Agar Diffusion Test for Serum Cholinesterase Typing and Influence of Temperature on Dibucaine and Fluoride Numbers*

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Some people exhibit prolonged apnoea following the administration of the muscle relaxant, suxamethonium. It has been shown that these people have an atypical serum cholinesterase. This atypical enzyme hydrolyses suxamethonium, and all other substrates against which it has been tested, less effectively than the usual type of serum cholinesterase (Kalow and Genest, 1957; Kalow and Staron, 1957; Kalow and Davies, 1959; Davies, Marton, and Kalow, 1960). The usual and the atypical phenotypes (U and A) of the enzyme can be distinguished by the use of the local anaesthetic, dibucaine, as an inhibitor under certain standard conditions. The percentage of inhibition is known as the dibucaine number or DN (Kalow and Genest, 1957). The usual enzyme is inhibited approximately 80% by dibucaine, and the atypical enzyme is inhibited approximately 20%. A third phenotype of serum cholinesterase has been detected by means of the inhibitor technique. People of this intermediate type (phenotype I), like those of the usual type, are not suxamethonium sensitive: they have dibucaine numbers around 60. The frequencies of these three phenotypes of serum cholinesterase are approximately equal among various populations in Europe and Canada, namely, about 97% for the U phenotype, 3-4% for the I phenotype, and 1 in 4000 (0.025%) for the A phenotype.

Family studies indicate that these types of serum cholinesterase are genetically determined (Kalow and Staron, 1957) by two co-dominant alleles. One gene, E_1^u , controls the formation of the usual enzyme; the other gene, E_1^a , directs the formation of the atypical enzyme. Therefore, subjects with

phenotypes U and A are homozygous for genes E_1^u and E_1^a , respectively; subjects with phenotype I are heterozygous (E_1^u , E_1^a).

Serum cholinesterase types can also be detected by using other differential inhibitors in the same manner as with dibucaine. One of these differential inhibitors is RO2-0683 (dimethylcarbamate of 2-hydroxy-5-phenylbenzyl trimethylammonium bromide, 'Roche'). This compound is more selective than dibucaine, but it does have the disadvantage of requiring pre-incubation with the enzyme (Kalow and Gunn, 1959; Kalow and Davies, 1959). Two other differential inhibitors are fluoride and succinylcholine. The percentages of inhibition by these compounds have been termed fluoride number or FN (Harris and Whittaker, 1961) and succinylcholine number (McComb, LaMotta, and Wetstone, 1965).

McComb *et al.* (1965) determined the succinylcholine numbers and the dibucaine numbers of 80 individuals and plotted dibucaine numbers against the succinylcholine numbers. The A and I phenotypes were separated distinctly from each other into groups by both systems. The separation of I and U phenotypes was also clearly evident in the succinylcholine system, but the separation of the phenotypes in the dibucaine system was less distinct.

Harris and Whittaker (1961) used fluoride as an inhibitor and determined the fluoride numbers of sera of 285 people already classified by dibucaine numbers. When they plotted the dibucaine numbers against the fluoride numbers, it was observed that the groups of the U and I phenotypes each had a small subgroup in which the fluoride inhibition was relatively less marked than the dibucaine inhibition. These fluoride resistant phenotypes were called UF and IF. Family studies revealed that the fluoride variant was also

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genetically determined. It is believed that its synthesis is directed by a third gene, E_I^f , at the same autosomal locus of genes E_I^u and E_I^a . There is also evidence for a fourth gene, E_I^s , a 'silent' gene which is responsible for the complete absence of serum cholinesterase activity. The homozygote for E_I^s may be detected by the spectrophotometric method, but the heterozygote for E_I^s can be detected only by family studies (Liddell, Lehmann, and Silk, 1962; Simpson and Kalow, 1964).

The standard method for the differentiation of serum cholinesterase by determining dibucaine numbers spectrophotometrically is precise and highly reliable, but it is tedious when a large number of sera is to be tested. Harris and Robson (1963) devised a screening test, the agar diffusion test, which requires only simple equipment and permits rapid testing of a large number of sera. This method depends on the use of the differential inhibitor, Ro2-0683. The usual enzyme is inhibited almost completely by this compound, whereas the atypical enzyme is inhibited to a much lesser degree.

Two agar trays are used in the agar diffusion test: a control tray which contains no inhibitor and a tray which contains the inhibitor. Sera to be tested are suitably diluted and inserted into wells in the trays. After incubating overnight at 37° C. the agar is flooded with a mixture of buffer, substrate, and a diazo-reagent for about two hours. In the control tray, sera with normal activity give a sharp-edged brown circular zone about 1.3 cm. in diameter. In the inhibitor tray two types of reactions can be observed: (1) negative-sera (phenotype U) which show marked inhibition resulting in faint, diffuse zones, and (2) positive-sera (phenotypes I and A) which yield sharp-edged, brown circular zones comparable in diameter to the corresponding zones in the control tray. In a 'blind' test on 250 sera on which the dibucaine numbers had been determined spectrophotometrically, Harris and Robson (1963) found that 69 I and 14 A sera gave positive reactions and 167 U sera gave negative reactions. There were no discrepancies in the results from the two tests.

Similarly, Simpson and Kalow (1965) used the agar diffusion test to screen about 6500 Brazilians (1000 families). When an inhibitor-resistant enzyme was detected in a family, dibucaine and fluoride numbers were determined on all members of the family. The results of the screening test were as follows. Of the 435 U phenotypes, 78% were negative, 13% gave 'false positive' reactions, and 6% were 'undecided'. Two UF and one F phenotypes were negative. Of the 202

I phenotypes, 93% were positive, 3% gave 'false negative' reactions, and 4% were 'undecided'. One IF and four A phenotypes were positive.

This report is concerned with (1) further comparison of the agar diffusion test with the spectrophotometric method for typing serum cholinesterase, (2) the demonstration of the effect of temperature on the inhibition of serum cholinesterase by dibucaine and sodium fluoride, and (3) family studies of dibucaine and fluoride resistant variants.

Materials and Methods

The population from which the blood samples were drawn was described by Dublin, Bernanke, Pitt, Massell, Allen, and Amezcua (1964). It consisted of Caucasians who had had rheumatic fever or clinically proven rheumatic heart disease in the past, their non-rheumatic spouses, and their children.

The screening test of Harris and Robson (1963) was used, with the modification that the zones of reaction were observed at half-hour intervals for two hours instead of the single observation after two hours.

The spectrophotometric tests were performed at 26°C., instead of 21–25°C. as previously described (Kalow and Genest, 1957; Harris and Whittaker, 1961). A Beckman DU spectrophotometer equipped with thermal plates was used. DN was determined by the method of Kalow and Genest (1957), and FN was determined by the method of Harris and Whittaker (1961).

The effect of temperature on the inhibition reaction was studied by obtaining DN and FN values at 23, 26, 30, and 37° C. Except for the changes in temperature, the usual procedures for DN and FN were used.

Results and Discussion

Of the plasmas of 320 couples (320 people who had had rheumatic fever + 320 non-rheumatic spouses) screened by the agar diffusion test, 68 were classified as negative and 22 as positive (3.4%). There were 13 positive reactors among the rheumatics, and 9 among the non-rheumatics.

Spectrophotometric determinations (DN and FN) were performed on 383 plasmas randomly selected from the 640 plasmas. 370 plasmas were found to be U or UF phenotype, and 13 were All U or UF phenotype and all I phenotype were classified as negative and positive, respectively, by the agar diffusion test. In addition, DN and FN were determined on the 9 positive plasmas which had not been included in this sample. All were found to be I phenotype. There was no disparity between the classification with the agar diffusion test and the typing with the spectrophotometric method. Since no A phenotype was observed with the spectrophotometric method, it

TABLE I

FREQUENCY OF SERUM CHOLINESTERASE PHENOTYPE I

Method	No. Examined	No. of Phenotype I	Frequency Phenotype I (%)	E ₁ ^a Gene Frequency
Spectrophotometric determination	383	13	3.4	0.0170
Agar diffusion test	640	22	3.4	0.0172

clear that plasmas classified as positive in the agar diffusion test were I phenotype.

The frequency of I phenotype for the population studied spectrophotometrically was found to be 3.4%. Table I summarizes the frequencies calculated from the data from the agar diffusion test and from the spectrophotometric method. These results further substantiate the observations of Harris and Robson (1963) that the agar diffusion procedure is a reliable and accurate screening test for the classification of serum cholinesterase types.

The means and the standard deviations of DN and FN of plasmas of 392 (370 U + 22 I) subjects surveyed by the spectrophotometric method were calculated and compared with the findings of Harris and Whittaker (1961). Table II shows that there is good agreement in the values of the dibucaine

TABLE II

COMPARISON OF DIBUCAINE NUMBERS OBTAINED BY PRESENT AUTHORS WITH THOSE OF HARRIS AND WHITTAKER (1961)

Phenotype	No. of Individuals	Dibucaine Number		Investigators
		Mean	SD	
U	370	79.73	1.80	Present
	179	80.06	1.56	Harris <i>et al.</i>
I	22	61.50	3.33	Present
	84	61.93	4.17	Harris <i>et al.</i>

numbers. However, the means of our fluoride numbers in Table III are slightly lower than theirs. The initial suspicion was that the low fluoride inhibition was caused by the anticoagulant in the blood samples. It was believed possible that the ethylenediamine tetra-acetic acid (EDTA) might exert an indirect effect on the substrate-enzyme binding through formation of a chelation compound with a positively charged metallic ion in the plasma.

Fluoride numbers were determined on sera and plasmas collected in acid-citrate-dextrose (ACD), EDTA, heparin, or oxalate. From Table IV it can be seen that there was no significant difference among the means of sera and plasmas.

TABLE III

COMPARISON OF FLUORIDE NUMBERS OBTAINED BY PRESENT AUTHORS WITH THOSE OF HARRIS AND WHITTAKER (1961)

Phenotype	No. of Individuals	Fluoride Number		Investigators
		Mean	SD	
U	370	56.80	2.76	Present
	179	61.35	3.21	Harris <i>et al.</i>
I	22	44.64	2.06	Present
	84	44.79	4.48	Harris <i>et al.</i>

Since the determinations of dibucaine numbers and fluoride numbers in this study were performed at 26° C. instead of 21–25° C., as previously described (Kalow and Genest, 1957; Harris and Whittaker, 1961), studies were made of the effect of temperature on the inhibition of serum cholinesterase by dibucaine and sodium fluoride. When dibucaine numbers of a U subject were determined at various temperatures, a linear relation of the inhibition numbers to temperature was observed (Fig. 1). It can be seen from the graph that for an increment of one degree there is a decrease of nearly 0.6 in the dibucaine number ($\frac{\Delta DN}{\Delta t} = 0.6$) and a decrease of approximately 2.2 in the fluoride number ($\frac{\Delta FN}{\Delta t} = 2.2$). During the preparation of our manuscript, McComb *et al.* (1965) reported the temperature effect on the inhibition of serum cholinesterase by dibucaine and sodium fluoride.

TABLE IV

FLUORIDE NUMBERS OF SERA AND PLASMAS OF 6 SUBJECTS

Subjects	Sera	Plasmas			
		ACD	EDTA	Heparin	Oxalate
J.C.R.	56.41	55.56	57.00	56.57	55.36
J.P.	56.41	57.33	55.56	56.25	57.81
S.V.	54.29	54.39	54.84	54.54	55.77
G.L.	53.26	55.56	55.26	55.68	53.12
F.A.B.	58.14	56.32	56.79	55.13	57.59
T.L.	55.88	54.66	55.13	55.22	55.38
Mean	55.73	55.64	55.76	55.57	55.50

They found that dibucaine numbers were moderately affected by temperature, whereas fluoride numbers which averaged 59 at 25° C. were 22 when measured at 37° C, i.e. $\frac{\Delta FN}{\Delta t} = \frac{37}{12} = 3.1$.

This is similar to our finding. The difference in the means of FN obtained by Harris and Whittaker (1961) at 21–25° C. and by us at 26° C. can possibly

Fig. 1. The effect of temperature on the dibucaine and fluoride numbers of serum from a subject with phenotype U. Each point represents the average of triplicate determinations.

be attributed to the temperature effect. The necessity for precise control of temperature in the determination of FN is emphasized by these data.

The bimodal distribution of dibucaine numbers in our material is evident in Fig. 2 and Fig. 3. These data were obtained on the 383 randomly selected plasmas and the 9 positive plasmas detected in the screening tests. Subjects with DN < 69 were classified phenotype I (Harris and Whittaker, 1961). The low fluoride numbers in this group are also consistent with this phenotype. The family data (Table V), which include all parents with DN < 69, support the designation of phenotype I for DN < 63. Unfortunately, no family material was available to check the critical portion of the distribution (DN 64-68).

Indistinct clustering of U and UF phenotypes is shown in Fig. 3. The family data (Table VI), which include all parents with FN < 55, suggest that the separation of the two groups occurs at approximately FN 53. The overlap of the groups is probably related to the differential effect of temperature on the fluoride numbers of the two phenotypes (Fig. 4). The fluoride number of a UF individual was observed to be less affected by

temperature ($\frac{\Delta FN}{\Delta t} = 1.5$) than the fluoride number of a U individual ($\frac{\Delta FN}{\Delta t} = 2.6$). It can be seen from Fig. 4 that the separation of the fluoride numbers of the two phenotypes is less marked at

TABLE V
STUDY OF FAMILIES IN WHICH ONE PARENT WAS A DIBUCAINE VARIANT

	Parents		Children							
	Father	Mother	1	2	3	4	5	6	7	8
DN	80.88	54.79*	61.67*	77.86	63.51	78.85	56.25*	63.24	64.29	78.89
FN	57.35	39.73	45.00	55.73	45.95	55.77	51.56	48.53	46.43	58.89
DN	57.50*	80.67	80.46							
FN	48.75	56.30	55.17							
DN	58.49*	79.75	80.36	59.68*	80.70	81.52	78.75			
FN	45.28	56.96	57.14	51.61	61.40	57.61	57.50			
DN	60.17*	78.69	78.82	84.06						
FN	42.37	59.02	56.47	55.07						
DN	80.18	61.43*	78.38	78.57	78.19					
FN	55.86	44.29	55.90	57.14	61.65					
DN	81.48	61.76*	62.35*	65.79	79.00	63.16				
FN	55.67	44.12	37.65	53.93	56.00	44.21				
DN	62.03*	84.21	57.78*	78.79						
FN	44.30	56.14	51.11	56.06						
DN	78.48	62.34*	58.33*	79.12						
FN	56.96	46.75	39.58	54.95						
DN	79.35	62.67	80.00							
FN	55.44	44.00	55.38							

* Subjects with DN < 63.

FIG. 2. Frequency distribution of U and I phenotypes. People with DN < 69 are considered to be phenotype I; the others, U.

FIG. 4. The relation between temperature and fluoride numbers of two people, one of phenotype U, the other of phenotype UF.

FIG. 3. Diagram illustrating the correlation of dibucaine and fluoride numbers in 392 people. The separation of U and I dibucaine variants is clear, but the distinction between U and UF is less evident.

high temperatures. In fact, at 37–40° C. the numbers are almost identical. Since our measurements were made at a higher temperature (26° C.)

TABLE VI

STUDY OF FAMILIES IN WHICH ONE PARENT HAD FLUORIDE NUMBER 54 OR LESS

	Parents		Children			
	Father	Mother	1	2	3	4
DN	54·08	44·90*	53·60	52·34*	52·38*	
DN	80·61	75·51	79·20	76·64	76·87	
FN	55·36	46·03*	59·21	57·29	50·00*	
DN	80·36	74·60	80·26	78·12	77·36	
FN	46·74*	57·14	49·16*	48·18*		
DN	76·90	81·32	77·10	77·27		
FN	46·96*	56·99	49·03*	58·39		
DN	73·91	78·49	72·55	83·90		
FN	48·56*	60·00	55·04	51·61*	56·99	55·08
DN	78·03	80·00	82·17	75·27	80·65	83·05
FN	50·00*	61·25	53·09			
DN	76·00	80·00	77·78			
FN	52·22*	57·50	52·31*	56·90		
DN	73·33	77·50	78·46	81·03		
FN	52·83	61·03	59·00	55·06		
DN	82·08	79·49	81·00	80·81		
FN	53·00	55·56	54·17			
DN	73·00	81·48	82·50			
FN	53·03	57·14	49·33*	50·91*		
DN	77·27	79·12	78·67	79·39		
FN	55·32	53·33	53·21	53·09		
DN	80·85	79·17	78·90	81·48		
FN	53·36	64·29	59·42	54·93		
DN	76·75	81·43	84·06	77·46		
FN	53·61	54·19	54·40	55·17		
DN	80·12	80·18	82·40	79·31		
FN	57·38	53·66	53·54			
DN	81·15	80·49	82·68			
FN	54·02	56·25	59·26	56·32		
DN	78·16	77·50	79·63	81·49		
FN	54·17	54·02	55·46	55·96	55·08	
DN	80·00	79·31	82·35	80·73	81·02	
FN	54·17	57·50	56·12	61·40	55·56	56·12
DN	80·83	77·50	81·63	82·46	84·44	80·61
FN	55·26	54·25	53·60	55·08		
DN	80·70	79·74	82·40	83·05		
FN	54·37	57·31	52·38*	53·70	52·75	
DN	79·61	79·31	80·95	82·10	83·51	
FN	57·97	54·44	54·55	52·83		
DN	82·61	78·89	80·81	79·58		

* Subjects with FN < 53.

than that at which previous investigators have made their determinations (21–25° C.), a less distinct separation of phenotypes was observed in our material.

Summary

The results in the cholinesterase typing of 392 plasmas by the agar diffusion test were fully confirmed by the spectrophotometric determination of dibucaine numbers.

The main advantage of the agar diffusion test is the simplicity with which dibucaine variants can be detected. In our hands the test was of no value in detecting the UF phenotype.

The dibucaine number and the fluoride number were found to be inverse functions of temperature. The change in fluoride number per degree was roughly 4 times as great as the change in dibucaine number. Furthermore, the magnitude of the change of the fluoride number with temperature was greater with the U than with the UF plasmas.

By using the spectrophotometric method, the distinction between U and I dibucaine variants was apparent at 26° C., but the U and UF groups could not be clearly separated. Since previous investigators have determined fluoride numbers at 21–25° C., and since the distinction between U and UF is less marked when fluoride numbers are determined at raised temperatures, our failure to distinguish clearly these phenotypes may be related to the temperature effect.

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