amorphous residue (8·00 g.) obtained from the second 0·1 N-HCl eluate from the Amberlite IR-4B column was dissolved in just sufficient water. Acetone was added at 0° almost to the point of precipitation. The clear solution became turbid on standing at room temperature. The colourless needles (7·0 g.) that separated at room temperature had a double m.p. 191–192°, 198–205° after two further crystallizations. Paper chromatography of this material, using butanol–acetic acid–water, gave the following spots with ninhydrin (R_F values and intensities): 0·20 (V), 0·23 (V), 0·26 (V), 0·52 (III), 0·65 (I).

The mixture (500 mg.) was subjected to chromatography on a column (70 × 4 cm.) of powdered cellulose (318 g.) using butanol–acetic acid–water (20 ml./hr.) and a procedure similar to that employed in the case of hypoglycin A. The ninhydrin-positive material was collected in 18 × 10 ml. fractions. Paper chromatography showed that fractions 2–8 (203 mg.) contained only one of the original constituents (R_F 0·67). There were also traces of fast-running impurities (R_F 0·77, 0·86, 0·91) which were also present in all the other fractions collected. These appeared to have been extracted from the powdered cellulose by the hypoglycin B. Crystallization from acetone–water removed these impurities and gave hypoglycin B as fine needles, double m.p. 194–195°, 200–207°; $[\alpha]_D^{32} + 9{\cdot}6 \pm 2°$ in water (c, 1·12). [Found (material dried at 60°/0·2 mm.): C, 52·9; H, 6·9; N, 10·6%.] It was hydrolysed to a mixture of new ninhydrin-positive compounds and gave a water-insoluble copper salt. Paper chromatography using butanol–acetic acid–water and phenol–water–ammonia gave spots of R_F 0·61 and 0·60 respectively. The compound is an acid (neutralization equiv. 175).

Toxic constituents of the arillus

The ripe and unripe arilli of *Blighia sapida* were treated individually by the separation procedure used for the seeds. Hypoglycin A was identified in both cases. Hypoglycin B was not present. The estimation of the concentration of hypoglycin A in the arillus involved an elution procedure similarly to that developed by Moore & Stein (1948) and Boissonnas (1950) for amino acids.

The IR-4B unabsorbed fraction (6·9 g.) from unripe arillus (2·3 kg.) was applied to paper as spots of 20–480 μg. In the case of the ripe arillus (1·5 kg.), 120–720 μg. of the corresponding fraction (2·49 g.) were used. The ninhydrin colorations were compared with those produced with standard solutions of pure hypoglycin A, using a Klett-Summerson photoelectric colorimeter.

SUMMARY

1. Two peptides, hypoglycin A (a neutral compound, m.p. 280–284°; $[\alpha]_D^{32} + 9{\cdot}2 \pm 2°$) and hypoglycin B (an acid, double m.p. 194–195°, 200–206°; $[\alpha]_D^{32} + 9{\cdot}6 \pm 2°$; neutralization equiv. 175) have been isolated from the unripe seeds of *Blighia sapida*.

2. The aqueous extract of the seeds, which contains some fourteen ninhydrin-positive compounds in addition to hypoglycin A and B, was fractionated by means of the Amberlite ion-exchange resins IR-120, IRC-50 and IR-4B. The isolation of hypoglycin A and B was followed by toxicity tests using kittens and albino rats.

3. The hypoglycaemic activity of the peptides is circumstantial evidence of a connexion between the Jamaican disease 'vomiting sickness' and the practice of eating the fruit of *Blighia sapida*.

4. Hypoglycin A, but not hypoglycin B, occurs in the arillus of the fruit. The unripe arillus contains a very much higher concentration of hypoglycin A than the ripe arillus.

Grateful acknowledgement is due to the Government of Jamaica for financial support, to Mr E. V. Ellington for the determinations of the concentration of hypoglycin A in the arillus of the fruit, and to Dr P. Feng and the Department of Physiology, University College of the West Indies, for toxicity estimations. We are indebted to Mr Bryan Lutze-Wallace for technical assistance.

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Return of Cholinesterase Activity in the Rat after Inhibition by Organophosphorus Compounds

2. A COMPARATIVE STUDY OF TRUE AND PSEUDO CHOLINESTERASE*

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There are two distinct types of enzyme capable of the hydrolysis of acetylcholine. True cholinesterase is predominantly associated with nervous tissue and erythrocytes, while pseudo cholinesterase is found in the serum, liver and pancreas. The two enzymes can be distinguished not only by their distribution, but also by their substrate specificity (Mendel, Mundell & Rudney, 1943) and their sensitivity to certain selective inhibitors (Aldridge, 1953*a*; Fulton & Mogey, 1954). Furthermore, it has been shown that pseudo cholinesterase can be almost completely inactivated without the development of any significant signs of poisoning (Mazur & Bodansky, 1946; Hawkins & Gunter, 1946; Hawkins & Mendel, 1949), while complete inhibition of true cholinesterase is invariably fatal.

It has been shown in a previous paper (Davison, 1953) that the rates of reactivation of rat true and pseudo cholinesterases after inhibition by diethyl *p*-nitrophenyl phosphate (paraoxon) are markedly different. Indeed, it has been suggested that the rates of recovery are a characteristic of the inhibited enzyme. Recent work suggests that the organophosphorus compounds phosphorylate the enzyme's active centre (Aldridge, 1954) and that reactivation *in vitro* is the result of hydrolysis of the phosphorylated enzyme. The stability of the phosphorylated enzyme is, therefore, a measure of the strength of the link between the phosphorus of the inhibitor and the enzyme. The previous work on the reactivation of inhibited cholinesterase has been extended by the use of various organophosphorus compounds and different cholinesterases. The results have been interpreted in terms of the phosphorylation theory.

Not only do the rates of recovery of phosphorylated rat true and pseudo cholinesterases differ, but while the reactivation of inhibited pseudo cholinesterase is complete and kinetically first order, that for inhibited true cholinesterase is partial only. One explanation of the results is that there are two types of true cholinesterase, differing in the stability of their phosphorylated active centres. It has also been found that the reactivation of phosphorylated rat, horse and chicken pseudo cholinesterase is acid catalysed, whereas the recovery of phosphorylated rat and rabbit true cholinesterase activity is not. This interesting finding may well indicate a fundamental dissimilarity between the active groups of the two enzymes and thus account for the obvious differences between them. This work is part of a thesis which has been accepted by the University of London for the degree (external) of Doctor of Philosophy in the Faculty of Medicine.

* Paper 1 of this series: Davison, 1953, **54**, 583.

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MATERIALS AND METHODS

The inhibitors used in this work were: dimethyl, diethyl, di-*n*-propyl and di*iso*propyl *p*-nitrophenyl phosphates; di-*n*-propyl and di*iso*propyl phosphorofluoridates; tetraethylpyrophosphate; *NN'*-di*iso*propyl phosphorodiamidic fluoride and *NN'*-di*iso*propyl phosphorodiamidic anhydride. Diethyl and di-*n*-propyl *p*-nitrophenyl phosphates and di-*n*-propyl phosphorofluoridates were synthesized according to the methods of Fagerlind, Holmstedt & Wallen (1952) and McCombie, Saunders & Stacey (1948). The remaining compounds were obtained from Dr H. Coates, Albright and Wilson Ltd., and from Dr G. S. Hartley, Pest Control Ltd. Crystalline pepsin and trypsin were obtained from Armour Laboratories.