The Esterases of Skin

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The enzyme content of skin has received little attention; yet the risk of exposure of this tissue to toxic agents, a risk that is increasing with modern industrial processes, and the attendant possibility of damage to essential skin enzymes, makes any addition to knowledge in this direction desirable. Accordingly, when it was found that acetylcholine was rapidly hydrolyzed by rat-skin slices, it was decided to examine this activity in greater detail.

The presence of a cholinesterase in skin does not appear to have been reported previously, though from the work of Langley (1923), Lewis & Grant (1924), Dale (1933), Dale & Feldberg (1934) and others on the innervation and vascular reactions of the skin, it was to be expected that certain of the structures in skin would be associated with cholinesterase activity.

Since the work of Vahlquist (1935) there has been some doubt as to whether all cholinesterase activity is due to a specific enzyme. Richter & Croft (1942), Mendel & Mundell (1943), Mendel, Mundell & Rudney (1943) and Mendel & Rudney (1943) agree that the cholinesterase of red blood cells is specific, while that of the serum of certain species is non-specific. Mendel & Rudney (1943) also obtained a non-specific cholinesterase from dog pancreas, and have suggested the name pseudo-cholinesterase for this type of enzyme. Further, Alles & Hawes (1940), Glick (1941) and Richter & Croft (1942) have each described differences in the specificity and other properties of cholinesterase preparations from different sources, and the last-named workers have suggested that the cholinesterases should more properly be regarded as a group of related enzymes.

The first step in the further investigation of the cholinesterase activity of skin was therefore to decide whether or not it is due to a specific enzyme. In our opinion the evidence now to be presented supports the view that it is a true specific cholinesterase.

METHODS

Estimation of esterase activity

Esterase activity was determined manometrically with Ammon's (1933) adaptation of the Warburg technique, in which the acid produced by enzymic hydrolysis is measured by CO_2 evolution from bicarbonate buffer at pH 7·4. Skin slices were suspended in 0·025 M-$NaHCO_3$ (total volume after additions, 3 ml.), the gas space being filled with 95% N_2+5% CO_2. All measurements were made in duplicate at 38°, the majority lasting for a total period of 1 hr., intermediate readings being taken after 15 or 30 min.

Non-enzymic hydrolysis, which was in all cases small, was controlled by including an additional pair of bottles containing no substrate. A correction was also made in each experiment for the acid production of the tissue in the absence of added substrate, which again was always small.

Esterase activity is expressed as μl. CO_2/g. tissue (wet wt.)/hr., except where otherwise stated.

Substrates

Three substrates, acetylcholine chloride (ACh), methyl butyrate (MB) and tributyrin (TB), were used; the first two were added as solutions in 0·025 M-$NaHCO_3$ (final concentration 0·015 and 0·03 M respectively), while the third was pipetted directly into the bottles, 0·05 ml. being added unless otherwise stated. Under our conditions these concentrations were found to give a constant rate of gas evolution over two successive 30 min. periods.

Source of tissue

Experiments were carried out on three kinds of skin.

Adult rat skin. The fur was clipped from an area of 30–40 sq. cm. of the belly, flanks and chest of normal white rats (weighing not more than 250 g.) under light ether anaesthesia. 1–2 hr. after the end of the anaesthetic the rat was decapitated, the skin dissected away, scraped free from underlying adipose and connective tissue, and sliced with a razor. 150–200 mg. were added to each bottle. Histological examination of the skin prepared in this way showed that subcutaneous tissue had been effectively removed.

Young rat skin (hairless). After decapitation of the rat the skin was removed from the entire trunk, scraped and sliced as before. 50–100 mg. were added to each bottle.

Human skin. Two experiments were carried out with samples of normal skin from two human subjects; in each case the skin was obtained from the breast immediately after surgical removal.

RESULTS

Cholinesterase activity of skin. Table 1 shows the cholinesterase activity of rat-skin slices found in the original experiments. In these early experiments the activity was measured over only 30 min. It will be seen that the level of activity shows but slight variations among the different animals in the weight-range studied. The values, moreover, agree closely with those found for adult rats in the present work, which are presented in Table 2. This table also includes data from young rat skin and human skin.

Table 1. *Cholinesterase activity of rat-skin slices*

Wt. of rat (g.)	Enzyme activity (μl. CO_2/g./hr.)
33	1356
56	1156
58	1124
59	1220
60	1180
62	1066
65	1114
74	1038
84	1046

The former differs little from the adult skin; human skin is somewhat less active.

Hydrolysis of tributyrin and methyl butyrate. The first step in investigating the specificity of this activity against acetylcholine was to determine whether skin possesses esterase activity towards other esters, particularly simple aliphatic esters. In conformity with the earlier work on the specificity of the cholinesterases of blood (Stedman, Stedman & Easson, 1932; Stedman, Stedman & White, 1933), tributyrin and methyl butyrate were selected as substrates.

Table 2. *Hydrolysis of acetylcholine, tributyrin and methyl butyrate by skin slices*

(Enzyme activity expressed as μl. CO_2/g./hr.)

Acetylcholine	Tributyrin	Methyl butyrate
	Adult rats	
1079	4153	—
1492	3093	—
933	4427	—
1186	3938	—
1466	3706	—
1263	4922	—
1140	3863	—
1033	4687	—
1004	3001	—
1298	5051	—
1680	4952	8258
	Young rats	
1480	1777	1774
1377	2293	2597
1499	1626	5172
1871	2725	1529
1554	3227	1015
1182	3902	1112
1497	2170	4463
1445	2409	2538
	Human	
406	949	—
565	1142	—

Table 2 shows that tributyrin is split even more rapidly than acetylcholine by both adult and young rat skin as well as by human skin. In the case of methyl butyrate the hydrolysis is still more rapid, so that observations with this substrate were restricted to young rat skin, in which the hydrolysis proceeds at a more conveniently measurable rate.

Further, it will be seen from Table 2 that, by contrast with the relatively constant values obtained with acetylcholine, the hydrolysis rates of tributyrin and methyl butyrate by young rat skin showed considerable variation in the different experiments. This variation was seen to be related to the age of the animal, a point that will be dealt with in a later section.

Sensitivity to eserine. The first test to determine the specificity of the skin cholinesterase activity was a comparison of the sensitivity of the different esterase activities to eserine. Varying amounts of eserine (British Drug Houses Ltd., physostigmine sulphate) were dissolved in 0·025M-$NaHCO_3$, and added to the bottles immediately after the substrate.

Table 3. *Effect of eserine on hydrolysis of acetylcholine* (ACh), *tributyrin* (TB) *and methyl butyrate* (MB) *by skin slices*

(Enzyme activity expressed as μl. CO_2/g./hr.)

Concentration of eserine ($\times 10^{-5}$M)	ACh	ACh + eserine	TB	TB + eserine	Inhibition ACh (%)	Inhibition TB (%)
			Adult rats			
0·5	1263	297	3093	2584	77	16
	1235	396	3001	2509	68	16
1·0	1079	0	—	—	100	—
	1492	4	—	—	100	—
	933	0	—	—	100	—
	1186	52	—	—	96	—
	1140	98	4427	3700	92	16
	1147	0	5051	4898	100	3
5·0	1033	13	3706	3089	99	17
	1128	0	4952	4587	100	7
			Young rats			
0·5	1871	439	2725	2407	77	12
1·0	1480	121	1777	1670	92	6
	1377	9	2293	1629	99	29
5·0	1554	4	3227	2563	100	21
			Human			
1·0	406	0	949	756	100	20
	565	0	1142	1013	100	11

Concentration of eserine ($\times 10^{-5}$M)	ACh	ACh + eserine	MB	MB + eserine	Inhibition ACh (%)	Inhibition MB (%)
			Young rats			
1·0	1182	162	1774	1789	87	0
	1497	47	2597	2159	97	17

Table 3 shows the effect of 0·5, 1·0 and 5·0 $\times 10^{-5}$M eserine sulphate on the rates of hydrolysis of acetylcholine and tributyrin by adult rat skin. The cholinesterase activity is seen to be strongly inhibited at the lowest concentration, and completely at the other two, while the tributyrin activity is only slightly affected at all the concentrations used. This marked difference is also observed in young

rat skin and human skin. Two experiments with young rat skin and methyl butyrate included in Table 3 show the same insensitivity to eserine. In all the experiments quoted in Table 3 readings were taken over two successive 30 min. periods; no significant change was noted in the eserine inhibition of any of the substrates in the two periods, and the small and variable inhibition of tributyrin and methyl butyrate hydrolysis was not significantly increased by a tenfold increase in the concentration of eserine.

Experiments with mixed substrates. The second criterion of specificity was obtained from summation experiments in which the CO_2 evolution of bottles containing single substrates was contrasted with that of bottles containing two substrates.

If two separate esterases, specific for the two substrates, are present, the CO_2 evolution of the bottles containing the mixed substrates should equal the sum of the CO_2 evolutions of the bottles containing the single substrates, provided the substrate concentrations are adequate to saturate the enzyme systems present and the presence of the one substrate does not inhibit the hydrolysis of the other. If, on the other hand, one enzyme is responsible for the hydrolysis of the two substrates, no summation could take place at these substrate concentrations owing to saturation of the enzyme. The results presented in Table 4 show that the former type of behaviour is obtained with acetylcholine if either methyl butyrate or tributyrin is the second substrate.

was used (2 μl. pipetted into the bottles by means of a micropipette), a preliminary experiment having shown that this would give a CO_2 evolution approximately equal to that of the acetylcholine. A rough determination of the Michaelis constants for tributyrin and acetylcholine showed that they were approximately equal (0·003 M), so that an objection to Exp. 904 is that, if a single enzyme is involved, it would not be saturated by the tributyrin and so might bring about the hydrolysis of both substrates. In the next three experiments in Table 4, 0·02 M tributyrin was used (0·02 ml. pipetted), this concentration lying on the 'plateau' of the substrate concentration-activity curve. In Exp. 920 the skin from an 8 g. rat was used; with animals of this weight the tributyrin activity is so much lower that rates of hydrolysis comparable with those of acetylcholine are obtained; in this experiment 0·05 ml. of tributyrin was added. It will also be seen from Table 4 that the CO_2 evolution remains reasonably constant over the two 30 min. periods studied.

The rate of hydrolysis of methyl butyrate by adult rat skin is so much greater than that of acetylcholine that no attempt was made to carry out summation experiments with this tissue. Satisfactory experiments were, however, carried out with very young rats (6 and 7 g.); these are also given in Table 4.

The results of all these experiments clearly indicate summation with acetylcholine and either tributyrin or methyl butyrate, and provide further evidence that the cholinesterase is distinct from

Table 4. *Effect of mixed substrates on activity of skin*

(Enzyme activity expressed as μl. CO_2/g./30 min.; a = 0–30 min., b = 30–60 min. period of experiment.)

	Acetylcholine		Tributyrin		Acetylcholine + tributyrin		Comparison of found and calc. CO_2 evolution from mixed substrates (μl. CO_2/g./hr.)	
Exp.	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	Found	Calc.
904	513	—	693	—	1128	—	—	—
910	415	519	2125	2219	2754	2482	5236	5278
912	549	561	1247	1285	1528	1480	3008	3642
914	622	585	1561	1741	2354	2396	4750	4509
920	619	880	841	785	1425	1665	3090	3125

	Acetylcholine		Methyl butyrate		Methyl butyrate + acetylcholine		Comparison of found and calc. CO_2 evolution from mixed substrates (μl. CO_2/g./hr.)	
Exp.	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	Found	Calc.
932	529	755	752	777	1298	1476	2774	2813
933	656	708	499	516	1287	1123	2410	2379

In the case of the tributyrin experiments the much more rapid hydrolysis of this substrate by adult rat skin presented a difficulty. In the first summation experiment (Exp. 904) 0·002 M tributyrin the esterase (or esterases) splitting these aliphatic esters.

By contrast with the complete summation of acetylcholine with either tributyrin or methyl buty-

rate hydrolysis, three experiments with mixed tributyrin and methyl butyrate yielded inconclusive results, there being some evidence of a small degree of summation. On the other hand, mixed methyl and ethyl butyrate, substrates which might confidently be expected to be hydrolyzed by the same enzyme, showed no evidence of summation (Table 5), thereby confirming the interpretation put on the positive summation with acetylcholine as one of the substrates.

Table 5. *Hydrolysis of mixed methyl and ethyl butyrate by rat-skin slices*

(Enzyme activity expressed as μl. CO_2/g./30 min.)

	Period (min.)	
	0–30	30–60
Methyl butyrate	1074	1045
Ethyl butyrate*	1157	1168
Methyl butyrate + ethyl butyrate	1078	1055

* Ethyl butyrate, 0·01 ml./3 ml.

Variation of esterase activity with age. In the course of the foregoing experiments it became clear that the different esterase activities of rat skin showed a marked divergence in their respective variations according to the age of the animal. This is illustrated in Table 6. The rates of hydrolysis of acetylcholine and methyl butyrate are approximately equal in very young rats, while that of tributyrin is slightly higher; but whereas the cholinesterase activity does not significantly change with increasing weight, the tributyrin activity increases somewhat, and the methyl butyrate activity markedly. This may be regarded as still further evidence that the enzyme hydrolyzing acetylcholine is distinct from that affecting these other esters. Further, the markedly greater increase in activity towards methyl butyrate with increasing age over that towards tributyrin, together with the slight evidence of partial summation with these two substrates, suggests the possibility that two distinct enzymes may be attacking these two substances.

Table 6. *Variations of skin esterase activity with age*

(Enzyme activity expressed as μl. CO_2/g./hr. Numbers in brackets denote the number of experiments with rats of any one age group.)

Wt. of rat (g.)	Acetylcholine	Tributyrin	Methyl butyrate
3–6	1395 (3)	2293	1015
7–8	1545 (3)	2270 (4)	1449 (3)
10	1554	3227	2162 (3)
13–15	1345 (2)	—	2186 (2)
20	—	3885 (2)	4818 (2)
54	1680	—	8258
80–100	1168 (3)	—	—
145–200	1222 (6)	4335 (5)	—
225–230	—	4805 (2)	—

DISCUSSION

The numerous studies of the cholinesterase activity of various animal tissues (Plattner & Hintner, 1930; Gilman, Carlson & Goodman, 1939) do not appear to include skin. It was thought desirable to investigate the cholinesterase activity of skin, particularly as certain pathological conditions of skin may be associated with abnormal functioning of cholinergic nerves. Grant, Pearson & Comeau (1936), for example, have investigated a series of cases of urticaria of nervous origin, and have concluded that the condition is brought about by the release of acetylcholine in the skin as a result of stimulation of cholinergic nerve fibres. Alexander, Elliott & Kirchner (1940) have stated that eserine, introduced electrophoretically into the skin, is a powerful urticariogenic substance even in dilutions of 1:10,000 and occasionally 1:100,000.

The results described in this paper show that both rat and human skin possess an active cholinesterase. That this is a specific enzyme has been shown by comparing the sensitivities to eserine of the enzymic hydrolysis of acetylcholine, tributyrin and methyl butyrate, both of which latter substrates are also rapidly hydrolyzed by rat skin. Confirmation of this conclusion has been obtained by summation experiments with acetylcholine and either tributyrin or methyl butyrate.

It was also found that while the cholinesterase activity of skin remains approximately constant over the weight range of animals studied, the rates of hydrolysis of tributyrin and methyl butyrate increase with increasing age. This increase is in striking contrast with the reported changes in the respiration of skin, which diminishes with increasing age; Adams (1936), for example, found that the Q_{O_2} for skin from very young rats falls at 4–6 weeks to a persistently low value.

SUMMARY

1. Rat and human skin have been shown to have cholinesterase activity.

2. Rat skin also contains an esterase (or esterases) which hydrolyzes tributyrin and methyl butyrate.

3. The cholinesterase activity is very sensitive to eserine, being completely inhibited by 10^{-5} M eserine sulphate. The aliphatic esterase on the other hand is insensitive.

4. Summation experiments with mixed substrates confirm the conclusion that the cholinesterase and aliphatic esterase activities are due to different enzymes.

5. Whereas the cholinesterase activity remains approximately constant, the tributyrin activity increases somewhat, and the methyl butyrate activity increases markedly with age.

6. It is concluded that the cholinesterase of skin is a true, specific cholinesterase.

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Bacterial Tetrathionase: Adaptation without Demonstrable Cell Growth

(A Report to the Medical Research Council)

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In suitable conditions micro-organisms react to changes in their chemical environment by changes in enzymic activity. Two types of process may be distinguished. In the first, the new property develops slowly in successive cultures and, once developed, remains stable even in the absence of the specific substrate. In the second, the new property appears after a delay of only a few hours when the cells are brought into contact under appropriate conditions with the specific substrate, but is rapidly lost when they are subcultured in an environment not containing it (Dubos, 1940). Yudkin (1932) suggested that the term 'training' be used for the first process, and 'adaptation' for the second. 'Training' may well be brought about by natural selection. 'Adaptation' is almost certainly not, since the time for development of the new property is too short for appreciable cell division to have occurred. With the formic hydrogenlyase of *Esch. coli*, Stephenson & Stickland (1932) showed that adaptation could occur without evidence of cell division but only in an environment which was favourable for maximal growth, since they had to add full strength nutrient broth to their suspensions in order to produce adaptation. Stephenson & Yudkin (1936), however, found with the galactozymase of *Saccharomyces cerevisiae* that adaptation could occur slowly in the absence of added nitrogen source and without any significant cell division, while Hegarty (1939) found that washed suspensions of *Streptococcus lactis* could be adapted to ferment galactose in the absence of either nitrogen source or cell division. Pollock, Knox & Gell (1942) and Pollock & Knox (1943) showed that washed suspensions of *Bact. paratyphosum* B prepared from agar cultures could be adapted to reduce sodium tetrathionate rapidly to thiosulphate in accordance with the equation

$$Na_2S_4O_6 + 2H = Na_2S_2O_3 + H_2S_2O_3,$$

simply by preliminary treatment for 2–3 hr. at 37° with tetrathionate and a suitable hydrogen donator. The present paper gives in detail the evidence that this adaptation occurs without detectable growth.

METHODS

Several strains of *Bact. paratyphosum* B were used; some were freshly isolated from clinical cases, others were stock laboratory cultures maintained on egg slopes. One ml. of an 18 hr. broth culture was inoculated on to the surface of bottles containing 30–40 ml. of nutrient agar prepared with