Rapid estimation of serum cholinesterase activity using the Astrup micro equipment

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SYNOPSIS A rapid micro technique for the estimation of serum cholinesterase is described. Acetylcholine bromide is incubated with serum within the capillary of the Astrup electrode. The enzyme hydrolyses the substrate with the liberation of acetic acid. This causes a fall of *pH* which is seen on the galvanometer of the instrument and the rate of this fall is shown to be proportional to enzyme concentration.

The method has been calibrated in international units and compared with a more conventional technique. The values found in homozygotes with normal dibucaine-resistant enzymes and in heterozygotes are reported, together with their dibucaine and fluoride numbers.

Prolonged apnoea following the administration of scoline (succinyl-dicholine) in anaesthesia usually occurs in patients who are homozygous for the atypical type of serum cholinesterase (Bush, 1961). These patients all have a low concentration of the enzyme. Serum cholinesterase estimation is of most value in assessing the cause of apnoea and the result can be given within a few minutes. This method was evolved to measure rapidly and accurately the enzyme activity with the minimum delay and disruption of laboratory routine.

MATERIALS AND METHOD

REAGENT: 0.05 M. ACETYLCHOLINE BROMIDE (1.136 g./100 ml.) The solution was found to be stable for several months at -20°C . It was dispensed in 1 ml. amounts in polystyrene cups with polythene caps which had been purchased for use on the autoanalyser. The contents of one of these was thawed when required.

The solid acetylcholine bromide was kept in a desiccator over silica gel.

ASTRUP MICRO EQUIPMENT The Radiometer A.M.E. 1 apparatus was used. The *pH* meter has an extended scale between *pH* 6.6 and 8.0. The electrode was standardized using a buffer of *pH* 7.38 before every test and checked occasionally with a buffer of *pH* 6.98.

The equipment includes a tonometer in which up to 0.1 ml. of solution may be equilibrated with a humidified oxygen and carbon dioxide mixture. All parts of the apparatus are surrounded by water jackets at 38°C .

TECHNIQUE Into the tonometer of the Astrup micro

equipment 0.05 ml. of serum was introduced and equilibrated with 4% CO_2 , 96% O_2 mixture for two minutes.

To this was added 0.05 ml. of 0.05 M. acetylcholine bromide and the mixture shaken for 10 to 15 seconds. This mixture was sucked into the capillary of the electrode and the *pH* read. It was usually between 7.35 and 7.45 and immediately started to fall. The time (*t*) taken for the fall from *pH* 7.3 to 7.1 was measured in seconds. (This was normally between 30 and 65 seconds.)

CALCULATION
$$\frac{150}{t} \times 100 = \text{cholinesterase activity in international units per 100 ml. serum.}$$

NORMAL VALUES These were obtained from the sera of 25 male and 25 female, healthy blood donors. The sera were first screened by the agar diffusion technique (Harris and Robson, 1963), using R 0 2 - 0683 as inhibitor, to exclude homozygotes or heterozygotes for the atypical (dibucaine-insensitive) enzyme. Serum from a normal male was substituted for that from the one heterozygote found in the group.

For male adults the mean is 403 i.u. per 100 ml. serum, the standard deviation is 64 i.u., and the normal range (95% of the population) is 274 to 532 i.u. per 100 ml. serum.

For female adults the mean is 352 i.u. per 100 ml. serum, the standard deviation is 74 i.u., and the normal range (95% of the population) is 204 to 500 i.u. per 100 ml. serum.

The significance of difference between the means for males and females was calculated and the probability (*P*) that they were the same populations found to be between 0.01 and 0.02. This indicated that there was a significant difference between the two normal ranges which confirm-

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ed previous findings (Rider, Hodges, Swader, and Wiggins, 1957).

PRECISION Twenty-five single determinations of the cholinesterase activity of the same serum were performed. The mean value was 238 i.u. per 100 ml. serum, the standard deviation ± 3.02 i. u. per 100 ml. serum, and the mean ± 3 standard deviations (99.6% of all determinations) ± 9.06 i.u. per 100 ml. serum = $\pm 4\%$.

RESULTS OF TESTS PERFORMED TO DEVELOP AND INVESTIGATE THE METHOD

THE INITIAL pH OF SERUM The pH of separated serum rises during storage due to loss of carbon dioxide. Thus, a freshly taken sample may be close to pH 7.4 and 12 hours later have risen to pH 8.6. To bring the initial pH of the reaction mixture to within a reasonable range the serum was equilibrated with the 4% CO₂, 96% O₂ gas mixture used in the Astrup technique for CO₂ measurement. This brought the pH of serum with a normal standard bicarbonate to between 7.35 and 7.45.

OPTIMUM PROPORTIONS OF REACTANTS The rate of pH decrease at various substrate concentrations was

determined using pooled serum. The resulting curves were roughly parallel using substrate concentrations above that produced by adding 0.0125 ml. of 0.05 M. acetylcholine bromide to 0.05 ml. of serum. It was therefore decided to use a mixture of 0.05 ml. of serum and 0.05 ml. of 0.05 M. substrate for the test.

LINEARITY OF THE RATE OF pH FALL UNDER THE CONDITIONS OF THE TEST Clotted blood from 10 hospital patients was centrifuged within one hour of collection and the serum separated. Each specimen was equilibrated with 4% CO₂, mixed with substrate and drawn into the capillary of the electrode. The fall of pH was then plotted against time. Figure 1 shows five of the curves produced all of which were nearly linear between pH 7.3 and 7.1.

ALKALINE HYDROLYSIS OF ACETYLCHOLINE BROMIDE The capillary of the Astrup equipment was well washed with buffer and water. It was then filled with 1×10^{-3} M. dibucaine hydrochloride solution for three minutes to inhibit the cholinesterase present in any traces of serum on the capillary walls. The capillary was again washed with buffer followed

FIG. 1. Equal volumes of serum and 0.05 M. acetylcholine bromide incubated in the capillary of the Astrup micro equipment. Rates of fall of pH shown to be nearly linear between 7.3 and 7.1.

by water, and filled with a mixture of equal parts pH 7.38 buffer solution and 0.05 M. acetylcholine bromide. No fall of pH was observed in 30 minutes, showing that alkaline hydrolysis of the substrate does not occur under these conditions.

CALIBRATION OF THE METHOD IN INTERNATIONAL UNITS An international unit of enzyme activity is defined as 'that which will catalyse the transformation of one micro mole of substrate under defined conditions' (International Union of Biochemists Commission on Enzymes, 1961).

As the fall of pH in the test is measured in a closed system, without exposure to the atmosphere, the calibration had to be performed under the same conditions. The cholinesterase present in 12 ml. of pooled serum from 10 blood donors was inhibited with 0.05 ml. neostigmine (2.5 mg./ml.). Twenty minutes later, the cholinesterase activity was measured and the fall of pH found to be less than 0.01 pH units in three minutes. The serum was next equilibrated with 4% CO₂ at 38°C. for 90 minutes in a swirling tonometer. It was then anaerobically transferred to a 10 ml. syringe which was sealed until the next stage of the procedure.

Mixtures of 0.05 M. acetylcholine bromide and a solution containing 0.05 M. acetic acid and 0.05 M. choline bromide were prepared. The dead space in a

well-lubricated, glass, 2 ml. syringe and needle was filled with clean mercury. The needle of the 2 ml. syringe was introduced into a rubber tube on the end of the syringe containing the equilibrated serum and the plunger drawn up to the 1 ml. mark. The needle was then withdrawn, dipped into the substrate-acid-choline mixture and the plunger drawn up to the 2 ml. mark. A further spot of mercury was then drawn into the syringe to wash the mixture from the dead space. The syringe now contained 1 ml. serum, 1 ml. substrate mixture, and a drop of mercury. The contents were mixed by shaking, the mercury expelled from the dead space, and the pH measured on a sample sucked into the capillary of the electrode.

The results of this experiment, which was repeated on three different batches of pooled serum, are shown in Fig. 2 where the fall of pH is related to the changes in the concentration of acetic acid in the mixtures. The mean of the three results show a fall of pH from 7.3 to 7.1 to be due to 2.503 μ moles of acetic acid.

When equal volumes of serum and 0.05 M. substrate were mixed in a closed system, therefore, a fall of pH from 7.3 to 7.1 may be said to have been due to the hydrolysis of 2.503 μ moles of substrate. This, divided by the time taken in minutes for the fall, gave the enzyme activity in international

FIG. 2. *Equilibration in international units. The pH was obtained by adding mixtures of 0.05 M acetylcholine bromide, acetic acid, and choline bromide to an equal volume of serum in a closed system.*

FIG. 3. Comparison between results by the present method and Radiometer titrator method. Regression coefficient = 0.74. 95% confidence limits 0.63 to 0.85.

FIG. 4. Comparison between results by the present method and a modified titrator method using the same concentration of reagents in each case. The line with a slope of 45° is not a calculated regression line.

units per millilitre serum. If the time is measured in seconds, then $\frac{2.503}{t} \times 60 = \frac{150}{t} = \text{enzyme activity}$ in international units per millilitre serum.

COMPARISON WITH A CONVENTIONAL METHOD

Means of duplicate determinations of the activities of 26 sera obtained by the new method were not compared with those obtained using a conventional titration technique (Radiometer instruction leaflet). In this method a mixture of 25 ml. of saline, 1 ml. of serum, and 2 ml. of 10% acetylcholine bromide were incubated at 38°C . in contact with the electrodes of a pH meter. The apparatus could be adjusted so that N/10 sodium hydroxide was automatically added from a micrometer syringe burette to neutralize the acid produced and maintain the pH at 7.4. Figure 3 shows that the results obtained by the new method are about 74% of those obtained by the titrator technique.

It was considered that the difference in results was probably due to using a 1 in 2 dilution of serum in the new method compared with 1 in 28 in the titrator method. A further 15 samples were therefore compared. In this case the titrator method was modified. Equal volumes of serum and 0.05 M acetylcholine bromide were used as the incubation mixture and the pH was maintained at 7.2 with N sodium hydroxide. These conditions simulated those used in the micro method and the results obtained by both methods are seen to agree closely (Fig. 4).

The 'international units' obtained by the new method are not the same as those by other methods because, though each of them measures the number of micro moles of substrate transformed each minute, the conditions of the test differ in all cases. It is important, therefore, to give the normal range when expressing results.

RELATIONSHIP BETWEEN THE ENZYME CONCENTRATION AND ACTIVITY AS MEASURED BY THIS METHOD

A patient who developed scolone apnoea was found to have a cholinesterase activity of less than 20 i.u. per 100 ml. serum. A series of mixtures of this serum was made with serum having a normal cholinesterase activity. The activities of the mixtures were then measured. The calculated values (all very low) due to the abnormal serum were then subtracted from the total activity. The test was repeated using different specimens of the patient's and normal serum (Figs. 5 and 6). These results show that the activity as measured by this method is proportional to the concentration of the enzyme.

FIGS. 5 and 6. Enzyme activity related to the concentration of normal serum in a mixture of sera with normal and very low cholinesterase activities.

KEEPING PROPERTIES OF THE SERUM ENZYME

There was no change in the enzyme activity when serum was stored at 25°C. and 37°C. for 48 hours and for three months at -20°C.

RESULTS OBTAINED BY THE METHOD

In Table I the serum cholinesterase activities of patients are listed along with the dibucaine and fluoride numbers and the reason for performing the

TABLE I
SERUM CHOLINESTERASE ACTIVITIES OF PATIENTS IN PRESENT SERIES

Subject	Sex	Cholinesterase (i.u./100 ml.)	Dibucaine No.	Fluoride No.	Genetic Type	Reason for Test
1	M	65	15	15	Homozygote atypical	Scoline apnoea 2 hours
2	M	24	21	24	Homozygote atypical	Scoline apnoea 3 hours
3	M	66	16	31	Homozygote atypical	Scoline apnoea 2 hours
4	M	19	25	30	Homozygote atypical	Scoline apnoea 2½ hours
5	M	48	15	33	Homozygote atypical	Scoline apnoea 2 hours
6	F	72	24	30	Homozygote atypical	Sister of 5
7	M	55	14	25	Homozygote atypical	Scoline apnoea 2 hours
8	M	60	6	32	Homozygote atypical	Scoline apnoea 2½ hours
9	M	50	15	35	Homozygote atypical	Scoline apnoea 2 hours
10	M	384	60	58	Heterozygote	Brother of 1
11	M	295	65	58	Heterozygote	Brother of 1
12	F	177	53	48	Heterozygote	Daughter of 1
13	M	155	43	46	Heterozygote	Son of 1
14	M	234	51	41	Heterozygote	Son of 2
15	M	266	60	48	Heterozygote	Son of 2
16	M	300	61	50	Heterozygote	Son of 3
17	M	275	44	51	Heterozygote	Son of 4
18	M	305	55	48	Heterozygote	Son of 4
19	M	198	56	41	Heterozygote	Father of 5 and 6
20	F	238	65	50	Heterozygote	Mother of 5 and 6
21	F	210	60	50	Heterozygote	Sister of 5 and 6
22	M	240	55	54	Heterozygote	Son of 7
23	M	234	44	55	Heterozygote	Son of 7
24	M	300	65	28	Heterozygote	Son of 8
25	F	275	51	36	Heterozygote	Daughter of 8
26	M	138	66	37	Heterozygote	30 minutes' apnoea
27	F	440	77	65	Normal	Wife of 1
28	M	468	81	57	Normal	Father of 26
29	F	350	84	58	Normal	Sister of 26
30	M	330	81	67	Normal	Brother of 26
31	F	300	84	65	Normal	Wife of 3

test. Dibucaine and fluoride numbers were measured using the Gilford attachment to a Unicam SP 500 monochromator unit.

It should be noted that anticholinesterase drugs are sometimes given to patients before the cause of the apnoea is found. In these cases the cholinesterase activity will be found to be low and the dibucaine and fluoride numbers incorrect, due to the presence of the previously administered inhibitor.

The method may also be used as a screening test for poisoning by organic phosphorus compounds used as insecticides (Aldridge and Davies, 1952) or as long-acting pupil dilators in the treatment of glaucoma. Dibucaine and fluoride numbers on these patients may also be incorrect due to the circulating inhibitor.

DISCUSSION

The most useful application of this method is the investigation of prolonged apnoea during and after anaesthesia. This may be due to slow hydrolysis of the scoline (succinyl dicholine) used as a muscle relaxant for intubation. The scoline is usually rapidly hydrolysed by serum cholinesterase so that relaxation only lasts about three minutes. If there is a very low concentration of the enzyme or the presence of an atypical enzyme which, while able to hydrolyse acetylcholine has little effect on the scoline, there will be prolonged relaxation with consequent apnoea (Bush 1961; Lehmann, Liddell, Blackwell, O'Connor, and Daws, 1963).

The homozygotes for the atypical enzyme have a very low serum cholinesterase activity which may be rapidly discovered by this technique as the Astrup apparatus is generally in readiness for pH measurement throughout the day. Blood may be taken by venepuncture or by heel or thumb prick into capillary tubes without anticoagulant. While it is being centrifuged to separate the serum a tube of 0.05 M. acetylcholine bromide may be thawed. The enzyme activity is measured and the result available in less than 10 minutes. We have investigated eight patients with apnoea of longer than 30 minutes' duration following scoline. All had values below 75 i.u./100 ml. serum.

Heterozygotes for the normal and atypical enzymes occasionally develop apnoea following scoline (Tefler, MacDonald, and Dinwoodie, 1964) and may have a normal or only slightly reduced cholinesterase activity. These should be investigated by the measurement of the dibucaine and fluoride numbers (Kalow and Genest, 1957; Lehmann *et al.* 1963).

The principal disadvantage of the method is that it is only accurate when used for serum which has a reasonably normal standard bicarbonate since no buffer solution is used. Red cells and tissue extracts have different buffering capacities from plasma and a calibration curve would need to be performed in each case. Theoretical disadvantages are that the initial rate of enzyme activity is not measured and that the fall of pH will inhibit the enzyme. After equilibration with 4% CO₂ the fall to 7.3 from the initial pH of 7.5, the time the substrate is added usually only takes a few seconds. The fall of pH is only over 0.2 pH units and it has been shown that the time taken for this fall is proportional to enzyme concentration.

As acetylcholinesterase in red cells will also hydrolyse the substrate there may be some interference from this enzyme if there is any haemolysis or if the serum is left in contact with the clot for long time.

The method is only possible because of the extreme stability and sensitivity of the pH equipment. Any equally stable apparatus may of course be used in place of the A.M.E. 1. The principle may well be used for the investigation of other enzyme-substrate systems which result in a change of pH.

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