3. Urinary excretion of phosphorus and sulphur was lowered, but faecal excretion was increased.
4. Urinary excretion of uric acid and of creatinine increased on a zinc-containing diet.
5. These results are discussed in relation to the

systemic effects previously noted by the author as characteristic of zinc poisoning.

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The Preparation of Soluble Cholinesterases from Mammalian Heart and Brain

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In a previous paper (Ord & Thompson, 1950) evidence was presented in support of the presence of two different types of cholinesterase in certain tissues of the rat and of man. One enzyme hydrolyses benzoylcholine and is highly sensitive to inhibition by di-isopropyl fluorophosphonate (DFP), and therefore corresponds in these respects to the so-called 'non-specific' (Nachmansohn & Rothenberg, 1945) or 'pseudo' cholinesterase (Mendel & Rudney, 1943), and another, apparently a 'specific' or 'true' cholinesterase, is relatively insensitive to inhibition by DFP and inactive towards benzoylcholine, although readily hydrolysing acetyl- β -methylcholine ($[\text{Me}_3\text{N}^+\cdot\text{CH}_2\cdot\text{CHMe}\cdot\text{OAc}]\text{OH}^-$).

This earlier work was done with whole homogenates of the various blood-free tissues, and it seemed desirable, therefore, to attempt to obtain soluble, purified preparations in order to characterize more clearly the different types of cholinesterase in these tissues. Since rat heart hydrolysed benzoylcholine very actively, we have chosen this tissue as a source of the pseudo-cholinesterase. The rate of hydrolysis of acetyl- β -methylcholine by rat heart, however, is so low as to render it unsuitable for the purification of the true enzyme, and for this purpose we therefore chose rat brain.

A secondary purpose underlying the work described in this paper was provided by considerations as to the toxicity of cholinesterase inhibitors. On account of the selective action of certain inhibitors

on the two types of cholinesterase, and of the very different toxicological actions of different cholinesterase inhibitors in the whole animal, it would appear that at least two distinct lines of approach present themselves regarding the mechanism of poisoning: (1) an *in vitro* study of the action of these toxic compounds on the types of cholinesterase present in affected tissues, and (2) a study of these compounds in the whole animal, with a view to determining whether there exist any differences in the power of penetration of different inhibitors to the cholinesterases in different tissues, i.e. whether there are any 'barriers' of a physico-chemical or more complex biological nature that might influence the reactivity of inhibitors with the cholinesterases situated at certain sites in the body. With regard to the first of these alternatives, it is clearly desirable that the enzyme under study should be in a soluble form, so that difficulties of penetration and the influence of adjacent substances situated in cells or cell particles may be reduced to a minimum.

Much work has been done on the purification and behaviour of the soluble cholinesterase of plasma (Stedman & Stedman, 1935; McMeekin, 1939; Strelitz, 1944; Adams & Whittaker, 1949*a*), but some doubt has justifiably been felt about the relevance of such work to the problem of the particular cholinesterase at or near the nerve endings in the tissues. Similarly, purified preparations of erythrocyte cholinesterase have been

described (Mendel & Rudney, 1943; Adams, 1949), but the only purification of a cholinesterase from an innervated mammalian tissue, of which we are aware, is that reported by Mendel & Mundell (1943). These workers prepared a soluble, highly purified pseudo-cholinesterase from dog pancreas. As their source was a glandular tissue, whose exocrine secretion is rich in enzymes, it seemed important to study also a pseudo-cholinesterase derived from a muscular tissue devoid of secretory activity.

Nachmansohn & Rothenberg (1947) obtained highly purified preparations of the true cholinesterase of the electric organ of *Electrophorus electricus*, but no reports have yet been published of any real purification of the enzyme in mammalian brain. Indeed, until the work of Little (1948), it was generally thought that the cholinesterase of brain was firmly associated with the insoluble cell particles of homogenates of this tissue.

EXPERIMENTAL

White rats of either sex were used. They were killed by decapitation, and homogenates of brain or of heart ventricle were made as previously described (Ord & Thompson, 1950).

Estimation of esterase activity. Ammon's (1933) adaptation of the Warburg manometric technique was used. All measurements were made at 38° and at pH 7.4, and were corrected for non-enzymic hydrolysis of the substrate. Esterase activity is expressed as $\mu\text{l. CO}_2/\text{g. wet wt. of tissue/hr.}$ for the unpurified preparations, and as $\mu\text{l. CO}_2/\text{mg. trichloroacetic acid-precipitable N/hr.}$ for the purified preparations, unless otherwise stated.

Estimation of nitrogen. After precipitation by the addition of an equal vol. of 10% trichloroacetic acid, protein N was estimated by the micro-Kjeldahl technique, using the distillation apparatus described by Markham (1942).

Substrates. (1) Acetylcholine chloride (ACh) (British Drug Houses Ltd.); (2) acetyl- β -methylcholine chloride (MCh) (Savory & Moore, Ltd.); (3) benzoylcholine chloride (BCh), prepared by Dr A. H. Ford-Moore, Experimental Station, Porton; (4) propionylcholine perchlorate (PrCh), and (5) butyrylcholine chloride (BuCh), both given to us by Dr V. P. Whittaker, Department of Biochemistry, Oxford; (6) tributyrin (TB) (British Drug Houses Ltd.).

Each substrate, with the exception of TB, was dissolved in 0.025M-NaHCO₃ immediately before use. The TB was pipetted directly into the side arm of the Warburg flasks, and was covered with a small volume of 0.025M-NaHCO₃. The

final concentrations used throughout were 0.015M for ACh and BCh, and 0.03M for MCh, PrCh, BuCh and TB, these concentrations giving a steady rate of CO₂ evolution over the times studied.

Inhibitors (1) Di-isopropyl fluorophosphonate (DFP), kindly provided by the Experimental Station, Porton; (2) eserine sulphate (British Drug Houses Ltd.). Both inhibitors were dissolved in 0.025M-NaHCO₃ before use.

Other reagents. The reagents used in the course of the purification were all of A.R. standard (British Drug Houses Ltd., and Hopkins and Williams Ltd.). Cholic acid and sodium deoxycholate, prepared from hog bile, were given to us by Prof. G. A. D. Haslewood. Most of the other surface-active substances were kindly provided by Imperial Chemical Industries Ltd.

RESULTS

Rat-heart cholinesterase

Purification. The starting material was a 10% (w/v) homogenate of rat-heart ventricle in 0.025M-sodium bicarbonate, the final mixture having a pH of 8.4-8.6 and a cholinesterase activity, with acetylcholine as substrate, of about 3600 $\mu\text{l. carbon dioxide/g. wet wt. of tissue/hr.}$ If such a suspension is centrifuged at 3500-4000 rev./min. for 10 min., the greater part of the activity appears in the centrifuged deposit. Following the observation of McCance (1950) that the cholinesterase of dog colostrum is not destroyed by the enzymes of an acetone-dried preparation of pig pancreas, it was decided to attempt to release the cholinesterase from the bulk of material thrown down on centrifuging by carrying out a brief tryptic digestion of the heart homogenates.

A volume of the homogenate, varying in different experiments from 10 to 100 ml., was therefore incubated at 37° for 1 hr. with 1-2 ml. of an aqueous suspension of trypsin (British Drug Houses Ltd.), to give a final concentration of 50 mg. trypsin/100 ml. homogenate. After incubation the overall cholinesterase activity was unchanged, but 88% of it was now present in the supernatant after centrifuging, as compared with only 39% initially (Table 1). In the concentration employed the preparation of trypsin showed no cholinesterase activity.

The supernatant (*S*₁), which was still very opaque, was separated from the centrifuged deposit; on

Table 1. *Effect of tryptic digestion (1 hr. at 37°) on the cholinesterase of rat-heart ventricle*
(Activity as $\mu\text{l. CO}_2/1.5 \text{ ml. homogenate/15 min.}$)

Whole homogenate			Supernatant from centrifuged homogenate			
A	B	B as % of A	C	D	C as % of A	D as % of A
Initially	After tryptic digestion		Initially	After tryptic digestion		
178	167	93	72	149	40	84
163	158	97	64	147	39	90
141	157	111	52	128	37	91
	Mean	97			39	88

Table 2. *Stages in purification of rat-ventricle cholinesterase*(Activity expressed as $\mu\text{l. CO}_2/\text{mg. trichloroacetic acid-precipitable N/hr.}$ For details of purification procedure see text.)

Fraction	Original homogenate	S_1	S_2	F_{50}	F_{65}
Activity	150	744	1128	1869	4520
Percentage of original cholinesterase content	100	81	33	26	24
$\mu\text{g. N/fraction derived from 1 ml. original homogenate}$	2390	446	119	51	19
Purification	—	$\times 5$	$\times 8$	$\times 12$	$\times 30$

bringing to pH 6.0 (glass electrode) a heavy protein precipitate developed, from which, on centrifuging, a clear brownish-yellow supernatant (S_2) could be decanted. Fraction S_2 was then treated with an equal volume of saturated ammonium sulphate solution, and the small precipitate which formed was discarded after centrifuging. The supernatant (F_{50}) was brought to 65% saturation by the addition of solid ammonium sulphate; after standing for 10 min. the floccular precipitate containing the cholinesterase was thrown down by centrifuging. It was then dissolved in distilled water and dialysed overnight (16 hr.) against tap water. The small precipitate which formed at this stage was removed by filtering (Whatman no. 42 paper); the filtrate (F_{65}), which was a clear straw-coloured liquid, contained about 24% of the total cholinesterase activity (estimated with acetylcholine as substrate) present in the original homogenate, and showed about a 30-fold purification when assessed as activity/mg. protein nitrogen. The degrees of purification obtained at the different stages are shown in Table 2.

Properties of the purified enzyme from heart. From our earlier experiments (Ord & Thompson, 1950) on the sensitivity to inhibition by DFP of the hydrolysis by rat heart of benzoylcholine and acetyl- β -methylcholine, it was concluded that the enzyme

soluble cholinesterase (F_{65}) is given in Table 3, the relative rates of hydrolysis of the different substrates being expressed as percentages of the hydrolysis rate of acetylcholine. It will be seen that in the purified heart preparation the activity towards both acetyl- β -methylcholine and tributyrin is very significantly reduced, whereas the relative rates of hydrolysis of benzoyl-, propionyl- and butyryl-choline are almost unchanged. This finding supports the conclusion that at least two distinct esterases are present in these purified homogenates.

Summation experiments with mixed substrates were next carried out to obtain further evidence of the independence of the enzymes hydrolysing acetylcholine and tributyrin in this heart preparation, and also to discover whether acetyl-, propionyl- and butyryl-choline are each hydrolysed by the same enzyme. These results are summarized in Table 4.

It was found with the unpurified homogenate and also with the purified F_{65} fraction that the carbon dioxide output in the presence of tributyrin and either acetylcholine or butyrylcholine as the second substrate amounted to 86–107% of the sum of the carbon dioxide output in the presence of each substrate separately, indicating that an 'ali-esterase' and a cholinesterase are present in the purified preparation. On the other hand, no summation of activity was obtained in the presence of acetylcholine and either propionyl- or butyryl-choline, indicating that these substrates are hydrolysed by the same enzyme.

The presence of a separate ali-esterase was confirmed by inhibition experiments in which it was found (Table 5) that concentrations of either eserine or DFP which inhibited completely or almost completely the cholinesterase, left the ali-esterase relatively unaffected.

The mean concentration of DFP required to cause 50% inhibition of esterase activity (I_{50}) under our *in vitro* conditions was $0.32 \times 10^{-8} \text{ M}$ for the purified F_{65} fraction, as compared with $1.4 \times 10^{-8} \text{ M}$ for the original homogenate. This reduction in the I_{50} concentration for the purified as contrasted with the unpurified heart preparation probably reflects the removal of inert protein present in the whole homogenate, which had reacted with and thereby lowered the effective concentration of the DFP.

Table 3. *Relative rates of hydrolysis of choline esters and tributyrin by the original and the purified rat-ventricle preparations*

(Acetylcholine rates in each case taken as 100.)

	ACh	BCh	MCh	PrCh	BuCh	TB
Original preparation	100	29	9	227	138	70
Purified preparation (F_{65})	100	27	2	239	137	27

hydrolysing benzoylcholine was a pseudo-cholinesterase in the sense defined by Mendel & Rudney (1943). As confirmatory evidence of this it was decided to carry out more detailed studies of substrate specificity with various choline esters and with an aliphatic non-choline ester, tributyrin. A comparison of the results obtained with the unpurified homogenate (H) and with the partially purified,

Table 4. *Hydrolysis rates of mixed substrates by rat-ventricle preparations*

(μ l. CO ₂ /ml. preparation/30 min.)					
1st ester TB	2nd ester		Both esters		'Summation' (%)
	ACh	BCh	Found	Calculated	
		Unpurified preparation			
114	157	—	233	217	86
114	—	59	158	173	91
PrCh					
323	150	—	263	473	0
		Purified preparation (F_{65})			
TB					
50	230	—	264	230	94
42	181	—	224	223	100
42	—	52	101	94	107
BuCh					
199	231	—	220	430	0

The above results obtained with a partially purified preparation of rat-heart ventricle confirm our earlier conclusion that this organ contains at least two enzymes hydrolysing choline esters, and reveal in addition the presence of an aliphatic esterase, hydrolysing tributyrin, but inactive against choline esters. The stages of purification have almost completely removed the true cholinesterase hydrolysing acetyl- β -methylcholine (Ord & Thompson, 1950), and have yielded a preparation

Table 5. *Effect of inhibitors on purified rat-ventricle preparations*

Inhibitor	Concn. (M)	Inhibition (%)		
		ACh	PrCh	TB
Eserine sulphate	0.1×10^{-6}	80	—	0
Eserine sulphate	1.0×10^{-6}	96	—	0
DFP	3.5×10^{-9}	49	48	—
DFP	90×10^{-9}	100	—	32

containing an esterase highly active towards acetyl-, propionyl- and butyryl-choline. This second cholinesterase, however, although resembling the pseudo-cholinesterase present in many animal sera in its high sensitivity to inhibition by DFP and its inactivity towards acetyl- β -methylcholine, differs from it with regard to the optimal acyl group of choline esters hydrolysed by it; thus, it hydrolyses propionyl-choline more rapidly than either acetyl- or butyryl-choline, differing, therefore, both from the cholinesterase of human or horse erythrocytes (Adams, 1949; Mounter & Whittaker, 1950) and from the cholinesterase of human or horse plasma (Adams & Whittaker, 1949*b*; Sturge & Whittaker, 1950).

Rat-brain cholinesterase

Effect of homogenizing medium on the extraction of brain cholinesterase. If homogenates of rat brain in 0.025M-sodium bicarbonate are centrifuged at 3500–4000 rev./min. the greater part of the cholin-

esterase activity appears in the precipitate. Little (1948) has shown that the same is true for mouse brain, but found that if, instead of using sodium chloride-sodium bicarbonate mixtures, the brain was homogenized in distilled water, about 90 % of the activity was present in the centrifuged supernatant; further, if sodium chloride solutions were used as the homogenizing medium the amount of enzymic activity in the supernatant decreased as the sodium chloride concentration was raised. Using human erythrocytes as their source of cholinesterase, Mentha, Sprinz & Barnard (1947) found that the enzyme can be extracted from the cells with ammonium hydroxide at pH 8.3, while Arragon & Sala (1948) succeeded in eluting it from kieselguhr with ammonia at pH 8.0–8.2. As a first step towards the purification of brain cholinesterase we therefore decided to study in greater detail the effect of differences in composition of the homogenizing medium on the proportion of cholinesterase extracted into the centrifuged supernatant.

Homogenates (10 %, w/v) of whole rat brain were prepared in a variety of different aqueous solutions and in distilled water. The conditions of homogenizing and centrifuging were kept as far as possible identical. The pH of the homogenate was determined (glass electrode), and a sample taken for estimation of cholinesterase activity. The remainder was centrifuged for 10 min. at 4000 rev./min., and the activity of the supernatant estimated. The results of these experiments are shown in Table 6. No correlation was observed between the percentage of cholinesterase appearing in the supernatant and the pH of the medium. It will be seen, however, that those media, namely dilute sodium, ammonium or barium hydroxides or distilled water, in which a high proportion of cholinesterase is extracted into the supernatant, are all lacking in the physiological anions, Cl⁻ or HCO₃⁻. The nature of the cations used, Na⁺, K⁺, NH₄⁺, and Ba⁺⁺ seemed insignificant.

Table 6. *Effect of homogenizing medium on the extraction of rat-brain cholinesterase*(1 in 10 (w/v) homogenates used throughout; activity expressed as $\mu\text{l. CO}_2/0.3 \text{ ml./30 min.}$)

Medium	pH of medium	Enzyme activity in		Percentage of total cholinesterase activity in supernatant
		Whole homogenate	Centrifuged supernatant	
0.025 M-NaHCO ₃	8.6	114	26	23
0.025 M-KHCO ₃	8.6	123	21	18
0.025 M-NH ₄ Cl	5.1	154	46	30
0.9 % (w/v) NaCl	7.2	103	12	12
0.45 % (w/v) NaCl	7.1	117	17	15
0.025 M-NaHCO ₃ + NaOH*	10.5	105	40	38
Glass-distilled water	6.0	101	74	74
NaOH*	10.6	87	76	87
NH ₄ OH*	10.5	104	98	94
Ba(OH) ₂ *	10.6	149	108	74

* Dilute NaOH, Ba(OH)₂ or NH₄OH added to water or, where stated, to 0.025 M-NaHCO₃ to bring to pH 10.5-10.6.

The dependence of the partition of the brain cholinesterase on the level of the anions Cl⁻ and HCO₃⁻ was confirmed by a series of experiments using water, dilute sodium bicarbonate, ammonium hydroxide, sodium hydroxide and sodium hydroxide/sodium bicarbonate mixtures as media in which the concentration of the brain in the homogenate ranged from 5 to 40 % (w/v) (Fig. 1).

Fig. 1. *Effect of dilution of brain homogenates on the degree of cholinesterase extraction.*

The more concentrated brain homogenates will contain a higher concentration of Cl⁻ and HCO₃⁻ derived from the dispersed tissue, and it will be seen that with the concentrated preparations (20 %, w/v) none of the media extracted more than 50 % of the overall cholinesterase activity into the centrifugable supernatant. As the brain concentration is reduced, the anion concentration also falls, and the more dilute the preparation the greater is the percentage of the total cholinesterase present in the supernatant derived from water, NaOH or NH₄OH homogenates. The effect of the HCO₃⁻ ion is demonstrated

strikingly, as it is only with the most dilute homogenate and in the presence of sodium hydroxide that a significant increase in the extraction of cholinesterase into the supernatant is obtained.

As a result of these experiments it was decided to use glass-distilled water (pH 6.0) as the homogenizing medium and a brain concentration of 10 % for the subsequent experiments.

Effect of surface-active agents on rat-brain cholinesterase. An initial problem that presents itself in the purification of cholinesterase from brain is the separation of an enzyme-containing protein fraction from the large amount of lipid material present in this tissue. The supernatant (W) obtained by centrifuging homogenates of brain in water, even though it contains the greater part of the cholinesterase activity, is still opaque. In the hope of obtaining optically clear solutions on which to attempt further purification, the effect of the addition of various surface-active agents on these milky suspensions was next studied (Table 7).

The effect of these substances, present in a final concentration of 1 % (w/v), on the 'solubilization' of these brain supernatants was striking. Of the twenty compounds studied fifteen produced some degree of clarification of the milky supernatant, and eleven of them produced yellowish solutions which were almost optically clear. The degree of solubilization was largely independent of the ionic nature of the surface-active agent, although the non-ionic compounds appeared to be slightly less active. On the other hand, the different ionic types of agent produced very different effects on the cholinesterase activity of the preparation. None of the six non-ionic agents showed any inhibitory influence on the enzymic activity; indeed in each case slightly more than 100 % of the original activity was present. The four cationic agents and seven out of the ten anionic agents, however, caused an almost complete inhibition of the cholinesterase. 'Lissapol LS paste'

Table 7. *Effect of surface-active substances on the cholinesterase in centrifuged supernatants of water homogenates of rat brain*

(All surface-active agents present in final concentration of 1% (w/v). *N*=non-ionic, *A*=anionic, *C*=cationic. × × ×, rapidly and effectively cleared. × ×, cleared, more slowly; ×, slightly cleared.)

Surface-active agent	Ionic nature	Constitution	Degree of 'solubilization'	Cholinesterase activity as percentage original level
Permal COL	<i>A</i>	Na salt of highly sulphonated oil, with fatty alcohol-polyethylene oxide condensate	× ×	1
Calsoene oil, HS	<i>A</i>	Highly sulphonated oil	× × ×	1
Dispersol L	<i>A</i>	Na salt of naphthalenesulphonate with H.CHO in aqueous solution	—	1
Sulphosal	<i>A</i>	Alkyl sulphate	× × ×	1
Teepol	<i>A</i>	Alkyl sulphate	× × ×	0
Permal BX	<i>A</i>	Na salt of alkylated naphthalene-sulphonate	× × ×	3
Lissapol C paste	<i>A</i>	Sodium oleyl sulphate with water	× × ×	10
Lissapol LS paste	<i>A</i>	Sodium oleyl- <i>p</i> -anisididesulphonate with water	×	81, 77
Sodium cholate	<i>A</i>		× × ×	94, 86
Sodium deoxycholate	<i>A</i>		× × ×	53, 66
Cetrimide	<i>C</i>	Cetyltrimethylammonium bromide	—	0
Fixanol C	<i>C</i>	Cetylpyridinium bromide	× × ×	1
Fixanol VR	<i>C</i>	Tetradecylpyridinium bromide	× × ×	1
Lissolamine A50	<i>C</i>	Cetyltrimethylammonium bromide with water	—	2
Lubrol MO*	<i>N</i>	Fatty alcohol-ethyleneoxide condensate	—	101
Lissapol N	<i>N</i>	Polyoxyethylene condensate	×	114
Lissapol Nx	<i>N</i>	Anhydrous Lissapol N	×	108
Crill No. 6	<i>N</i>	Polyoxyethylene sorbitan monolaurate	—	112
Lubrol W	<i>N</i>	Cetyl alcohol-polyoxyethylene condensate	× ×	107
Saponin†	<i>N</i>		×	112

* Insoluble in water, therefore dispersed in supernatant.

† Small amount added.

(sodium oleyl *p*-anisididesulphonate with water), sodium cholate and sodium deoxycholate, although causing some inhibition, did yield active preparations. As 'Lubrol W' (a cetyl alcohol-polyethylene oxide condensate) was the most effective of the non-ionic substances as regards clarification, highly translucent preparations being obtained with it, further work was carried out with this compound and also with cholic and deoxycholic acids. It was shown that 1% Lubrol W did not affect the rates of hydrolysis of acetylcholine, benzoylcholine or acetyl- β -methylcholine, nor the degree of inhibition produced by DFP.

Purification of brain cholinesterase: (a) *In the absence of surface-active agents.* Various attempts were made to purify the supernatant (*W*) obtained after homogenization in distilled water. Fractional precipitation by increasing the acidity resulted in insoluble but fully active precipitates (at about

pH 5.5), and it was not possible to obtain a clear supernatant with appreciable activity as in the case of the rat-heart preparations. Fractional precipitation with ammonium sulphate yielded three distinct protein fractions: (1) precipitated at 7% saturation, and containing 58% of the cholinesterase activity of the whole fraction *W*; (2) precipitated at 23% saturation, and (3) a fraction precipitated at 46% saturation. Both these last fractions contained small amounts of cholinesterase activity.

The precipitate obtained at 7% saturation (*P*₇) was washed once with water to remove excess ammonium sulphate, and could then be resuspended to give a stable white suspension in water. On bringing to pH 5.5 a floccular precipitate formed; on resuspending this in water and enzymic activity was fully recovered; on bringing to pH 12 the precipitate dissolved completely, but was irreversibly inactivated. The neutral aqueous sus-

pension of P_7 was also soluble in the presence of 0.5 % Lubrol W .

This solution retained its full cholinesterase activity, but, in contrast to the stable suspension without Lubrol W , was completely inactivated on bringing to pH 5.5. 0.3 % Sodium deoxycholate behaved similarly, yielding solutions containing 60 % of the original cholinesterase activity, the solution again being inactivated by hydrochloric acid at pH 5.5.

The effect of short periods of digestion with proteolytic enzymes (trypsin, pepsin, papain) on P_7 was also studied; although trypsin caused some measure of solution of the suspension, all three proteinases inactivated the preparation. The neutral aqueous suspension of fraction P_7 showed an activity of 2480 μ l. carbon dioxide/mg. total nitrogen/hr., compared with an activity of 280 μ l. carbon dioxide/mg. total nitrogen/hr. for the original homogenate, i.e. a ninefold purification is achieved by this single ammonium sulphate precipitation. We next attempted the purification of homogenates previously treated with surface-active agents.

(b) *In the presence of 1 % Lubrol W .* On treatment of the milky supernatant (W) from the centrifuged water homogenate of rat brain with 1 % Lubrol W translucent yellowish solutions were obtained. On adjusting the pH to 4.5 a bulky precipitate formed. After centrifuging a clear yellow supernatant was obtained, containing 39 % of the original cholinesterase activity towards acetylcholine. This solution was then brought to pH 8.0, and saturated ammonium sulphate solution added to about 24 % saturation, when the first permanent cloudiness appeared. The preparation was filtered (Whatman no. 42), the precipitate discarded, and the filtrate brought to 50 % saturation. The precipitate which now formed separated on top of the centrifuged solution, and, after decanting the latter, was found to be immediately soluble in water. After dialysis overnight a yellowish solution (SB) was obtained, which contained 25 % of the original cholinesterase activity, and was ten times purer than the original homogenate when assessed on mg. total nitrogen basis.

Attempts at further purification of this preparation by adsorption or reprecipitation were unsuccessful.

Properties of purified preparations of brain cholinesterase. Substrate specificity studies with acetylcholine, benzoylcholine, acetyl- β -methylcholine and tributyrin have been carried out on the purified fraction SB derived from a Lubrol-treated water homogenate of brain. Table 8 shows the relative rates of hydrolysis of these substrates expressed as percentages of the hydrolysis rate of acetylcholine.

It will be seen that while the original brain homogenate readily hydrolysed tributyrin, the purified

fraction SB did not, although retaining its full activity towards acetyl- β -methylcholine. The level of hydrolysis of benzoylcholine is too low to permit any conclusion as to whether this compound is hydrolysed by the acetylcholine-hydrolysing enzyme or not.

Table 8. *Relative rates of hydrolysis of choline esters and tributyrin by a whole homogenate of brain and by the soluble, purified fraction SB*

(ACh rates in each case taken as 100.)

	ACh	BCh	MCh	TB
Whole homogenate	100	6	75	44
Fraction SB	100	4	81	0

The evidence from the above experiments for the presence of a distinct enzyme hydrolysing tributyrin is confirmed by the finding that whereas 1 % Lubrol W has no effect on the cholinesterase activity of these brain homogenates, it produces about 40 % inhibition of hydrolysis of tributyrin.

DISCUSSION

As stated earlier, the purpose of this work was to obtain soluble preparations of true and pseudo-cholinesterases from innervated tissues. Since the highest centrifuging speed which we had available was 4000 rev./min. no claims can be made that the cholinesterase present in the centrifuged supernatants of water homogenates of brain is in true colloidal solution; it is very possible that it is still attached to small particles which would sediment at slightly higher speeds of centrifugation. Our final preparations from heart (F_{65}) and from brain (SB), however, were obtained by the further purification of optically clear solutions, which had themselves been obtained by the previous removal of heavy protein precipitates first by acidification and subsequently by salting-out. As they have not been tested by high-speed centrifugation it is not possible to state that their contained proteins are fully dispersed. From the earlier precipitation stages and the physical appearance of the final preparations (F_{65} and SB), however, we think it reasonable to conclude that we have obtained colloidal solutions of these cholinesterases, although in the case of brain a 'solubilizing' agent is present so that we cannot exclude the possibility that our solution may contain relatively large solubilized lipoprotein or other complexes. Although no high degree of purification of these enzymes has been achieved (30-fold for the heart cholinesterase, and 10-fold for the brain), the partially purified, soluble preparations reveal certain further differences among the tissue cholinesterases, apart from the well known difference in their activity towards benzoylcholine and acetyl- β -methylcholine.

For example, the high rate of hydrolysis of propionylcholine by the purified preparation from rat heart indicates that there is a cholinesterase which belongs neither to the aceto-cholinesterase nor to the butyro-cholinesterase types described by Sturge & Whittaker (1950); while such a classification on grounds of substrate specificity evidently holds for the enzymes in the three tissues (brain, erythrocytes and plasma) studied by these workers, it would seem that we have not yet sufficient evidence to generalize from these cases to the cholinesterases present in other tissues of the body.

The cholinesterases of different tissues may in fact comprise a group of related enzymes showing a range of differences in their specificity towards artificial substrates. Thus, in addition to this enzyme in rat heart which differs from others so far studied in respect of its activity towards propionylcholine, Sawyer (1945) has described a benzoylcholinesterase, and Levine & Suran (1950) have recently reported a further type of cholinesterase in swine serum which is inactive towards both benzoylcholine and acetyl- β -methylcholine.

A further point of difference is shown by the relative resistance of the heart and brain enzymes to digestion by trypsin; while the cholinesterase in rat heart which is active against benzoylcholine is not affected by short periods of tryptic digestion of the crude tissue homogenate, the cholinesterase in brain is inactivated by such treatment. That this is not a peculiarity common to so-called true cholinesterases, however, is shown by the fact that in further preliminary experiments we have found that the cholinesterase in rat diaphragm can be extracted unimpaired by tryptic digestion.

The effect of surface-active agents on brain cholinesterase does not appear to have been studied hitherto, although Huidobro & Atria (1949) have reported on the effects of five different detergents on human serum cholinesterase; all of the compounds studied by them were ionic, and only one, 'Zephiran', a high-molecular alkyl dimethylbenzylammonium chloride, had any effect on cholinesterase activity, causing only approximately 50% inhibition under their conditions. Their results with the serum enzyme seem to be in striking contrast to those obtained here with the brain enzyme, in which eleven out of fourteen ionic surface-active substances caused a complete or almost complete inhibition of activity. Rossiter (1949) has also used surface-active substances (saponin, 'alkyl sulphate' and bile salts) to liberate the esterase from rabbit polymorphonuclear leucocytes, and has found that none of these substances inhibits this enzyme.

The action of these surface-active agents in bringing into solution the brain cholinesterase raises the question of the mechanism of this effect. It is

unlikely that it is a liberation of the enzyme from the brain cells, as in the lysis of red blood cells or in the less drastic action of detergents on intact polymorphonuclear leucocytes (Rossiter, 1949), since our starting material was a centrifuged supernatant of water homogenates of brain, in which, presumably, the larger formed elements will no longer be present. It is therefore more probably a true solubilizing effect on lipoprotein complexes suspended in solution and containing the cholinesterase. It should be pointed out that not only are the centrifuged homogenates grossly opaque unless clarified by a surface-active substance, but that the partially purified precipitate, P_7 , salted out with only 7% saturated ammonium sulphate, is also insoluble in water at pH 7.0 unless Lubrol W or sodium deoxycholate is present. We think that these findings are consistent with the view that the brain cholinesterase may itself be a lipoprotein, or that it occurs in intimate association with other lipoprotein complexes. If this should be true it may be expected to influence the relative ease of penetration of different cholinesterase inhibitors to the enzymes in brain and, for example, in serum, quite apart from any differences of affinity that such inhibitors may have for the active centres of the proteins in question.

SUMMARY

1. Soluble preparations of cholinesterases from rat heart and brain have been obtained, the enzyme from heart being purified approximately 30-fold and the brain enzyme approximately 10-fold.
2. Specificity and inhibition studies on the heart preparation have revealed the presence of at least two cholinesterases and an ali-esterase. The cholinesterase present in greatest amount exhibits certain differences from both the true and pseudo-cholinesterases described hitherto.
3. A study of the effect of twenty surface-active substances on the extraction and activity of rat-brain cholinesterase has been made. Out of fourteen different ionic surface-active substances eleven caused a complete or almost complete inhibition of activity. All six non-ionic substances left the activity unimpaired.
4. The mechanism of the 'solubilizing' action of certain of these surface-active agents on the brain enzyme is discussed.

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The Relative Metabolic Inertia of Tendon Collagen in the Rat

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The protein collagen, various polysaccharides and other substances have been known for many years to be present in the extracellular spaces of the body. Changes in these tissue components in disease have been demonstrated by pathologists, and recent clinical and anatomical studies on rheumatism and allied diseases have greatly stimulated further work on the 'ground substances' of the extracellular spaces. Very little is known, however, about the metabolism of these substances even in the normal subject. Information, therefore, on the metabolism of collagen would seem to be of interest. This protein is widely distributed throughout body tissues and is found in large amounts in bone, cartilage, tendons and skin. At an estimate, about one-third of the total mass of the protein of the mammalian body consists of collagen and it is thus important to establish whether this extracellular protein is, like most intracellular proteins, constantly broken down and resynthesized, even in the adult animal. Collagen is particularly rich in glycine, and glycine labelled with ^{14}C in the methylene group was therefore used to determine the turnover of collagen in three series of rats. For preliminary communication, see Perrone & Slack (1951).

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EXPERIMENTAL

General

Treatment of animals and administration of labelled glycine. Male albino rats of the Institute stock were used throughout. Series A consisted of four rats with body weight ranging from 358 to 390 g.; their ages were not known exactly, but all animals of this group were more than 14 months old. Series B consisted of five young adults (initial body wt. 203–207 g.), whilst series C consisted of five young rats with initial body weight ranging from 50 to 80 g. All animals were apparently in normal health and had not been used for other experiments. Throughout the experiment they were given the Institute stock diet. The labelled glycine, which was kindly supplied by Dr H. R. V. Arnstein, had an activity of 1 $\mu\text{C}/\text{mg.}$; the required dose was mixed with about 30–50 mg. non-isotopic glycine and fed in the diet over a period of 3 days at the beginning of each experiment. The rats of groups A and B each received 10 $\mu\text{C.}/100$ g. initial body weight. Three of the rats of group C (nos. 99, 100 and 104) received 40 $\mu\text{C.}/100$ g. initial body weight, whilst the other two (nos. 115 and 116) received 6.25 $\mu\text{C.}/100$ g. initial body weight. The animals were killed by stunning at varying times during the experiment.

Growth of animals. The old rats of group A showed no marked or consistent change in body weight (Table 1). The young adults of series B showed a steady increase of weight during the experiment, the rate varying somewhat with different animals (Table 2). The young rats of series C increased their body weight very markedly during the experi-