A FIELD TEST FOR THE ASSAY OF HUMAN WHOLE-BLOOD CHOLINESTERASE

BY

D. R. DAVIES, M.Sc., F.R.I.C.

AND

J. D. NICHOLLS

*The Chemical Defence Experimental Establishment,
the Ministry of Supply, Porton*

The level of cholinesterase (ChE) in the blood is a valuable index of the amount of anticholinesterase which has been absorbed into the system, and it has in fact been used for this purpose (Metcalf, 1951; Davies, 1952a; Marchand, 1952). Since anticholinesterases are being used extensively in agriculture and for insect vector control—very often far removed from laboratory facilities—the determination of such an index under field conditions would be useful. The essential requirement of a field test is simplicity. The procedure and equipment required should be simple, few reagents be necessary, and the time taken for the performance of the test be reduced to a minimum. Finally, the test should be sufficiently uncomplicated to be carried out by non-specialist personnel.

Aldridge and Davies (1952) have discussed the possibility of applying the generally used techniques for ChE assay to field conditions, and came to the conclusion that, for reasons given, these were unsuitable. They did, however, suggest that the electrometric method of Michel (1949) was satisfactory under certain circumstances. They based their suggestion on the fact that this technique lent itself to the rapid and easy estimation of considerable numbers of samples. While subsequent experience has shown this to be true, there are two major disadvantages. The pH meter is not reliable enough to use in the field; and the glass electrode is most certainly too fragile for this purpose.

Fleisher and Pope (1954) have applied the Hestrin (1949) colorimetric method to the estimation of whole-blood ChE. Their method possesses the advantage that it is a colorimetric technique and that single estimations can be completed, including the collection of the blood sample, in twenty-five minutes. Its disadvantages as a field test are (1) relatively few tests can be performed in a given time, and (2) at least six reagents and an equally large number of manipulative processes are involved. Furthermore, the technique requires the use of a spectrophotometer.

Limperos and Ranta (1953) have described a test which they have used under field conditions and which approximates to the requirements. The principle is the same as that of the electrometric method. The differences are (1) that changes in pH are followed by changes in a bromthymol blue (B.T.B.) solution incorporated in the reaction mixture, and (2) the test is carried out in an unbuffered medium. The levels of enzyme activity are assessed by the colour of the solution after a given interval of twenty minutes, and these colours are green, olive green, olive brown, and orange. In practice the distinction between the first two and the second two is difficult. This is a serious disadvantage, since (as is shown below) this is the important range. A second major disadvantage is that under certain conditions of illumination the combination of blood and B.T.B.

appears to fluoresce. When this happens colour assessment is almost impossible. Despite these difficulties a relatively slight modification of procedure has been shown to give a workable and satisfactory test. If a fixed colour change is taken—for example, from green to orange—and the time for the change to occur is observed, these difficulties are overcome.

Experimental

Apparatus.—Tubes 3 by $\frac{3}{8}$ in. (7.5 by 1 cm.) (one for each test). Pipettes: 10 ml. graduated in 1 ml.; 5 ml. graduated in 0.5 ml.; 20 c.mm. haemoglobin pipette. Rack to hold ten 3 by $\frac{3}{8}$ -in. tubes. Watch to read accurately in minutes. Triangular needles, 1 packet. Thermometer.

Reagents.—Two solutions only are needed. (1) Acetylcholine chloride ampoules (Roche Products), 0.6% solution. Dissolve the contents of six 0.1-g. ampoules in 100 ml. of distilled water. This solution can be stored at 20° C. for a period of five or six days without deterioration, and provides sufficient solution for 200 tests. (2) Bromthymol blue: (a) Stock solution, 0.04 g. of powder is dissolved in 1 ml. of N/20 sodium hydroxide. Approximately 90 ml. of distilled water is added and then sodium hydroxide, dropwise, until the solution just turns blue. The total volume is made up to 100 ml. The pH of this solution should be 7.7, and if it is possible, since this solution can be made up in the laboratory, it should be checked with a pH meter. (b) For use, dilute two volumes of stock solution with one volume of distilled water.

Technique.—Collect 20 c.mm. of blood in a haemoglobin pipette and wash it out into 1 ml. of dilute B.T.B. solution contained in one 3 by $\frac{3}{8}$ -in. tube. Add 0.5 ml. of acetylcholine chloride solution and note the time of addition. Note the exact time at which the colour of the reaction mixture becomes deep orange. The interval is a measure of the enzyme activity. To facilitate the determination of the end-point, a standard* is easily prepared as follows: Blood and B.T.B. solution in the above proportions are added to a tube and then 0.15 ml. of N/100 acetic acid. The volume is then made up to 1.5 ml. (The pH should be 6.7.)

Results

If the time interval over which the requisite colour change occurs is determined precisely, then the assessment of whole-blood activity correlates closely with determinations made by the electrometric method. This is shown in Fig. 1. In

this figure the reciprocal of the time (T) taken to effect the given colour change at temperature (t) is plotted against the activity of the sample in $\Delta\text{pH}/\text{hour}$ always determined at 25° C. (For convenience the actual ΔpH value has been multiplied by 100 throughout.) In this case t was between 19 and 22° C. The close correlation between $\Delta\text{pH}/\text{hour}$ and T is obvious from the graph.

The relative activities of different specimens at varying temperatures have been plotted against the temperature, the activity at 20° C. being taken as equivalent to 100. The relative activity increases with temperature in a non-linear fashion from 10° to 40° C. Above 40° C. results become uncertain. At 10° C. the relative activity is about 65, whilst at 30° C. it is 137.

*A permanent glass standard is obtainable from Tintometer Ltd., Salisbury.

FIG. 1.—The relationship between ChE activity measured as (1) the reciprocal of the time necessary to effect given colour change (that is, $1/T$), and (2) in electrometric units (that is, ΔpH). Ar determined at temperatures between 19 and 22° C., ΔpH determined at 25° C.

The information yielded by these two series of experiments allows us to simplify the test for field conditions. If in addition it is possible to relate enzyme levels to zones which will permit clear-cut executive action, then a very useful test may be evolved. The latter idea is possible, for three critical levels of whole-blood ChE can be defined: (1) The lower level of normal whole-blood ChE activity; (2) the level of hypersensitivity; (3) the clinical danger level.

Lower Level of Normality.—There have been very many determinations of normal values of red-cell, plasma, and whole-blood ChE, and the limits of variation have been extensively worked out (Callaway *et al.*, 1951; Wolfstie and Winter, 1952; Fleisher and Pope, 1954). These are consistent with each other. For the present purpose the values for the whole-blood ChE as determined in this laboratory have been used to define the lower limits of normal activity. On a recently determined series the mean value of whole-blood ChE was 133 (Δ pH/hour) with a standard deviation of 12 (Δ pH/hour). The lower fiducial limit of normality is therefore 109 (Δ pH/hour).

Level of Hypersensitivity.—No symptoms of systemic poisoning are obvious until a very significant reduction of the blood ChE has occurred. Callaway *et al.* (1954), however, have shown that when the whole-blood ChE of rabbits has, by previous exposure to anticholinesterase, been reduced to half the pre-exposure value, there is a significant reduction in the LD₅₀, thus demonstrating that a reduction in the blood ChE level is indicative of an increased sensitivity towards further doses of anticholinesterase. Their data did not permit an extrapolation which would have indicated the exact point at which this increased sensitivity is first obvious. In the absence of such precise data, and bearing in mind the main purpose for which this test is being evolved, we therefore suggest that the level of clinical significance could be set at 50% of the mean normal level—that is, at Δ pH/hour of 67.

Clinical Danger Level.—Davies (1952b) noticed that the blood cholinesterase of animals dosed with anticholinesterase by the intravenous, intramuscular, or percutaneous route had to be reduced to at least 20% of normal before the onset of serious clinical symptoms. In his experience the critical whole-blood cholinesterase level is about 20% of normal, although it does not follow that symptoms automatically appear when this level of blood cholinesterase is reached (Frawley *et al.*, 1952). The clinical danger level may therefore be set at 20% of the mean normal level—that is, at Δ pH/hour of 26 units.

Effect of Temperature

The most difficult problem has been to evolve a method of temperature compensation without the use of a constant temperature bath or enclosure. This was done as follows. As indicated above, several simultaneous observations were carried out on the same sample at various temperatures, together with electrometric determinations at 25° C. These results were then summarized in a family of straight lines corresponding to each temperature and relating $1,000/A_T$ and Δ pH/hour, where T is the time taken for the completion of the colour change. From these, T was determined for each critical level at varying temperatures, and a new family of curves constructed relating T and temperature. By plotting these on one diagram, as in Fig. 2, a chart showing various zones has been constructed, each zone corresponding to the interpretation shown on the right side. (See also Table I.)

TABLE I

	Lower Level of Normality Δ pH 109	Upper Level of Hypersensitivity Δ pH 67	Level of Serious Poisoning Δ pH 26
10° C.	41 minutes	63 minutes	111 minutes
15° C.	32 "	49 "	98 "
20° C.	26 "	40 "	87 "
25° C.	22 "	34 "	74 "
30° C.	19 "	30 "	62 "
35° C.	14 "	23 "	53 "

The *modus operandi* of the test is therefore as follows: The temperature of the ambient air is noted. An ordinate of the chart is then drawn corresponding to this temperature, and the time read off at the point where it cuts each curve.

The tubes are prepared as described earlier—that is, 1 ml. of B.T.B., 20 c.mm. of blood, and 0.5 ml. of acetylcholine chloride—and then the colours noted

FIG. 2.—Chart relating time of colour change with temperature and degree of poisoning.

at each of these times, obtained from the chart (Fig. 2). In all the tubes in which the colour has changed to deep orange at the time of inspection the reaction is complete. For example, if the temperature was 23° C., the test with all normal blood samples would be complete in 21 minutes. Blood which changed colour in less than

34 minutes but longer than 21 minutes would possess whole-blood ChE levels lower than normal, and the level would indicate a slight absorption of anticholinesterase. Such levels would, however, be of little clinical significance. They would, on the other hand, indicate indifferent safety procedures or personal carelessness on the part of the individual.

If the time taken is longer than 34 minutes this indicates that the individual is hypersensitive, and will come into the appropriate zone. If even after 72 minutes the test is not complete, at least 80% inhibition is indicated, implying danger of clinical symptoms.

It should be emphasized that an exact colour match with the standard is seldom necessary, since the criterion at the critical time is whether the colour change is complete at that time.

One other point with reference to temperature needs to be discussed. The test may in extreme circumstances—that is, at 10° C.—take 100 minutes to complete, and if no constant temperature bath is to be used it is rather important to know what temperature fluctuation may be permitted during the period of the test itself. The experiments on which the relationship between field test and the electrometric method (shown in Fig. 1) were based were carried out at 19° C., 20° C., and 22° C., hence a variation of 3° C. during the period of the test would seem to be permissible. Lowry *et al.* (1954) confirm this suggestion, for they state that to measure cholinesterase to within 3% the permissible temperature range is 3° C. This is, however, a point that ought to be checked under a wide variation of conditions, or at least kept in mind during the period of the test.

A series of experiments were carried out at different temperatures to check the chart that has been produced. The procedure was exactly as described. Forty-eight samples of blood were prepared by diluting normal blood with inactivated blood. Thirty samples (obtained from eight individuals) were examined on different occasions in the climatic chamber at 31° C. and 85% relative humidity. A further 16 samples (from four individuals) were tested in a constant temperature bath at 31° C. The results are shown in Table II.

Of the 47 samples so tested, 45 conform precisely to the pattern described in the test. Of the discrepant samples those with Δ pH/hour—116, T₃₁—19 minutes are only just outside the expected zone. A sample of Δ pH—116 should have a reaction time of 18 minutes or less.

TABLE II.—The Confirmation of Reaction Times at 31° C.

Normal Range		Range of Slight Absorption		Hypersensitive Range	
Δ pH/hr.—109		Δ pH/hr.—109–67		Δ pH/hr.—67–26	
T ₃₁ °	18 min.	T ₃₁ °	18–29 min.	T ₃₁ °	29–67 min.
130	15	108	20	66	31
126	15	104	20	63	32
146	14	103	21	59	42
132	15	103	20	58	39
123	15	102	20	56	40
140	14	100	22	55	33
129	15	100	22	53	40
148	15	96	22	52	40
136	16	95	23	49	35
133	15	88	23	36	47
127	15	87	23	36	50
146	15	85	26	33	50
116	19	81	25	30	52
116	19	78	27		
110	18	76	27		
		75	27		
		75	28		
		73	28		
		67	27		

Δ pH/hour values and reaction times grouped according to the clinical zones described in Fig. 2.

Summary

The field test of Limperos and Ranta for the assay of human whole-blood ChE has been modified to facilitate its more extensive use. A chart has been constructed which permits adjustments in the conditions of the test to compensate for temperature variation and to relate ChE levels to the degree of poisoning.

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Speaking at a largely attended council meeting of the Invalid Children's Aid Association on May 13, Dr. F. S. COOKSEY, director of the department of physical medicine at King's College Hospital, London, described the rehabilitation of the physically handicapped child under three heads: medical care, education of the mind and body, and "settlement," both at home during school life and later at work. With regard to the first, Dr. Cooksey stressed the importance of early diagnosis, particularly in cases of cerebral palsy, and of integrating the medical care under one doctor. "Only one man can integrate the treatment of the child, and he is the family doctor—who frequently does not," said Dr. Cooksey. So far as education is concerned, he thought that too many children with potentially normal brains were suffering from lack of education because they are segregated on account of their physical handicaps. "We should realize that the physically handicapped child should earn its living with its brain, not necessarily with its hands." Craft work was emphasized too much. Speaking of settlement in the home, Dr. Cooksey drew attention to permissive regulations under the National Assistance Act which allow for alterations to be made in the home so that the handicapped child can live more comfortably—such things as the widening of doors, the erection of ramps, and special appliances and furniture.

Medical Memorandum

Female Circumcision and the Age of the Menarche

From previous observations (Ellis, 1950; Wilson and Sutherland, 1953) it is evident that in tropical countries the onset of menstruation may be early or late compared with that for English children, but that it is not determined solely by the climate.

Studies in African ethnopsychiatry (Carothers, 1953) suggest that environmental factors may be of importance in the development of personality and constitution. It is of interest, for example, that exophthalmic goitre (Graves's disease), which may be associated with personality type (Martin and Fisher, 1945), is rare in Africans (Gillman and Gillman, 1951; Carothers, 1953; Davidson, 1954; Wilson, 1954). It is well known, too, that changes in environment which directly or indirectly modify glandular function may affect menstruation in English girls (Browne, 1950).

During nutritional investigations in Sierra Leone which extended from December, 1953, to February, 1954, the opportunity arose to investigate the influence of initiation rites, which include female circumcision, on the age at menarche of Africans. A comparison was made between Creole girls who are Colony-born Africans, among whom no circumcision is practised, and girls belonging to native tribes in the Protectorate, who at the age of about 12–13 years retire into seclusion for ceremonies of initiation. These rites are characterized by an operation on the clitoris and labia minora, which may vary from small incisions to total excision, with the object of restricting sexual pleasure. After this the girl is ready for marriage; without the operation she will not obtain a husband. This initiation and operation usually precedes the menarche; in view of the high emotional content of the ritual it therefore seemed possible that the onset of menstruation might be affected.

Records for the tribal girls were particularly difficult to obtain, and data were available for only 33. The number of Creole girls studied was 129. The present age of each girl was recorded, together with a note whether or not her periods had started. With this information the average age of the menarche and its standard deviation may be calculated, using the statistical technique of probit analysis. The results obtained may be compared with corresponding results for 2,590 girls in the South of England (Wilson and Sutherland, 1953). None of the differences is statistically significant (see Table). Although the number of tribal girls is small, it may be concluded that there is no evidence that the initiation and operation has any substantial effect upon the age of the menarche.

	No. of Girls	Average Age of Menarche (years)	Standard Deviation (years)
Tribes	33	13.44	1.30
Creoles	129	13.65	0.94
S. England	2,590	13.49	1.19

DAGMAR C. WILSON, M.D., M.R.C.P.,
 F.R.C.O.G., D.P.H.,
 Laboratory of Human Nutrition,
 University of Oxford.

IAN SUTHERLAND, M.A., D.Phil.,
 Medical Research Council's Statistical
 Research Unit, London.

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