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Cholinesterases of the Tissues and Sera of Rabbits

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Ten years ago Mendel and associates (Mendel, Mundell & Rudney, 1943) clearly distinguished between two types of cholinesterase (ChE) in mammalian tissues, and utilized the selective substrates acetyl- β -methylcholine (MeCh) and benzoylcholine (BzCh) to measure the activities of 'true' and 'pseudo'-ChE, respectively. Other investigators have employed different nomenclature and criteria for this purpose, including over-all substrate patterns (Nachmansohn & Rothenberg, 1945; Augustinsson, 1948, 1949; Goldstein, 1951), differences in kinetics of hydrolysis (Alles & Hawes, 1940) and selective inhibition (Hawkins & Gunter, 1946; Adams & Thompson, 1948; Hawkins & Mendel, 1949). By means of these methods, the distributions of the two enzymes have been studied extensively in rat tissues (Sawyer & Everett, 1947; Ord & Thompson, 1951); their occurrence in cat tissues has been investigated histochemically (Koelle, 1950, 1951).

In recent years, evidence has accumulated for the existence of several choline ester-hydrolysing enzymes which do not conform with Mendel's classical descriptions of true and pseudo-ChE's. Sawyer (1945) reported an enzyme of guinea pig and rabbit liver which splits BzCh but not acetylcholine (ACh), and assigned to it the designation 'benzoylcholinesterase' (BzChE). Evidence of a similar enzyme has been noted in guinea pig kidney (Nachmansohn & Rothenberg, 1944) and beef kidney (Gunter, 1946; Blaschko, Chou & Wajda, 1947). The occurrence and characteristics of other choline ester-hydrolysing enzymes have been reviewed by Augustinsson (1948) and Whittaker (1951).

Ellis and associates (Ellis, Sanders & Bodansky, 1947; Ellis, 1947) found that the plasma of certain rabbits contains BzChE in addition to true or specific ChE, whereas in the plasma of others only the latter ChE is present in significant amounts. This observation was confirmed by Levine and associates (Levine, Hoyt & Suran, 1950). Levy (1951) reported on the presence of several other esterases in rabbit plasma, including a possible trace of pseudo-ChE.

With the exception of the ruminants, the plasma of most species contains predominantly non-specific or pseudo-ChE and varying amounts of specific or true ChE (Gunter, 1946; Augustinsson, 1949). In the cat, Goldstein (1951) has shown that the ChE's of the plasma are identical with those of the liver and gastric mucosa. Studies of the distribution (Koelle, 1950, 1951) and effect of inhibition (Koelle, Koelle & Friedenwald, 1950) of the non-specific ChE of the cat's ileum have indicated that this enzyme has an important role in the regulation of intestinal motility. The same conclusion was reached by Burn and associates (Burn, Kordik & Mole, 1952) in the course of an investigation of the effects of irradiation on rats. Therefore, it seemed of interest to determine whether the absence of appreciable amounts of an ACh-hydrolysing non-specific ChE in rabbit plasma was reflected by a similar situation in the gastro-intestinal tract and other tissues. Information on this point might be expected to have a bearing on the function of non-specific ChE in other species as well. To this end, the objects of the present study have been: (1) the determination of the rates of hydrolysis of ACh and other choline esters by the plasma and tissues of

rabbits with and without appreciable plasma BzChE activity; (2) the partial purification of some choline ester-hydrolysing enzymes of rabbit plasma and tissues to determine their individual substrate patterns; (3) the identification of the partially purified enzymes with those of the plasma and tissues by means of the selective action of inhibitors; and (4) the histochemical localization of the choline ester-hydrolysing enzymes in certain tissues of the rabbit.

Terminology in the field of ChE's has become increasingly confusing, as the foregoing discussion would suggest. In the present report, the term 'BzChE', as defined by Sawyer (1945), is used for the chief BzCh-hydrolysing enzyme of the sera of certain rabbits in keeping with the reports of the authors cited. The other ChE's are referred to as 'aceto-ChE' (true, specific or acetyl-ChE) and 'butyro-ChE' (pseudo- or non-specific ChE) in accordance with the general resemblance of their substrate patterns to those of human erythrocytes or plasma, respectively (Sturge & Whittaker, 1950), although in the present study no attempt was made to determine the substrates exhibiting maximal rates of hydrolysis. This classification carries no implications concerning natural substrates or functions.

EXPERIMENTAL

I. *The hydrolysis of choline esters by the sera, intestines and livers of rabbits*

Of the thirty rabbits used during the present investigation, the sera of eleven hydrolysed BzCh at significant rates. As noted by previous investigators, there was no correlation between this characteristic and sex or breed. The rates of hydrolysis of BzCh, butyrylcholine (BuCh), ACh and MeCh by the sera and exsanguinated intestines and livers were determined for four rabbits of each type.

Materials and methods. Rabbits were anaesthetized with ether, and 50–100 ml. of blood were taken by cardiac puncture and allowed to clot. The serum was removed by

centrifugation. The abdominal aorta was exposed and a cannula inserted, directed cranially, immediately central to the bifurcation. The thoracic aorta was rapidly exposed and clamped just above the diaphragm, the right ventricle incised, and approximately 1 l. of 0.85% NaCl solution allowed to flow by gravity through the cannula. At the end of the perfusion, requiring approximately 5 min., the small intestine and liver appeared nearly free from blood. The whole liver was removed, freed from the gall bladder, blotted and weighed. The entire small intestine was removed, cleaned of adherent fat, slit open, cut into short segments, and passed rapidly through three saline rinses before being blotted and weighed. Both organs were minced with scissors then homogenized in the Waring Blender for 5 min. with a mixture of ice and distilled water to give a final suspension of 1 part wet weight in 4 vol. Homogenates and sera were stored at approximately 5°.

Rates of hydrolysis were determined in duplicate by a conventional modification of Ammon's (1933) method, using the Warburg apparatus. The final concentrations of substrates, tipped in from the side arm, were: 0.006M-BzCh, 0.01M-BuCh, 0.005M-ACh and 0.03M-MeCh. The volume of enzyme preparation was varied according to its activity in the presence of a given substrate. The vessels contained a final concentration of 0.03M-NaHCO₃ and 0.033M-MgCl₂ in a total volume of 3.0 ml., and were equilibrated with 5% CO₂-95% N₂ (v/v). Rates of hydrolysis were determined on the basis of CO₂ production during the 30 or 60 min. period beginning 10 min. after the addition of substrate. Values were corrected for non-enzymic hydrolysis of substrate and acid production in the absence of substrate.

Results. Table 1 lists the individual rates of hydrolysis by the serum, small intestine and liver of each rabbit, and the average values for the rabbits with and without significant serum BzCh-hydrolysing activity. The hydrolysis of BuCh by the sera in general paralleled that of BzCh, although the ratios of the velocity of hydrolysis differed significantly for the two groups. A relatively constant ratio for the hydrolysis of ACh and MeCh held throughout both groups, and there was no significant difference between the absolute values of the two groups for these substrates. The average rates of hydrolysis of all four substrates by the intestinal homogenates

Table 1. *Hydrolysis of choline esters by rabbit sera, intestines and livers*

Rabbit	$\mu\text{moles CO}_2/\text{g. (or ml.)}/\text{hr.}$											
	Sera				Intestines				Livers			
	BzCh	BuCh	ACh	MeCh	BzCh	BuCh	ACh	MeCh	BzCh	BuCh	ACh	MeCh
A	1.0	12.5	22.8	10.1	—	—	—	—	63.0	191	182	78.1
B	1.1	8.1	22.3	8.0	7.2	51.4	109	41.6	18.0	142	260	110
E	0.7	7.1	21.4	7.1	4.5	68.0	105	36.7	15.8	33.4	112	53.7
F	1.1	9.2	31.3	10.9	6.7	96.0	129	35.7	8.8	185	195	75.5
Av.	1.0	9.2	24.5	9.0	6.1	72.0	114	38.0	26.4	138	188	79.0
C	16.8	61.2	21.6	7.6	—	—	—	—	60.8	245	69.1	26.8
D	13.0	40.1	22.2	6.4	11.4	79.0	125	47.0	97.5	159	230	110
G	12.7	51.0	30.6	10.2	10.9	89.9	110	34.4	21.5	69.7	81	35.2
H	15.7	55.5	22.2	8.4	5.2	61.2	114	19.4	67.5	192	119	52.2
Av.	14.6	51.9	24.2	8.1	9.2	77.0	116.5	33.6	61.8	167	124	56.3

were approximately the same for the two groups. Again, the rates for ACh paralleled those for MeCh, and the same was true for BzCh and BuCh. The ratio between BzCh and BuCh hydrolysis more closely approached that seen in the sera showing insignificant BzCh hydrolysis than in the sera with high BzCh-hydrolysing activity. The hydrolysis of BzCh by the liver homogenates tended to reflect the activity of the sera, as was noted by Ellis (1947). However, rabbits *A* and *G* were notable exceptions in their respective groups. Furthermore, there was no suggestion of constancy in the ratio between BzCh and BuCh hydrolysis. As with the sera and intestines, the ratio for ACh and MeCh hydrolysis was relatively constant.

The parallelism between the hydrolysis of ACh and MeCh by each of the preparations suggests that the same enzyme was responsible for most of these activities; by Mendel's criteria, this would be considered a 'true' (aceto-) ChE. The differences in the ratios of hydrolysis of BzCh and BuCh by the sera of the two groups indicated that more than one enzyme hydrolyses one or both of these esters, a possibility which Levy (1951) suggested. One of these serum enzymes might be identical with an enzyme in the intestines of both groups which hydrolyses the two substrates. It seems unlikely that the intestinal enzyme corresponds with the serum BzChE which is high in one group, because of the difference in the ratios of BzCh and BuCh hydrolysis by those sera and by the intestines of both groups. The great variations among the hydrolysis ratios and absolute values with BzCh and BuCh by the livers affords little evidence concerning the possible identity of the enzymes concerned with those of the sera or intestines.

II. The partial purification and substrate patterns of rabbit ChE's

Ellis (1947) and Levy (1951) reported unsuccessful attempts to separate the BzCh- and atropine-hydrolysing activities of rabbit plasma by fractionation with ammonium sulphate, although they presented convincing evidence that different enzymes acted on these substrates. The latter author was likewise unable to separate, by the same technique, the plasma enzymes responsible for the hydrolysis of BzCh and MeCh. In developing a histochemical method for localizing ChE's in cat tissues, it was found that specific (aceto-) and non-specific (butyro-) ChE's were precipitated to considerably different degrees at various concentrations of sodium sulphate (Koelle, 1951). The same procedure was therefore applied to rabbit serum and a homogenate of ileum.

Method. To each of a series of centrifuge tubes were added 2.0 ml. of pooled rabbit serum with high BzCh-hydrolysing activity, maleate buffer (Temple, 1929), and varying

amounts of distilled water and 40% (w/v) anhydrous Na_2SO_4 solution to produce a final volume of 10.0 ml. and pH of 8.65 ± 0.20 , with Na_2SO_4 concentrations ranging from 12 to 28%. After standing for 30 min. at room temperature, the tubes were centrifuged for approximately 20 min. at 4000 rev./min. The clear solutions were removed from the precipitates and discarded after determining the pH values by means of a Beckman glass-electrode instrument. The precipitates were dissolved in 0.85% NaCl solution and brought to a volume of 5.0 ml. It was found that amounts of Na_2SO_4 and buffer equivalent to those carried over with the

Fig. 1. The precipitation of BzCh- and MeCh-hydrolysing activities of pooled rabbit sera (upper chart) and of BuCh- and MeCh-hydrolysing activities of the supernatant of a homogenate of rabbit ileum (lower chart) at different concentrations of Na_2SO_4 . Serum precipitations done at pH 8.65 ± 0.20 , ileum homogenate precipitations at pH 6.00 ± 0.20 .

precipitates did not cause significant changes in apparent enzymic activity when added directly to the Warburg vessels; hence, dialysis of the redissolved precipitates was not performed. Samples were assayed for BzCh and MeCh hydrolysis by the method described in the preceding section; 2.0 ml. of pooled plasma brought directly to 5.0 ml. with saline solution served as the control. Essentially the same procedure was conducted using the supernatant fluid of a centrifuged sample of the homogenate of ileum *F* as the source of enzyme. Here, the mixtures were adjusted to pH 6.00 ± 0.20 , and BuCh and MeCh were used as substrates in the assays. In running recovery tests of serum ChE's at this pH, the fineness of the resulting precipitates rendered separation by centrifugation impracticable. Accordingly, precipitates were collected on no. 3 Whatman filter paper and recovered quantitatively by immersing the papers in saline solution in graduated centrifuge tubes overnight, bringing to the desired final volume with

allowance for the volume of the filter paper, comminuting, centrifuging, and decanting the enzyme solutions.

Results. The results of the Na_2SO_4 -precipitation recovery tests are shown in Fig. 1. The values obtained when the serum enzymes were precipitated at $\text{pH } 6.0 \pm 0.2$ differed only slightly from those illustrated for $\text{pH } 8.65 \pm 0.20$.

The percentage recoveries of the BzCh-hydrolysing activity of serum and the BuCh-hydrolysing activity of the ileum differed considerably from the recoveries of the MeCh-hydrolysing activity at concentrations of Na_2SO_4 from 8 to 20%. These differences permitted the separation of the enzymes concerned, as described below.

Methods. A 93.0 ml. sample of sera with high BzCh-hydrolysing activity, pooled from three rabbits, was used for the separation of the enzyme responsible for this effect from the MeCh-hydrolysing enzyme. At each stage, the mixture was brought to $\text{pH } 8.9 \pm 0.6$ by the addition of 40% Na_2SO_4 solution and maleate buffer. Each step is listed below in terms of the final concentration of Na_2SO_4 , the time it was allowed to stand at room temperature prior to centrifugation, and the portion used in the succeeding step (precipitate or solution). All precipitates were dissolved in 0.85% NaCl and stored in the refrigerator until being brought to room temperature for subsequent precipitation. Steps: (1) 16%, 20 min., solution; (2) 24%, 20 min., precipitate; (3) 20%, 30 min., solution; (4) 24%, 30 min., precipitate; (5) 20%, 30 min., solution; (6) 24%, 30 min., precipitate. The final precipitate was dissolved and brought to a total volume of 20.0 ml. with 0.85% NaCl and designated as 'serum BzChE'.

Serum (93 ml.), pooled from three rabbits with low BzCh-hydrolysing activity, was used for the isolation of the MeCh-hydrolysing enzyme. The procedure employed was similar to that described above, and the steps were as follows: (1) 16%, 20 min., precipitate; (2) 16%, 20 min., precipitate; (3) 8%, 30 min., solution; (4) 16%, 30 min., precipitate; (5) 16%, 30 min., precipitate. The final precipitate was dissolved and brought to a total volume of 20.0 ml. with 0.85% NaCl and designated as 'serum aceto-ChE'.

The homogenates of the small intestines of four rabbits, prepared as described in the preceding section, were pooled and centrifuged, and a 193 ml. portion of the supernatant was used for the isolation of the BuCh-hydrolysing enzyme. Subsequent to the first step, precipitates were collected on Whatman no. 3 filter paper; otherwise, the procedure was similar to that described above. Steps: (1) 5%, 30 min.,

solution; (2) 25%, 30 min., precipitate; (3) 15%, 120 min., solution; (4) 25%, 16 hr., precipitate. The final precipitate was dissolved and brought to a total volume of 42.0 ml. with 0.85% NaCl and designated as 'intestinal butyro-ChE'.

The protein concentrations of the starting materials and final preparations were determined by the trichloroacetic acid precipitation method described by Nachmansohn & Rothenberg (1947).

Velocities of hydrolysis at various substrate concentrations were determined by the same method as described in §I. CO_2 production was followed for 5–60 min., depending upon the period during which rates remained constant. As a basis for comparison, similar determinations were conducted with a preparation of horse serum non-specific (butyro-) ChE carried to the third stage of purification by the method of Strelitz (1944).

Results. Relatively low degrees of purification, in terms of enzymic activity/mg. protein, were achieved by the foregoing procedures (Table 2). However, the fractionations gave nearly complete separation of MeCh- from BzCh- or BuCh-hydrolysing activity, as indicated by the accompanying substrate-activity curves (Fig. 2). The curves obtained with the serum aceto-ChE (Fig. 2a) are characteristic of those reported for aceto- (specific) ChE in other species (Augustinsson, 1949). ACh was hydrolysed at the greatest velocity of the four esters tested, and showed a sharp peak at a concentration of 0.003M. MeCh was hydrolysed at increasing rates with increasing substrate concentration; the hydrolysis of BuCh and BzCh was insignificant at all concentrations tested. The patterns for 'serum BzChE' (Fig. 2b) and 'intestinal butyro-ChE' (Fig. 2c) are quite similar and present a marked contrast with that for horse serum butyro-ChE (Fig. 2d). All three enzymes hydrolysed BuCh at the greatest velocity and BzCh at considerably lower rates. However, the curve for ACh with the horse serum enzyme occupies an intermediate position, whereas both the rabbit enzymes split ACh at rates considerably below those for BzCh. None of the three hydrolysed MeCh significantly. In order to test the possibility that the hydrolysis of ACh by the rabbit enzyme preparations might have been due to the presence of another esterase, summation studies were conducted with ACh, BuCh and BzCh at a concentration of 0.01M. In Table 3 the sums of the

Table 2. Activities of crude and partially purified enzyme preparations

Enzyme preparation	Substrate	Protein	Activity	Activity per	Concentration
		(mg./ml.) (1)	($\mu\text{moles CO}_2$ /ml./hr.) (2)		
A Pooled sera	BzCh	74.0	7.69	0.104	3.4
B BzChE	BzCh	11.2	4.01	0.358	
A Pooled sera	MeCh	74.4	7.85	0.105	6.7
B Aceto-ChE	MeCh	16.7	11.7	0.701	
A Supernatant of intestinal homogenates	BuCh	15.5	9.11	0.588	2.9
B Butyro-ChE	BuCh	3.8	6.52	1.72	

individual rates of hydrolysis and the rates observed with the mixtures are listed for each pair of substrates. In all cases, the observed rates were considerably below the sums for the substrates individually, and with one exception lower than the rate for the more rapidly hydrolysed ester of each pair.

neither is inhibited by either substrate (Adams, 1949); furthermore, saturation concentrations of BuCh were not used.

It will be noted that the ratio of BuCh to BzCh hydrolysis by the whole intestinal homogenates (Table 1) considerably exceeded that with the

Fig. 2. Substrate concentration-activity patterns of partially purified rabbit serum aceto-ChE (a) and BzChE (b), rabbit intestinal butyryl-ChE (c) and horse serum butyryl-ChE (d).

Table 3. Summation studies of the hydrolysis of choline esters by purified rabbit and horse enzymes

Enzyme	Hydrolysis rates (μ moles CO_2 /ml./hr.)								
	ACh	BuCh	BzCh	ACh + BuCh		ACh + BzCh		BuCh + BzCh	
	(1)	(2)	(3)	(1) + (2)	Obs.	(1) + (3)	Obs.	(2) + (3)	Obs.
Serum BzChE	1.88	9.90	3.50	11.78	9.30	5.38	3.34	13.40	5.14
Intestinal butyro-ChE	3.34	12.20	4.60	15.54	11.80	7.94	4.98	16.80	8.04
Horse serum butyro-ChE	536	1550	230	2086	1350	766	208	1780	314

(The value for the combination of ACh and BzCh with intestinal butyryl-ChE is slightly in excess of that for BzCh alone.) These data indicate that in each case the same enzyme is responsible for the hydrolysis of all three substrates. However, it should be noted that this type of study is not completely conclusive, since it is based on the assumption that if more than one enzyme is present,

partially purified intestinal enzyme in the same range of substrate concentration (Fig. 2c). If it is assumed that the same enzyme in each type of preparation was chiefly responsible for the hydrolysis of both esters, an explanation for the discrepancy must be sought. One possibility is that an activator or inhibitor which affected the rate of hydrolysis of one substrate more than that of the other was

removed during the purification; the matter is under investigation at present.

The close resemblance between the substrate patterns of the partially purified serum BzChE and intestinal butyro-ChE suggests that the two enzymes are identical, in spite of the differences in the ratios of BzCh and BuCh hydrolysis found with the whole preparations. This question was investigated further in the inhibition studies which follow.

III. The effects of inhibitors on the choline ester-hydrolysing enzymes of rabbit sera and tissues

The sensitivity to eserine of both aceto- and butyro-ChE's, as compared with other esterases, has been demonstrated by Mendel and associates (Mendel & Rudney, 1943; Myers & Mendel, 1949). Diisopropyl fluorophosphonate (DFP) has been shown to inhibit most butyro-ChE's at lower concentrations than those required for the inhibition

both BzChE and butyro-ChE activities, and 0.03 M-MeCh for aceto-ChE. In addition to the enzyme preparations described above, a homogenate was prepared from the pooled superior cervical ganglia of four rabbits by grinding with powdered silica and distilled water in a porcelain mortar and bringing to a final concentration of 29 mg. wet weight of ganglia/ml. It has been shown that the degree of ChE inhibition obtained with TEPP *in vitro* is modified by the concentration of enzyme and the presence of proteins and other tissue constituents which can combine with the inhibitor (Hobbiger, 1951). Hence, results with the purified preparations and the various homogenates probably cannot be considered strictly comparable. However, inhibition studies conducted with the three reversible inhibitors and a specimen of pooled rabbit serum indicated that over a ten-fold range of concentration of enzyme and protein, the differences of inhibition obtained (Table 4) did not approach those seen with the different preparations of enzymes. This factor therefore probably played a minor role in the results obtained, with the probable exception of the ganglion homogenate (see below).

Table 4. Effect of enzyme concentration on inhibition by reversible inhibitors

(All determinations conducted with pooled serum containing high BzChE activity, using 0.01 M-BuCh as substrate; CO₂ production measured during 70 min. period for A, 40 min. for B, 10 min. for C.)

Inhibitor	Concentration (M)	Percentage control activity		
		A 0.1 ml. serum	B 0.3 ml. serum	C 1.0 ml. serum
Eserine	10 ⁻⁵	38.4	39.0	54.9
Nu-1250	10 ⁻⁶	86.5	76.9	78.1
Nu-683	3 × 10 ⁻⁷	45.5	43.5	58.2

of aceto-ChE's (Hawkins & Mendel, 1947). The neostigmine analogues Nu-683 (the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide) and Nu-1250 (the *N-p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide) inhibit selectively butyro- and aceto-ChE's, respectively (Hawkins & Gunter, 1946; Hawkins & Mendel, 1949). The effects of the foregoing inhibitors on the initial and purified enzyme preparations were studied in an attempt to clarify further their identities.

Method. ChE determinations were conducted as described in § I. DFP was prepared as a 0.1 M stock solution in anhydrous propylene glycol; the other inhibitors were stored in the refrigerator as 0.01 M-aqueous stock solutions, freshly prepared each week. Dilutions were prepared immediately prior to use and were added to the enzymes in the main wells of the vessels approximately 30 min. before tipping in the substrates; during half of this time they were at room temperature and the other half at the temperature of the water bath (38°). In determinations with the irreversible inhibitor DFP and the purified enzyme preparations, 0.006 M-BzCh and 0.01 M-BuCh were employed as substrates for the serum aceto-ChE and intestinal butyro-ChE, respectively. However, in the presence of the reversible inhibitors, the inhibition of BzChE was markedly influenced by the substrate employed (Table 4). Hence, BuCh was used throughout for measuring the inhibition of

Results. The partially purified serum BzChE and intestinal butyro-ChE were inhibited practically to the same degrees by DFP over a wide range of concentrations (Table 5). The serum aceto-ChE was

Table 5. The inhibition of partially purified rabbit ChE's by DFP

DFP concentration (M)	Percentage control activity		
	Serum BzChE (BzCh)	Intestinal butyro-ChE (BuCh)	Serum aceto-ChE (MeCh)
10 ⁻⁶	0	2	0
3 × 10 ⁻⁷	1	0	10
10 ⁻⁷	6	7	46
3 × 10 ⁻⁸	31	24	80
10 ⁻⁸	72	74	90

more resistant to the inhibitor, although the difference was not as marked as with the specific and non-specific ChE's of other species (Hawkins & Mendel, 1947). In the presence of the three reversible inhibitors, the intestinal butyro-ChE was considerably more inhibited than the serum BzChE. The concentrations at which the differences were most marked were selected for comparison with the whole homogenates and sera (Table 6). The serum aceto-ChE exhibited characteristic sensitivity to inhibition by eserine and Nu-1250 and relative resistance to Nu-683.

Table 6. *Inhibition of partially purified cholinesterases and cholinesterases of sera and homogenates of rabbit tissues*

Enzyme	Substrate	Percentage control activity			
		Eserine (10 ⁻⁵ M)	Nu-1250 (10 ⁻⁶ M)	Nu-683 (3 × 10 ⁻⁷ M)	Nu-1250 (10 ⁻⁸ M)
Partially purified:					
Serum aceto-ChE	MeCh	1	0	62	18
Serum BzChE	BzCh	84	87	57	100
Serum BzChE	BuCh	41	64	50	97
Intestinal butyro-ChE	BuCh	3	19	15	62
Serum A	BuCh*	2	8	5	13*
Serum B	BuCh	0	4	7	16
Serum C	BuCh	47	83	67	15
Serum D	BuCh	42	77	52	17
Liver A	BuCh	8	68	55	17
Liver B	BuCh	9	83	12	21
Liver C	BuCh	13	71	34	25
Liver D	BuCh	35	82	80	22
Ileum B	BuCh	5	31	20	16
Ileum D	BuCh	13	15	16	13
Sup. cerv. ganglia	BuCh	0	3	2	18

* With sera and homogenates, butyrylcholine was used as substrate in presence of all inhibitors excepting 10^{-8} M-Nu-1250, where acetyl- β -methylcholine was used.

The hydrolysis of MeCh by the sera and various homogenates was inhibited practically to the same extent by Nu-1250 as was the activity of the purified preparation of serum aceto-ChE. This indicates that the same type of aceto-ChE is present in all the tissues studied.

The BuCh-hydrolysing activities of the sera of rabbits A and B (insignificant BzChE activity) and of the ileum of a rabbit of each group (B and D) were inhibited approximately to the same extent by the three reversible inhibitors as was the purified intestinal butyro-ChE, suggesting the common identity of the enzyme concerned. The degrees of inhibition of the ganglionic BuCh-hydrolysing enzyme were somewhat greater, although this might have been due to the extremely small amount of tissue present in the reaction mixtures.

As would be expected, BuCh hydrolysis by sera C and D (high BzChE activity) was inhibited to the same degree as the partially purified serum BzChE preparation.

The inhibition of BuCh hydrolysis by the liver homogenates followed the pattern of neither of the partially purified BuCh-hydrolysing enzymes. Three of the four homogenates were relatively sensitive to eserine, all were relatively resistant to Nu-1250, and wide variation of inhibition was encountered in the presence of Nu-683. These findings confirm the indication of the earlier part of the study, that the livers contain at least one BuCh-hydrolysing enzyme distinct from both the purified preparations.

IV. *The histochemical localization of the ChE's of rabbit tissue*

The histochemical localizations of specific (aceto-) and non-specific (butyro-) ChE's in several tissues of the cat have been described and discussed in terms of their possible physiological roles (Koelle, 1951). The present finding of the relatively low rate of hydrolysis of ACh by the butyro-ChE of the rabbit tissues examined made it of interest to determine its cellular localizations, as well as those of the aceto-ChE.

Method. The procedure employed was similar to that described for cat tissues (Koelle, 1951) with the modifications mentioned below. It was found in preliminary Warburg determinations that the purified enzyme preparations hydrolysed acetylthiocholine (AThCh) and butyrylthiocholine (BuThCh), the histochemical substrates, at considerably greater velocities than their respective oxygen analogues, and that the relative substrate selectivities of the aceto- and butyro-ChE's remained the same with the thio-esters. Fresh frozen sections of ileum, jejunum and superior cervical ganglion were cut at 10μ and incubated for 60 min. in media containing AThCh (following preliminary treatment with 10^{-8} M-DFP for 30 min.) or BuThCh, for the demonstration of aceto- or butyro-ChE's respectively. The left superior cervical ganglia of four rabbits (two with high, two with low serum BzChE) were examined 14 days after sectioning of the preganglionic trunks; the right ganglia of the same animals were sectioned and stained at the same time. All storage and incubation media contained a final concentration of 24% Na_2SO_4 to eliminate enzymic diffusion artifacts. When 10^{-5} M-eserine was incorporated in the incubation medium containing BuThCh or

10⁻⁶M-Nu-1250 was included with the AThCh medium, sections of all the above tissues remained unstained, confirming the identity of the stained areas as sites of butyryl-ChE and acetyl-ChE activities, respectively. Following precipitation of CuS, the sections were gold-toned to intensify staining.

Results. The tissues examined from rabbits with high or low serum BzChE activities were identical in respect to the distributions and activities of acetyl- and butyryl-ChE's.

heavily stained fibres throughout the ganglion as ramifications of the preganglionic trunk was confirmed by their absence in the ganglia which had undergone preganglionic sectioning 2 weeks previously (Fig. 3b). As in the cat, butyryl-ChE activity was confined to the glial and sheath cells (Fig. 3c) and showed no significant difference in the denervated ganglia.

The distributions of the ChE's in the ileum and jejunum were similar, and also exhibited certain

Fig. 3. Histochemical localizations of ChE's of rabbit superior cervical ganglion. (a) Normal ganglion, aceto-ChE; (b) opposite ganglion of same rabbit, preganglionic trunk sectioned 14 days previously, aceto-ChE; (c) normal ganglion, butyryl-ChE.

Fig. 4. Histochemical localization of ChE's of rabbit intestine. Transverse sections. (a) Jejunum, aceto-ChE; (b) jejunum, butyryl-ChE; (c) ileum, butyryl-ChE.

In the superior cervical ganglion, the two types of enzyme showed the same general distributions as noted previously in the cat, with one quantitative difference. In the latter species, only occasional ganglion cells showed significant aceto-ChE activity, and most of the enzyme was associated with the preganglionic fibres and their ramifications (Koelle, 1951). In the rabbits, practically all the ganglion cells exhibited moderate aceto-ChE activity, although occasional cells stood out as having much higher activity (Fig. 3a). The identification of the

quantitative differences from the distribution patterns previously reported in the cat. In both species, aceto-ChE was found in the ganglion cells of the myenteric and submucosal plexuses and in the nerve fibres running between the plexuses and within the muscular layers, submucosa and villi. In the rabbit, aceto-ChE was also present in low concentrations in cells throughout the muscular layers and around the plexuses which appeared identical with the so-called interstitial cells (Fig. 4a). The latter elements seemed to represent the chief site of

butyro-ChE activity in the rabbit intestine, whereas the smooth muscle fibres showed relatively low activity. In addition, the enzyme was present in varying concentrations in the ganglion cells and in the mucosal cells (Fig. 4b, c). In the cat's ileum, much greater concentrations of non-specific or butyro-ChE were found in the membranes of the muscle fibres themselves, particularly in the longitudinal layer.

DISCUSSION

The foregoing findings indicate that rabbit tissues and sera contain an aceto-ChE which is similar in its properties and distribution to those of other species. The chief butyro-ChE of the intestinal tract and superior cervical ganglion resembles the corresponding enzyme of other mammals studied, in respect to its susceptibility to inhibitors and qualitative distribution, but differs in its extremely low velocity of ACh hydrolysis. In addition, the presence of a BzChE in the serum of certain rabbits has been confirmed, and evidence has been obtained for the presence in liver of at least one more enzyme, distinct from the three mentioned, which hydrolyses choline esters at significant rates.

The physiological significance of aceto-ChE has been reviewed exhaustively, and the present results contribute no evidence to modify existing concepts of its function. Of greater interest are the questions raised by the substrate patterns obtained with rabbit butyro-ChE and BzChE. In view of their total substrate patterns, previous investigators (Augustinsson & Nachmansohn, 1949) have minimized the likelihood that the butyro-ChE's of other species function primarily for the hydrolysis of ACh; present findings in the rabbit strengthen this viewpoint. References cited in the introduction indicate that in the gastro-intestinal tract butyro-ChE is of importance in modifying motility. In other autonomic effector organs in the rat, it is present in far greater concentrations than aceto-ChE (Ord & Thompson, 1950). The question thus arises as to the physiological substrate and function of this enzyme. Chang & Gaddum (1933) originally subjected extracts of various tissues to a number of pharmacological assays which indicated that ACh was the chief choline ester present. However, they stressed the limitations imposed on the identification by the presence of interfering substances in tissues and by the lack of specificity of most of the tests employed. Subsequent studies of the ACh content of various tissues have been based largely upon assays with a single test-object, usually the eserized frog's rectus muscle, which does not distinguish between ACh and other choline esters or closely related compounds. It is of interest to note in this respect that propionylcholine has recently

been identified in ox spleen by a chromatographic method (Banister, Whittaker & Wijesundera, 1951) and a ChE which hydrolyses this ester with maximal velocity has been obtained from the rat's heart (Ord & Thompson, 1951). Likewise, Nachmansohn and associates (Nachmansohn, Hestrin & Voripaieff, 1949) presented evidence that an acetylating system of rabbit brain forms a compound with ACh-like biological activity which is chemically distinct from ACh. These findings indicate that the cholinergic mediator may not consist of ACh alone but of ACh and one or more related compounds, a possibility which Whittaker (1951) has suggested. The analogy with the demonstration in recent years of the dual nature of the adrenergic transmitter is obvious. The search for other ACh-like compounds and the elucidation of their roles as transmitting agents or 'local hormones' (Burn, 1950) should produce significant developments in the field of autonomic physiology in the near future.

The histochemical localizations of butyro-ChE in the superior cervical ganglion and intestine deserve comment. Recent experiments have indicated that the inactivation of the comparable enzyme of the cat has little or no effect on transmission in the superior cervical ganglion, in contrast to the marked potentiation obtained when the aceto-ChE is inhibited (Kamijo & Koelle, 1952). The early suggestion of Feldberg & Vartiainen (1934) that ganglionic ChE may serve primarily as a 'buffer' against the spread of the transmitting agent may apply in a modified sense to the butyro-ChE at ganglionic sites. The controversial subject of the nature of the interstitial cells of the intestine was reviewed by Li (1940), who considered them in all probability to be primitive ganglion cells which played some role in the regulation of intrinsic conduction and rhythmic contractions. The present finding of their high content of butyro-ChE lends support to this opinion.

SUMMARY

1. The rates of hydrolysis of acetylcholine (ACh), acetyl- β -methylcholine (MeCh) benzoylcholine (BzCh) and butyrylcholine (BuCh) by the sera, intestines and livers of rabbits have been studied. Sera of rabbits with high benzoylcholinesterase (BzChE) activity also hydrolysed BuCh at a high rate in comparison with the sera of rabbits with insignificant BzChE activity. The livers of the former group likewise showed higher average values for BzCh and BuCh hydrolysis, but this finding was not constant. There was no significant difference between the sera of the two groups in respect to ACh or MeCh hydrolysis, or in the rates of hydrolysis of all four substrates by the intestines.

2. By means of fractional precipitation with sodium sulphate, the aceto-ChE and BzChE of the sera and the butyro-ChE of the intestines were partially purified and their substrate patterns determined. The pattern of the aceto-ChE was similar to that of other species. The patterns of the serum BzChE and intestinal BuChE were nearly identical, and contrasted with that of horse serum butyro-ChE in their relatively low rates of ACh hydrolysis.

3. Inhibition studies indicated that the serum BzChE and intestinal BuChE are two distinct enzymes. The liver appears to contain at least one additional enzyme capable of hydrolysing choline esters at significant rates.

4. The qualitative distributions of aceto-ChE

and butyro-ChE in the ileum, jejunum and superior cervical ganglion were found by histochemical examination to be similar to those of the corresponding enzymes of the cat. Certain semi-quantitative differences were noted.

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