The Determination of Ergothioneine. The Non-occurrence of Ergothioneine in Urine

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The problem of the quantitative determination of ergothioneine in biological fluids presents many difficulties. Methods based on the reduction of the Folin-Marenzi phosphotungstic acid reagent are not specific enough (cf. Potter & Franke, 1935); attempts to determine ergothioneine in urine, using this reagent, are unsuccessful even when quite elaborate precautions are taken to remove interfering substances (cf. Sullivan & Hess, 1933).

The diazotized sulphanilic acid-sodium hydroxide reagent employed by Hunter (1928) gives coloured products with a number of compounds including all the thiolimidazoles examined, but has a great advantage over other reagents in that the intense magenta colour obtained with ergothioneine appears to be given only by this compound, the closely related thiolurocanic acid and 4-phenyl-2-thiolimidazole. The method, however, has the disadvantage of being rendered unreliable by a number of interfering factors and, in particular, cannot be employed with urine and with certain bloods such as ox blood, for example, owing possibly to the presence of 'combined uric acid' (Hunter, 1949).

The Kraut reagent (potassium bismuth iodide) used by Klein & Linser (1933) as a precipitant in the determination of alkaloids in plant material seemed worthy of investigation and has been applied by us to the ergothioneine problem. In the work to be described we have investigated the ergothioneine content of normal urine, and of blood. It would appear that the use of the bismuth reagent has certain advantages over the direct determination by the Hunter (1949) method, and can be successfully applied in the case of ox blood.

Work (1949) has reported an ergothioneine content in normal urine of 130–300 mg./l., a finding which could not be confirmed by Woolf (1949). In the work described below we have demonstrated that ergothioneine does not occur in normal human urine in amounts sufficient to be detected (limit of detectability less than 1 mg./100 ml.). Experiments with rats have shown that it is extremely improbable that ergothioneine would be excreted in the urine in any significant quantity.

MATERIALS AND EXPERIMENTAL

Human urine. Pooled urine samples were used throughout. These were brought to pH 3.0 by the addition of HCl and allowed to stand at 0° for 24 hr. The precipitate was then removed and the clear filtrate used in the tests.

For direct paper chromatography this urine was treated by procedures (a) or (b). (a) Freeze dried, taken up in a little water, an equal volume of ethanol added, and the filtrate applied to the paper in the usual way in amounts equivalent to up to 600 μ l. of the original urine. (b) Desalted in the apparatus described by Consden, Gordon & Martin (1947), concentrated *in vacuo*, and applied to the paper in amounts equivalent to 200, 400 and 800 μ l. of the original urine. Urine to which a known amount of ergothioneine had been added was always, after treatment according to (a) or (b), run alongside the similarly treated original urine.

Urochrome. This was prepared from pooled samples of normal human urine, apparently free from protein, by the methods of Garrod (1894), Dombrowski (1908) and Drabkin (1930).

Potassium bismuth iodide. This reagent, known as the Kraut or Dragendorff reagent, can be made up in different ways. The use of HNO₃ was not considered advisable, so the solution was made up by dissolving 16 g. (BiO)₃CO₃ in 21 ml. conc. HCl. After filtration 41 g. KI in 125 ml. water were added and the deep red solution made up to 200 ml., the solution being then diluted with an equal volume of glycerol to inhibit hydrolysis of the reagent.

Iodobismuthous acid. A modified form of the above reagent used for the blood ergothioneine determinations was made by dissolving 8 g. (BiO)₃CO₃ in 10 ml. HCl and adding 28 ml. of colourless HI, sp.gr. 1.7, freshly distilled over red P, to the filtered solution.

Folin-Marenzi reagent. Used as described by Rimington (1930).

Hunter diazo reagent. Prepared and used as described by Hunter (1949).

Determination of ergothioneine in simple solution

The solution of ergothioneine (4 ml.) was acidified with 1 drop of 3N-HCl and treated with 8 drops of the KBiI₄ reagent. After standing at 0° for 5 min. a few drops of 0.5% aqueous choline chloride (sufficient to produce a visible precipitate) were added and the solution was centrifuged. The supernatant liquid was discarded and the tube allowed to drain thoroughly. The red precipitate was treated with water (2 ml.), and the buffer (2 ml.), followed by the diazo-

tized sulphanilic acid reagent (1 ml.) and 10N-NaOH (5 ml.) as described by Hunter (1949) to give a total volume of 10 ml. After centrifuging to remove any Bi_2O_3 produced as a result of hydrolysis of the precipitating reagent, the colour density was determined in a photoelectric colorimeter using Ilford filter no. 404. Alternatively, the ergothioneine precipitate was treated with Na_2SO_3 (0.5 ml.), Na_2CO_3 (4 ml.), urea (2.5 ml.) and phosphotungstic acid (2.0 ml.) as described by Rimington (1930), the colour density of the resulting solution after centrifuging being determined using Ilford filter no. 204.

Determination of ergothioneine in potassium chloride solution

The determination of ergothioneine dissolved in 0.16% aqueous KCl solution (the K ion concentration corresponding to that obtained after 1 in 5 dilution of normal blood corpuscles) was also carried out as described above. In another series of experiments 1 drop of the iodobismuthous acid reagent was used as precipitant instead of the KBiI_4 .

When simple solutions of ergothioneine containing less than 0.3 mg./100 ml. are precipitated with KBiI_4 or iodobismuthous acid, it is difficult to collect by centrifuging the small amount of precipitate formed. After quite long periods of centrifuging the supernatant liquid still remains cloudy. To overcome this difficulty all precipitations in simple solution were carried out in the presence of just sufficient choline to produce a visible precipitate. Creatinine or betaine are equally effective. All three are precipitated by the BiI_3 reagents, but are without effect on the subsequent colour development by the Hunter reagent. Such coprecipitation considerably improves the recovery of the ergothioneine, doubtless owing to the mechanical carrying down of the suspended ergothioneine BiI_3 complex.

Determination of ergothioneine in blood

The methods of deproteinization and colour development were essentially those of Hunter (1949). Oxalated blood (2 ml.) was centrifuged and the plasma removed. The corpuscles were then made up to a volume of 5 ml. by the addition of 0.01N-acetic acid and the deproteinization carried out by heating in a boiling-water bath. After centrifuging, 1 drop of 25% (w/v) basic lead acetate solution was added to the tube and the contents again centrifuged. A drop of 10% (w/v) H_3PO_4 was then added and after centrifuging a clear supernatant liquid was obtained. This was removed and the volume determined (3.7 ml. usually). After addition of 1 drop of 3N-HCl, the solution was cooled to 0° and 1 drop of the iodobismuthous acid was added. After 3 min. the small quantity of red precipitate obtained was collected by centrifugation and the tube drained thoroughly to remove the supernatant liquid. The ergothioneine in the precipitate was then determined as described above.

Ergothioneine determinations were also carried out on blood plasma, but no ergothioneine could be detected. Recovery determinations were made using blood corpuscles to which different amounts of ergothioneine had been added.

No addition of choline or creatinine was found necessary, as blood filtrates prepared as described gave a small amount of precipitate not due to ergothioneine, which served to bring down the ergothioneine complex satisfactorily.

Paper chromatography

Paper chromatography was carried out by the 'one-dimensional' method as described by Consden, Gordon & Martin (1944). The filter paper used was Whatman no. 531; this resists the action of 10N-NaOH with which the paper is sprayed during colour development. The solvent mostly used was a mixture of 2:6-lutidine (2 parts) and 2:4:6-collidine (3 parts), nearly saturated with water. Phenol and *p*-cresol were also used on occasion.

The substances run on the chromatograms were: (1) human and rat urines; (2) human blood corpuscles, haemolysed, deproteinized and desalted (vol. applied to paper $\equiv$ 0.5, 1 and 2 ml. of corpuscles); (3) human blood corpuscles, haemolysed and deproteinized, treated with the Kraut reagent, the resulting precipitate decomposed with H_2S , the H_2S removed by a stream of N_2 , and the solution desalted (vol. applied $\equiv$ 0.5, 1 and 2 ml. of corpuscles); (4) the precipitate from human urine treated with the Kraut reagent, treated as in (3) (vol. applied $\equiv$ up to 800 μl . original urine); (5) human urine to which ergothioneine had been added, treated as in (4) (vol. applied $\equiv$ up to 800 μl . urine); (6) urochrome preparations (up to 160 μg .). Ergothioneine and 2-thiolurocanic acid were always run alongside the test solution, as was the test solution plus ergothioneine.

After the solvent had run a convenient distance the paper was dried and the positions of ergothioneine and other reactants determined by using the Hunter reagent or the Folin-Marenzi reagent. For the Hunter method, 2 vol. of the diazotized sulphanilic acid reagent (Hunter, 1928) were mixed with 1 vol. of the sodium acetate- Na_2CO_3 solution and the paper immediately sprayed with the mixture. About 30 sec. later the paper was sprayed with 10N-NaOH. For the Folin-Marenzi method the paper was sprayed with 3N- Na_2CO_3 containing 5% Na_2CO_3 and then, when nearly dry, with the Folin-Marenzi reagent.

When phenol or *p*-cresol was used as solvent the paper was washed with ether before using the Hunter reagent.

Ergothioneine in rat urine. A young rat (weighing 100 g.) was injected subcutaneously with 10 mg. ergothioneine and the urine collected over the following 24 hr. period. This urine, after desalting and concentrating twenty times *in vacuo*, was chromatographed on filter paper, using lutidine-collidine, alongside a similarly treated sample of urine from the same rat collected previous to the ergothioneine injection; 20 μl . of the concentrate were used each time.

RESULTS

Ergothioneine in simple solution

Fig. 1 shows the calibration curves for the determination of ergothioneine by the two direct methods and after precipitation with the bismuth iodide reagents.

Precipitation of ergothioneine under the conditions described involves a loss due to the solubility of the precipitate. It would appear that for maximum precipitation some alkali metal ions are necessary as low results were obtained in simple solution using the iodobismuthous acid reagent. The smallest loss was given using iodobismuthous acid with 0.16% potassium chloride, or iodobismuthous

acid with 0.13% sodium chloride, or potassium bismuth iodide with water, the loss in these cases amounting to 3 μ g. of ergothioneine/ml. of solution. With potassium bismuth iodide in 0.16% potassium chloride the loss amounted to 4 μ g./ml., and this value increased with increase in the potassium chloride concentration. As these losses are quite constant, the accuracy of the method is not affected. In view of these results the iodobismuthous acid reagent was used in all blood determinations.

Fig. 1. Calibration curve for ergothioneine determination. $\odot$ — $\odot$, using Hunter diazo reagent and NaOH; $\times$ — $\times$, after precipitation from 4 ml. water with KBI_4 and using Hunter diazo reagent and NaOH; $\square$ — $\square$, after precipitation from 4 ml. 0.16% aqueous KCl with iodobismuthous acid, Ilford filter no. 404; $\triangle$ — $\triangle$, after precipitation with KBI_4 from 5 ml. water and using Folin-Marenzi reagent, Ilford filter no. 204.

The corresponding curve for the ergothioneine determination, using the Folin-Marenzi reagent after precipitation from simple solution, again does not go through the origin. In this case a small but constant colour value is given by the blank test without ergothioneine. It will be seen that the method employing the diazo reagent is considerably more sensitive than the other.

Ergothioneine in urine

Human urine. When human urine was treated with the bismuth iodide reagent a bulky precipitate was obtained. This precipitate contains substances which interfere with the Hunter diazo reaction, and small quantities of ergothioneine added to the urine before precipitation cannot be detected when the diazo reagent is used for the determination.

When, however, the bismuth iodide precipitate from normal urine is treated with the Folin-Marenzi reagent, the characteristic blue reduction product is always formed, corresponding to apparent ergo-

thioneine levels of 1–5 mg./100 ml., and ergothioneine added to the original urine can be nearly quantitatively recovered when allowance is made for this blank value (Table 1).

Table 1. *Recovery of ergothioneine added to urine using Folin-Marenzi reagent on KBI_4 precipitate*

Amount of ergothioneine added to urine (mg./100 ml.)	Apparent ergothioneine content (mg./100 ml.)	Ergothioneine recovered (mg./100 ml.)
0	2.0	—
1.6	3.4	1.4
3.2	4.9	2.9
4.8	6.4	4.4

In order to test if the material in the original urine reacting with the phosphotungstic acid was, in fact, ergothioneine, the Kraut reagent precipitate was decomposed with hydrogen sulphide to remove bismuth, hydrogen sulphide was driven off by a stream of nitrogen, and the resulting filtrate chromatographed one-dimensionally on filter paper after desalting. All R_f values given in this section are in lutidine-collidine.

No ergothioneine (R_f 0.38) or thiolutrocanic acid (R_f 0.77) could, however, be detected on the paper after spraying with the Hunter diazo reagent followed immediately by 10N-sodium hydroxide. Urines, to which as little as 1.0 mg. ergothioneine/100 ml. had been added, gave chromatograms with the characteristic ergothioneine spot after similar treatment.

Paper chromatograms of urines which had been freeze-dried and taken up in 50% ethanol, and of urines which had been desalted and concentrated *in vacuo*, showed no trace of ergothioneine on development with the Hunter reagent. Control experiments in which ergothioneine (1 mg./100 ml.) had been added to the original urine gave the expected characteristic spot.

Freeze-dried urine concentrates (from urine kept at 0° and pH 3 for 24 hr.), prepared as above, were chromatographed on filter paper in lutidine-collidine, *p*-cresol, or phenol and developed by the Folin-Marenzi reagent. A number of blue spots were produced, one corresponding to uric acid, and another to urochrome. At least one of these spots overlay the position of ergothioneine, a finding which may explain the high levels of this substance in urine reported by other workers. When the urine was concentrated using the potassium bismuth iodide precipitation method, the number of blue spots was greatly decreased, those due to uric acid and urochrome being now the main ones.

Rat urine. No ergothioneine or thiolutrocanic acid was detectable by the Hunter reaction in the chromatograms of rat urine either before or after ergothioneine injection. Rat urine specimens, to which

ergothioneine was added, gave the characteristic magenta spot in the expected position.

With the Folin-Marenzi reagent urine from normal rats and from injected animals gave spots due to uric acid (R_f 0.48) and urochrome (R_f 0.55). In addition, a diffuse spot from faster constituents (R_f 0.8–0.98) was observed. With the Hunter reagent, corresponding spots, one orange-yellow (R_f 0.55) the other light-yellow (R_f 0.8–0.98), were obtained from the urine specimens, the second being present in greater amount in the urine of the ergothioneine-injected rat.

Ergothioneine in blood

Recovery of added ergothioneine. Table 2 shows the results for the determination of different amounts of ergothioneine added to blood corpuscles after removal of the plasma. These results, together with the proportionality, which was shown to exist between different volumes of the same blood filtrate and the ergothioneine content of the precipitates obtained from them with iodobismuthous acid, justify the allowance for solubility (3 μ g./ml. of filtrate) which the findings above show to be associated with the precipitation of ergothioneine.

Table 2. *Recovery of ergothioneine added to blood corpuscles using the Hunter method of colour development after precipitation with iodobismuthous acid*

Subject	Original blood	Ergothioneine (mg./100 ml. blood)		
		Added	Found	Calculated
A	1.75	0.58	2.3	2.33
		1.15	2.9	2.90
		1.73	3.3	3.48
B	1.7	0.33	2.0	2.03
		0.65	2.5	2.35
		0.98	2.85	2.68

Ergothioneine levels in normal blood. Table 3 gives the levels of ergothioneine in the blood of a group of medical students. For comparison the corresponding values for the determination made by the Hunter method are given. Included in the table are the values for ox, rabbit, cat and guinea pig blood. In the case of ox blood less than 0.5 mg./100 ml. ergothioneine was detectable by the Hunter method.

Paper chromatograms of the bismuth iodide precipitate from a specimen of human blood corpuscles, prepared as above, showed, on development with the Hunter reagent, a magenta spot in the correct position for ergothioneine. The size of the spot was in agreement with the value of 2 mg. ergothioneine/100 ml. found by colorimetric determination. (From the size of the spot one can estimate the amount

of a substance present with an accuracy of about $\pm 50\%$ by running suitable standards alongside.) Direct paper chromatography of the deproteinized blood corpuscles also gave the magenta ergothioneine spot, but the spot was distorted, probably by some substance of similar R_f giving no colour with the Hunter diazo reagent. There was, however, no obvious loss of ergothioneine on passing through the bismuth precipitation.

Table 3. *Ergothioneine levels in blood*

Subject	Amount in mg./100 ml. blood	
	By Hunter method	After precipitation by iodobismuthous acid
P. A.	2.8	3.1
P. S. M.	1.4	2.45
K. W.	1.8	2.35
S. R. W.	1.5	2.0
J. B.	2.3	2.3
J. T. C.	2.1	2.3
J. E. C.	1.6	2.1
F. D.	—	2.25
J. R.	—	2.0
R. L.	—	2.0
H. M.	—	2.1
Ox (1)	<0.5	4.3
Ox (2)	<0.5	4.6
Guinea pig (1)	—	4.5
Guinea pig (2)	—	5.0
Cat (1)	—	<0.8
Cat (2)	—	1.25
Rabbit	—	2.2

The use of the Folin-Marenzi reagent to develop paper chromatograms of deproteinized haemolysed blood corpuscles reveals the presence of numerous reducing substances. If the filtrate from the deproteinized haemolysed blood corpuscles is treated with potassium bismuth iodide and the precipitate freed from bismuth as above, ergothioneine is left, in the resulting filtrate, as the main substance reacting with the Folin-Marenzi reagent on paper chromatograms. This proves that the potassium bismuth iodide precipitation removes interfering substances such as glutathione.

Urochrome

Paper chromatograms of the three urochrome preparations showed the presence of a main component of R_f 0.55 in lutidine-collidine, giving a pale-orange colour with the Hunter reagent, and a blue colour with the Folin-Marenzi reagent. Uric acid was also present in urochrome prepared by the methods of Garrod (1894) and of Dombrowski (1908). This was identified by the R_f values of 0.48 in lutidine-collidine and 0.05 in *p*-cresol, and by the characteristic intensity and speed of development of colour with the Folin-Marenzi reagent.

DISCUSSION

The use of the Kraut reagent or iodobismuthous acid offers a useful method of concentrating ergothioneine prior to its determination either by use of the Hunter reagent or by measuring the blue reduction product formed from the Folin-Marenzi reagent. By employing these reagents, one can remove interfering substances such as phenols, tyrosine or cysteine in the case of the Hunter reagent, and uric acid, cystine or glutathione in the case of the Folin-Marenzi reagent.

Blood

The use of iodobismuthous acid as a precipitant for ergothioneine in blood filtrates has certain advantages over the direct determination by the Hunter (1949) method. The yellow-colour development referred to by Hunter occurring with certain blood filtrates is to a considerable extent reduced. This yellow colour is a serious difficulty in the case of bloods of relatively low ergothioneine content such as those reported here. In ox-blood filtrates which gave scarcely detectable amounts of ergothioneine by the Hunter method satisfactory colour development is obtained.

The ergothioneine levels for human blood reported here are considerably lower than those previously published for Canadian subjects (Hunter, 1949). Hunter (1950) has also found lower values in subjects resident in England. Our lack of knowledge as to the origin of ergothioneine renders any explanation of this a matter of speculation. Blood ergothioneine levels for other species, except the ox, are in general agreement with those reported by Hunter (1928).

Urine

Not all substances interfering with the ergothioneine colour reactions can be removed by the bismuth iodide reagents; thus in the precipitate obtained from urine there are present substances of unknown composition that inhibit the Hunter reaction. These would prevent the determination of ergothioneine by this means if there were any present. The Folin-Marenzi reaction applied to these precipitates gives high false positive results owing to the presence of reducing substances, chiefly uric acid and urochrome.

No ergothioneine could be detected by paper chromatography of several specimens of human urine concentrated in three different ways. The absence on the chromatogram of inhibition of colour development by any substance in the urine was demonstrated by the production of a coloured spot from urine to which ergothioneine had been added before concentration. This spot was, at most, very slightly reduced in size and intensity as compared with a spot from an equivalent amount of ergo-

thioneine alone. The least amount of ergothioneine that could be detected was about 5 μ g. Having in mind the sensitivity of the method it can be concluded that if ergothioneine is present in normal urine the quantity must be less than 1 mg./100 ml.

This view is strengthened by the results obtained on rat urines. A subcutaneous injection of 10 mg. of ergothioneine in a 100 g. rat is a relatively enormous dose. Yet the urine of the next 24 hr. contained no trace of ergothioneine detectable by paper chromatography, that is less than 2 mg./100 ml. in the case of rat urine, or 0.25 mg. in all. It would seem that ergothioneine is not excreted even when the body is grossly overloaded, and it is therefore safe to deduce that it will not normally be excreted. Although obtained in a different animal species this is in conformity with our results with human urine.

The finding that urochrome is precipitated from urine by the Kraut reagent is not surprising, since heavy metal salts are known to precipitate urochrome. It is, however, rather surprising to find that some of the uric acid in urine is not precipitated in 24 hr. at pH 3 and 0°, that this is precipitated on adding the Kraut reagent, and then redissolves on treating the precipitate with hydrogen sulphide. It is difficult to account for this except by supposing that uric acid is in loose chemical combination with urochrome as suggested by Rangier (1935). The finding of uric acid in urochrome prepared by the methods of Garrod (1894) and Dombrowski (1908) supports this view. This chemical combination may be the reason for the unexplained high solubility of uric acid in urine.

Specificity of the Hunter reaction. The deep magenta colour in the Hunter reaction is, as far as we know, given only by ergothioneine, 2-thiolurocanic acid and 4-phenyl-2-thiolimidazole. All other substances examined, including about forty 2-thiolimidazole derivatives gave at most a yellow to orange colour under the conditions of the test (cf. Hunter, 1930).

SUMMARY

1. The use of the Kraut reagent (KBiI_4) and iodobismuthous acid respectively in the determination of ergothioneine is described.

2. By methods of paper chromatography it is found that ergothioneine does not occur in amounts greater than 1 mg./100 ml. in normal human urine.

3. In rats, a large subcutaneous dose of ergothioneine is not excreted in the urine as ergothioneine or thiolurocanic acid.

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The Esterases of Horse Blood

1. THE SPECIFICITY OF HORSE PLASMA CHOLINESTERASE AND ALI-ESTERASE

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The work of Adams & Whittaker (1948, 1949), Adams (1949) and Whittaker (1949*a*) has shown that the cholinesterases of human erythrocytes, pigeon brain and human plasma are able to hydrolyse many simple aliphatic esters. For a given acyl group, the aliphatic esters most rapidly hydrolysed are those of 3:3-dimethylbutanol (I), the uncharged carbon analogue of choline (II), other esters being hydrolysed at a rate mainly dependent on the degree of their deviation from this structure. The effect of

the acyl group depends on the cholinesterase. In any series, choline or non-choline, acetate is the optimum group for the erythrocyte and brain cholinesterases, and butyrate for the plasma cholinesterase. This difference provides a basis for a nomenclature which does not depend either on the assumption that one cholinesterase is more or less specific than another or upon its supposed physiological function or lack of function. We may term all those cholinesterases possessing a specificity pattern similar to the human erythrocyte and pigeon-brain cholinesterases, acetate-cholinesterases or aceto-cholinesterases, and those possessing a specificity pattern similar to that of human plasma cholinesterase, butyrate- or butyro-cholinesterases. (The terms aceto- and butyro-cholinesterase were suggested by Dr D. Richter at the May meeting of the Biochemical Society in 1948; cf. also Augustinsson & Nachmansohn (1949), whose publication appeared during the preparation of this paper.) It is possible that other cholinesterases at present regarded as belonging to the 'true' ('specific', 'e-type') or 'pseudo' ('unspecific', 's-type') classes will prove to possess one or

other of these two types of specificity pattern. To test this possibility further, the specificity of the cholinesterases of horse blood has now been investigated, the results obtained with the plasma (or serum) cholinesterase appearing in this paper, and those with the erythrocyte cholinesterase in the next (Mounter & Whittaker, 1950). We have also studied the specificity of the aliphatic esterase (ali-esterase of Richter & Croft, 1942) of horse serum with the object of defining more precisely the difference between these two types of esterase. A summary of this work has been given by Whittaker (1949*b*).

Horse serum has been a favourite source of cholinesterase for many workers, sometimes with misleading results. The native serum was found by Stedman, Stedman & Easson (1932) to hydrolyse both choline and non-choline esters, i.e. acetylcholine and butyrylcholine, methyl butyrate and tributyrin. Fractionation with $(\text{NH}_4)_2\text{SO}_4$ gave a preparation with a higher cholinesterase activity than the original serum but a lower aliphatic esterase activity. Easson & Stedman (1937) showed that suitable concentrations of prostigmine and eserine markedly inhibited cholinesterase activity while leaving the aliphatic ester hydrolysis unaffected, and also, that the rate of hydrolysis of a mixture of butyrylcholine and either methyl butyrate or tributyrin was greater than that of any of these substrates alone. They concluded that there were at least two enzymes present in horse serum, a cholinesterase and an aliphatic esterase, but admitted that the results did not exclude a small action on tributyrin by cholinesterase. Richter & Croft (1942) came to essentially the same conclusions as Stedman and his co-workers. They were able to confirm the separate identity of the aliphatic esterase (ali-esterase) by partial separation using adsorption, heat treatment or ageing and by inhibitor experiments. Glick (1938, 1939, 1941) carried out extensive specificity studies with native serum. He confirmed the increase in rate of hydrolysis found by Easson & Stedman (1936) as the homologous series of *n*-acylcholines is ascended as far as butyryl, and also showed that a further increase in acyl