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Research Article

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(*b*) Normal sera and all silent sera contain small amounts of a cholinesterase activity labeled the residual cholinesterase. The enzyme(s) responsible has properties similar to those of acetylcholinesterase rather than serum cholinesterase. It is estimated that about 1% of the activity of normal serum against acetylthiocholine is due to this enzyme. The source of the residual cholinesterase is not yet known.

Silent Cholinesterase Gene: Variations in the Properties of Serum Enzyme in Apparent Homozygotes

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ABSTRACT The cholinesterase activity of the sera of 25 subjects diagnosed as homozygotes for the silent cholinesterase gene was studied by a sensitive enzymatic method employing several thiocholine esters and various inhibitors, and by disc electrophoretic, immunochemical, and chromatographic methods.

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INTRODUCTION

Data in the literature indicate variation in the properties of serum cholinesterase (E.C. 3.1.1.8)[1] among individuals thought to be homozygotes for the silent cholinesterase gene. Many reports, including the original case (1), state that such sera have no detectable activity (1–10). Several sera, however, showed "trace" activity (2–4). In addition the report of Gutsche, Scott, and Wright (9) described a large group of Alaskan Eskimos with this genetic disorder; nine subjects had no cholinesterase activity and eight had very low, but "detectable" levels in their serum.

Hodgkin, Giblett, Levin, Bauer, and Motulsky (3) described a family in which two sibs had no serum cholinesterase activity; immunologic studies on the sera of these two subjects failed to show evidence of a protein cross-reacting with an antiserum to normal human serum cholinesterase. In contrast, Goedde, Gehring, and Hofmann (11) described two sera with a small amount of cholinesterase activity (2–3% of normal values) and found immunological evidence of cross-reaction with an antiserum to normal human serum cholinesterase. In a later paper, the same group (12) expanded their observations to five sera, two of which showed no enzymatic activity and no cross-reacting protein and three of which had both.

We have had the opportunity to assemble 25 sera from individuals thought to be silent gene homozygotes. In this report we present results of a study of these sera by a sensitive enzymatic method for serum cholinesterase utilizing various thiocholine iodide esters as substrates and by appropriate immunologic, electrophoretic, and chromatographic methods.

METHODS

Sera. One of the patients, L. C., was reported previously (4). J. P., D. P., and A. P. are three brothers discovered after J. P. had prolonged apnea after the administration of succinylcholine. Both parents emigrated from Sicily, but only the mother was available. Her serum had a normal dibucaine number and a cholinesterase activity 20% below

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[1] The nomenclature in this paper is that recommended by the Commission on Enzymes of the International Union of Biochemistry (Enzyme Nomenclature, Elsevier, Amsterdam, 1965). The systematic name for the serum enzyme is acetylcholine acyl-hydrolase, and the recommended trivial name is cholinesterase, formerly pseudocholinesterase. The systematic name for the enzyme of nerve, muscle, and red cell (E.C. 3.1.1.7) is acetylcholine acetyl-hydrolase, and the recommended trivial name is acetylcholinesterase, formerly "true cholinesterase."

the normal mean. The remainder of the silent gene sera were very kindly sent to us by Dr. Edward M. Scott of the Arctic Health Research Center, College, Alaska, and were obtained by him in the course of his studies on the silent cholinesterase gene in Alaskan Eskimos (9).

The atypical gene homozygotes were selected from a large group of patients found after succinylcholine-induced apnea. The sera used had low activities against acetylcholine or acetylthiocholine, low dibucaine numbers (13), high benzoylcholine: acetylcholine ratios (14), and in each case the pedigree showed no evidence for segregation of the silent cholinesterase gene.

Cholinesterase assay. The cholinesterase assay procedure is based on the Ellman reaction (15) and is modified from the method of Garry and Routh (16). The method utilizes phosphate buffer of pH 7.6, a substrate concentration of 2 mmoles/liter, diluted serum, and 2.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in a total volume of 5 ml. Serum and chromatographic fraction dilutions were made immediately before use. For the assay of normal serum, a dilution of 1:100 in 0.9% NaCl is satisfactory. Lower dilutions are used for lesser activities. The assay is usually for 3 min and is stopped by the addition of 1 ml of 0.5% quinidine sulfate.

When dibucaine or Mytelase inhibition of the reaction was measured the stock substrate and stock inhibitor were diluted together to the requisite strength. When dibucaine inhibition was determined propionylthiocholine was the substrate; acetylthiocholine was used for measurement of Mytelase inhibition. The inhibitor concentrations in the final assay mixture were dibucaine 0.03 mmole/liter, and Mytelase 3×10^{-8} mole/liter.

Quinidine inhibition was studied by adding a fourth assay tube prepared as above to contain DTNB-buffer, acetylthiocholine, and 1 ml of 0.5% quinidine sulfate. At zero time serum was added and the tube allowed to incubate the same time as the others, and compared to the tube in which the quinidine was added at the end of the incubation period.

Gel electrophoresis. Disc electrophoresis was done at 4°C with a 7.5% separating gel and a 1 cm stacking gel as given in the method of Davis (17). For normal serum 6 μl were applied at the top of the stacking gel and samples of up to 30 μl were used for serum and fractions of low activity. A current of 2.5 ma was applied for about 1.5 hr, until the albumin-dye front had migrated 35 mm into the separating gel. The gels were stained with acetyl- and butyrylthiocholine iodide by the method of Bernsohn, Barron, and Hess (18).

Antisera. To prepare rabbit anti-human serum cholinesterase, 15 mg of Cohn fraction IV-6-4 (Cutter Laboratories) was dissolved in 3 ml of 0.9% NaCl and homogenized with 3 ml of complete Freund's adjuvant. Rabbits were injected intramuscularly with 0.5 ml in each leg. The injections were repeated at 2-wk intervals, and the rabbits were bled 10 days after the fourth injection at which time they were given another injection. The bleedings and injections were repeated at 10- to 14-day intervals.

Antiserum free of rabbit serum cholinesterase was prepared by column chromatography on DEAE-cellulose and represented the fraction which eluted with the equilibrating buffer (0.01 M phosphate buffer, pH 6.7). This fraction was dialyzed against water to remove the buffer, lyophilized, and reconstituted with 0.9% NaCl when needed.

Immunoassays. The immunodiffusion studies were carried out with 1% Noble agar in 1% NaCl on microscopic slides prepared according to the method of Ouchterlony (19). The precipitates of cholinesterase-anticholinesterase were never visualized directly but were seen when the slides were stained for esterase activity with α-naphthyl butyrate and 5-chloro-*o*-toluidine (20).

For absorption studies the rabbit antiserum was absorbed three successive times with two volumes of appropriate serum concentrated four times by dialysis against polyvinylpyrrolidone. Each absorption was at 4°C for 4 days. The tubes were then spun at 15,000 *g* for 10 min and the supernatants recovered.

Precipitin studies in liquid media were done as follows: small amounts, 1–12.5 μl, of active rabbit immunoglobulin fraction devoid of cholinesterase and 150 μl of test serum or chromatographic fraction were diluted to 0.5 ml with 0.9% NaCl and incubated 1 hr at 37°C and 2–7 days at 4°C with continuous shaking. The tubes were spun at 15,000 *g* for 10 min and the supernatants recovered. The supernatants and appropriate controls were assayed for cholinesterase activity.

Chromatography on DEAE-cellulose columns. Whole serum was fractionated on purified DEAE-cellulose as outlined by Schulze and Heremans (21). Serum dialyzed against 1 liter 0.01 M phosphate buffer, pH 6.7, was added to 10 bed volumes of DEAE-cellulose which had been equilibrated against the same buffer. Fractions were eluted with increasing concentrations of buffer and NaCl. The fractions were pooled according to the peaks as measured by absorbance at 280 nm. The buffer concentrations for each peak for a typical normal serum are given in Table I and in Fig. 5. The fractions were dialyzed against distilled water at 4°C to remove the buffer and salt and were then lyophilized. The fractions were reconstituted in 0.9% NaCl just before use.

TABLE I

Eluting Solutions and Recovery of Protein in DEAE-Cellulose Column Chromatography of a Normal Serum

Peak No.	Buffer	NaCl	Total protein
	mmoles/liter	*mmoles/liter*	%
1	10		21.9
2	10		1.9
3	20		4.1
4	50		0.7
5	50		39.5
6	50 and 100		5.9
7	100		16.0
8	100	400	7.8
			97.8

A phosphate buffer of pH 6.7 was used.

RESULTS

Enzyme assays. Table II summarizes the results for all of the silent gene sera and compares them with values obtained from normal sera, sera of persons homozygous for the atypical cholinesterase gene. and red cell ghosts. The results show, with a single possible exception, L. C., that the silent sera may be divided

into two groups. The first group (type I), Nos. 1–7, representing 16 sera, is characterized by an activity against acetylthiocholine of about 1% of normal serum, a value of acetylthiocholine: propionylthiocholine activity generally greater than 1, and virtually no activity against butyrylthiocholine. Inhibition by dibucaine was very low, and inhibitions by quinidine and Mytelase were moderate. These properties resemble those of acetylcholinesterase of the red cell ghost rather than those of normal serum cholinesterase.

A second group of eight sera (type II), Nos. 9–13, is marked by higher activities against acetylthiocholine, averaging about 3% of normal serum, and a ratio of acetylthiocholine: propionylthiocholine or butyrylthiocholine of less than 1. Inhibitions by dibucaine and quinidine were considerably higher than in type I sera and inhibition by Mytelase was lower. These properties resemble those of normal serum cholinesterase rather than those of red cell ghost acetylcholinesterase.

Only one serum failed to fall clearly in either group.

TABLE II

Enzymatic Results with All Sera

Specimen*	Acetylthiocholine	Ac/Pr‡	Ac/Bu‡	Inhibition by		
				Quinidine§	Dibucaine‖	Mytelase§
	μmole/min per ml				%	
Silent homozygote sera						
1. J. P.	0.045	1.66	7.19	50	3	76
A. P.	0.037	1.68	9.28	51	6	73
D. P.	0.046	1.75	7.60	54	9	77
2. K. A.	0.035	1.33	6.56	57	5	83
B. A.	0.025	1.13	4.35	29	2	53
S. A.	0.034	1.00	3.47	51	8	60
J. A.	0.034	1.00	3.01	54	0	64
M. A.	0.047	1.21	3.65	58	7	66
N. A.	0.032	1.26	2.92	49	0	57
3. J. S.	0.044	1.24	4.68	51	16	57
R. S.	0.044	1.31	3.25	50	1	69
4. S. O.	0.035	1.08	3.54	50	8	64
E. O.	0.032	1.05	3.46	40	0	57
5. Af.	0.029	0.91	10.00	39	9	73
6. F.	0.034	1.20	3.91	65	5	63
7. K.	0.038	1.48	10.31	61	6	77
8. L. C.	0.040	1.35	2.60	63	25	64
9. L. L.	0.073	0.70	0.85	73	67	40
J. L.	0.12	0.63	0.73	85	68	24
P. L.	0.15	0.71	0.76	85	69	29
V. L.	0.14	0.67	0.77	81	67	31
10. P.	0.11	0.58	0.68	82	71	24
11. W.	0.11	0.57	0.66	86	72	12
12. E.	0.17	0.68	0.71	89	69	23
13. Ak.	0.096	0.80	0.74	82	58	25
Normal sera	3.94 (2.46–5.28)¶	0.52 (0.47–0.55)	0.61 (0.55–0.69)	99 (98–100)	85 (83–86)	3.6 (0–10.7)
Atypical homozygote sera	0.65 (0.46–0.78)	0.44 (0.41–0.46)	0.40 (0.37–0.44)	91 (85–95)	21 (18–31)	ND
Red cell ghosts		1.9 (1.5–2.1)	∞	45 (30–53)	14 (12–17)	97 (95–100)

ND = not done.

* The numbers indicate separate families; within each family the subjects are sibs except in family No. 2; in that family K. A. is the father of the remainder of the subjects. Subject No. 10 (P.) is the double half uncle of the four sibs of family No. 9.

‡ Activity against acetylthiocholine:activity against propionylthiocholine or butyrylthiocholine, (μmoles/min per ml at 37° C).

§ Substrate = acetylthiocholine iodide.

‖ Substrate = propionylthiocholine iodide.

¶ Numbers in parentheses give the range.

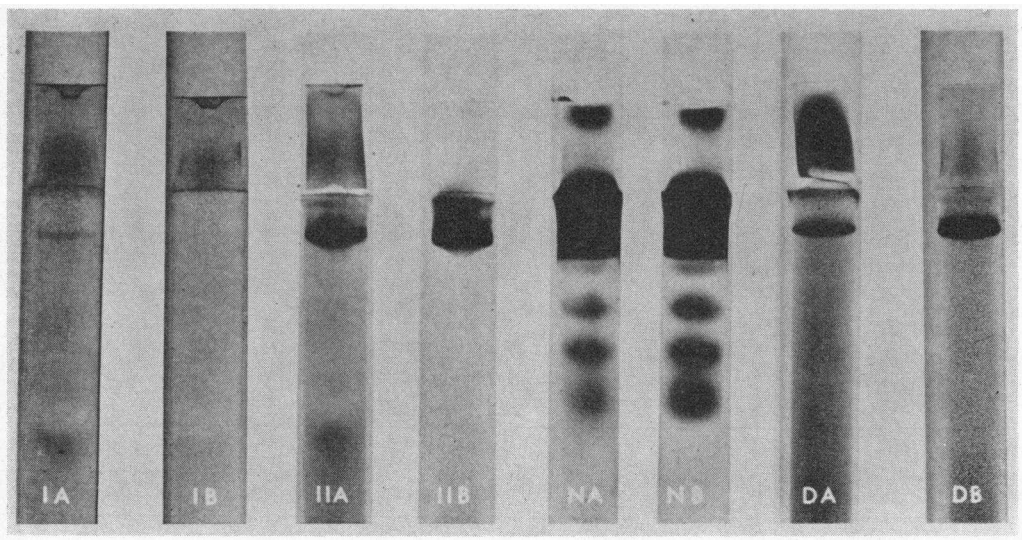

FIGURE 1 Gel electrophoresis of various sera stained for serum cholinesterase. Shown are sera representing type I, type II, normal (N), and diluted normal (D). Each type was stained with acetylthiocholine (A) and butyrylthiocholine (B) as substrate.

This is serum No. 8, L. C. Most of the properties of this serum resembled those of the type I sera. Two major differences were that the inhibition by dibucaine and the acetylthiocholine: butyrylthiocholine ratio were clearly intermediate between the two types. Sera from this patient have been studied many times, always with similar results.

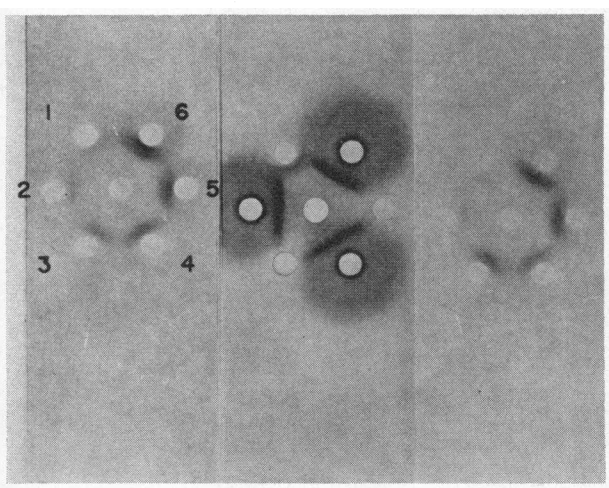

FIGURE 2 Immunodiffusion of rabbit anti-human serum cholinesterase with various types of sera. The center wells contain 10 μl of a 1:10 dilution of the rabbit antiserum. Each outer well of the left and right hand slides contains 10 μl of dilutions of two normal sera. From wells 1–6, the serum dilutions are 0.5, 1, 2, 3, 4, and 5%, respectively. In the center slide, wells 2, 4, and 6 contain 10 μl of a normal serum; well 1 contains 10 μl of a 4× concentrated type II serum (J. L.); well 3 contains 10 μl of a 4× concentrated type I serum (J. P.); and well 5 contains 10 μl of a 4× concentrated sample of the serum of L. C. Without concentration, the band at well 1 (type II), although present, would not reproduce well.

Electrophoresis. By acrylamide-gel electrophoresis the cholinesterase isoenzymes of normal human serum are resolved into at least five constant and several variable bands. Numbering from the anodal end, band 5 is the most intense, accounting for the major portion of the total activity. The bands cathodal to 5 are poorly resolved (*22*), particularly on acrylamide, are variable in number (*23*), and are known to be affected by storage (*24*). Our numbering system and results are identical to those of LaMotta, McComb, and Wetstone (*25*). The C4 band of Harris, Robson, Glen-Bott, and Thornton (*26*) is the same as our C5. The cholinesterase isoenzymes of normal serum with acetylthiocholine and butyrylthiocholine as substrates are shown in Fig. 1. As expected, there is greater activity against butyrylthiocholine.

When type I sera are studied by this method, using much larger amounts of serum because of the very low activities, the following pattern emerges. With acetylthiocholine as substrate there is a constant, though faint, band in the position of band 5 and a somewhat diffuse band anodal to band 1. These bands are inhibited by 10^{-5} M eserine. Neither of these bands is seen when butyrylthiocholine is the substrate. The first two gels in Fig. 1 depict a typical type I serum.

Type II serum gives a pattern similar to that of diluted normal serum. There is a distinct band at the 5 location and sometimes traces of bands 1–4; these bands are more prominent with butyrylthiocholine than with acetylthiocholine. These patterns are also given in Fig. 1.

The serum of L. C. was the only serum that was intermediate. With acetylthiocholine as substrate the intensity of the bands was similar to that of type I serum but the bands persisted, roughly equally, with butyrylthiocholine as substrate.

Immunologic studies. On the Ouchterlony immunodiffusion slides normal sera and sera from patients homozygous for the atypical cholinesterase gene gave prominent bands which fused completely. None of the type I sera, at any concentration, gave evidence of a cross-reacting protein. All of the type II sera did show a weak, but definite, band close to the antigen well. Several normal sera with varying cholinesterase activities were diluted in the range 0–5% and compared on slides. By visual comparison it was estimated that the type II sera contained some 1–3% of normal serum cholinesterase (Fig. 2). No cross-reacting band was seen in the serum of L. C.

Absorption of the antiserum with type I sera resulted in no loss of antibody (Fig. 3). Absorption of the antiserum with normal and atypical homozygote sera completely exhausted the antibody, and absorption with type II sera resulted in definite diminution (Fig. 4).

The various types of sera were incubated in liquid media with rabbit antibody devoid of cholinesterase. Under conditions which allowed for the removal of over

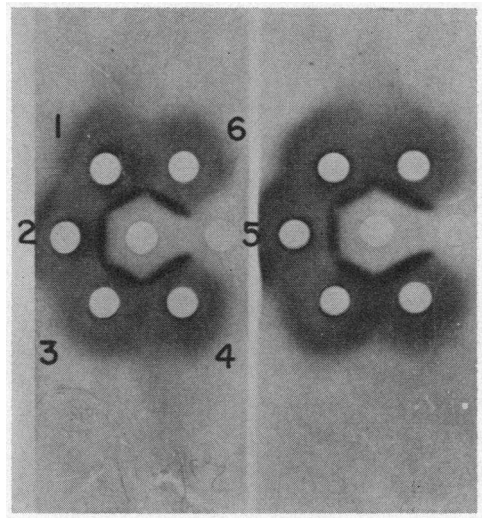

FIGURE 3 Absorption of rabbit antiserum with type I serum. The center well in the left hand slide contains 10 μl of a 1:25 dilution of rabbit antiserum. The center well in the right hand slide contains the same antiserum after absorption three times with two volumes of a 4× concentrated serum of a type I subject; the final antiserum dilution is the same in both slides. Wells 1, 4, and 6 contain 10 μl of a normal serum; wells 2 and 3 contain 10 μl of two different atypical homozygote sera, and well 5 contains 10 μl of a different type I serum (J. P.).

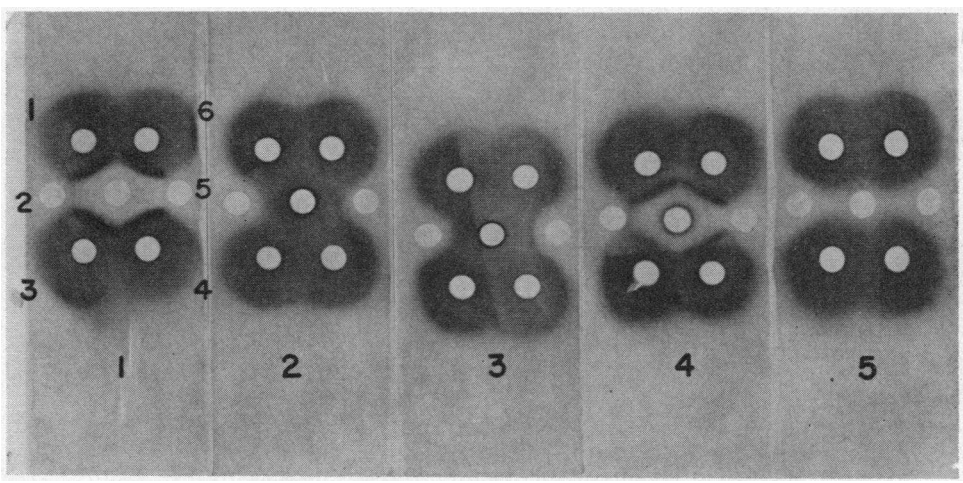

FIGURE 4 Absorption of rabbit antiserum with normal, atypical homozygote, and type II sera. The center wells contain the following: slide 1, 10 μl of a 1:25 dilution of the rabbit antiserum; slide 2, 10 μl of the same serum absorbed three times with two volumes of a 4× concentrated normal serum; slide 3, 10 μl of the same serum absorbed three times with two volumes of a 4× concentrated atypical homozygote serum; slide 4, 10 μl of the same serum absorbed three times with two volumes of a 4× concentrated type II serum, and slide 5, 10 μl of a 1:25 dilution of a normal rabbit serum as a control. The serum concentration in the center wells in slides 1–4 is the same. In each slide, outer wells 1, 4, and 6 contain 10 μl of a normal serum; well 3, 10 μl of an atypical homozygote serum; well 2, 10 μl of a different type II serum (J. L.); and well 5, 10 μl of a type I serum (J. P.). Note the faint band with the type II serum in slides 1 and 4.

TABLE III

Reaction between Purified Rabbit Anti-Human Serum Cholinesterase and Various Sera

Serum type		Cholinesterase removed
		%
Normal	1	98.2
	2	97.5
	3	98.1
	4	95.9
Type I	1 (J. S.)	4.4
	2 (J. P.)	2.1
Type II	1 (W)	46.2
	2 (Ak.)	26.2
	3 (E.)	41.8
L. C.	1	6.9
	2	3.5

The lyophilized purified antiserum was reconstituted in 0.9% NaCl to the original serum volume. 12.5 μl of antibody were incubated with 150 μl of test serum in a 100 × 10 mm test tube and made up to 0.5 ml with 0.9% NaCl. For remainder of details see Methods.

95% of the cholinesterase activity from normal serum, type I sera showed no significant loss; type II sera showed significant loss, although never to the extent seen with normal sera (Table III). The serum of L. C. behaved like type I serum in this experiment.

Chromatography. After fractionation on DEAE-cellulose columns, the cholinesterase activity of normal serum was obtained in two fractions (Fig. 5). A small amount of activity was found in peak 5; this material had properties similar to those of type I sera, with greater activity against acetyl- than butyrylthiocholine and lower inhibitions with quinidine and dibucaine than with the whole serum, and higher inhibition with Mytelase. Electrophoretically there was faint activity with acetylcholine and lesser activity with butyrylthiocholine. Fraction 6 contained no cholinesterase activity, and peaks 7 and 8 contained the remainder. Chemically and electrophoretically the properties of peak 7 and 8 material were identical with those of the whole serum.

Four type I sera (D. P., J. P., E. O., S. A.) were chromatographed and in each case all of the activity was found in peak 5. The electrophoretic and chemical properties of this material were identical with those of the unfractionated sera.

With type II sera the results were similar to those of normal serum in the recovery of peak 5 material but with a correspondingly lower activity in peaks 7 and 8. On electrophoresis the peak 5 material gave a band with acetylthiocholine as substrate, but there was no perceptible band with butyrylthiocholine.

The serum of L. C. behaved like type I serum on chromatography.

DISCUSSION

The results of this study clearly indicate that patients thought to be homozygotes for the silent cholinesterase gene may be divided into at least two groups. The sera of the first group (type I patients) contain a small amount of an enzyme(s) which can hydrolyze acetylthiocholine but which has other properties unlike those of normal serum cholinesterase. These include extremely low or absent activity against propionyl and butyrylthiocholine and a distinctly different pattern of inhibitions by dibucaine, Mytelase, and quinidine. Furthermore, this enzyme does not cross-react with an antiserum to normal human serum cholinesterase, and can be distinguished from normal serum cholinesterase by column chromatography. We suggest that the enzyme hydrolyzing acetylthiocholine which is present in the serum of type I patients be termed the residual cholinesterase. The residual cholinesterase is similar in its enzymatic properties to acetylcholinesterase; perhaps they are identical.

Although the enzymatic and immunologic properties of the serum cholinesterase of the second group (type II patients) resembled those of normal serum, chromatographically a fraction was found with properties similar to those of the residual cholinesterase. A similar result was obtained with normal sera. Both normal and type II sera seem, therefore, to be mixtures of two types of cholinesterase: serum cholinesterase and residual cholinesterase. Based on the values found in type I sera and the chromatographic results, we estimate that in normal serum about 99% of the activity against acetylthiocholine is due to serum cholinesterase and the re-

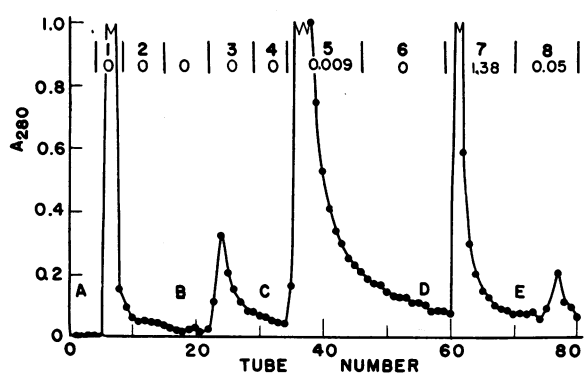

FIGURE 5 Chromatographic fractionation of normal serum on DEAE-cellulose. The letters indicate the point of change of pH 6.7 phosphate buffer concentration (see Table I). A = 10 mM; B = 20 mM; C = 50 mM; D = 100 mM; and E = 100 mM + 400 mM NaCl. The fractions were pooled on the basis of absorbance peaks; the upper set of numbers at the top gives the fraction number and the set of numbers immediately below gives the cholinesterase activity with acetylthiocholine as substrate (μmoles/min per ml of original serum).

mainder to the residual cholinesterase. In type II sera we estimate that about two-thirds of the total activity is due to serum cholinesterase and the remainder to the residual cholinesterase.

The tissue, or tissues, from which the residual cholinesterase originates remains unknown. Nerve, muscle, and red cell are particularly rich in acetylcholinesterase, but many other tissues contain small amounts. It is possible that it is derived from the red cell despite the fact that red cell acetylcholinesterase is tightly bound to the stroma. Perhaps there is a small labile fraction which can escape into the plasma either from an intact red cell or from an effete or dying cell. It is also possible that it is an artefact in serum and arises from platelets labilized during clotting. We have assembled some evidence against the latter hypothesis: (*a*) high-spun platelet-poor plasma from type I patients (J. P., A. P) collected in siliconized equipment gave the same enzymatic and electrophoretic results as serum; and (*b*) high-spun platelet-poor normal plasma similarly collected showed the same peak in fraction 5 as did serum after fractionation on DEAE-cellulose.

One hypothesis concerning the silent cholinesterase gene is that it results in the absence of synthesis of any recognizable gene product. This hypothesis fits in well with type I patients: presumably they are homozygous for this gene (S_1 gene) since there is no evidence by any criterion of any normal serum cholinesterase. The only cholinesterase present in the serum of type I patients is the residual cholinesterase, clearly a different protein presumably under different genetic control.

The findings in the type II patients cannot be explained by this hypothesis. These patients have about 2% of apparently normal serum cholinesterase. It is possible that this enzyme is qualitatively different from the normal enzyme, as in the case of the atypical (dibucaine-resistant) cholinesterase, but we found no evidence for this in our enzymatic, immunologic, or chromatographic results. Even if the predominant cholinesterase present in these patients is qualitatively abnormal, the good quantitative correspondence between the enzymatic and immunologic results would still indicate greatly retarded synthesis of gene product. Assuming the predominant cholinesterase in the serum of type II patients is "normal" serum cholinesterase, one must conclude that a different gene (S_2 gene) is present in these patients. Presumably this gene allows the synthesis of the normal gene product, but in very small amounts.

It is possible that the type II cases are homozygotes for the S_2 gene, or they may have one S_1 and one S_2 gene. The decision between these alternatives may not be possible until pedigrees are found in which both type I and type II patients occur. Similarly, it is not yet possible to prove that the S_1 and S_2 genes are allelic at the same locus.

The status of L. C., the only subject in the present study who is intermediate between type I and type II patients in several respects, is uncertain. When compared to the type I patients, the somewhat elevated inhibition by dibucaine and the band that reacted with butyrylthiocholine after electrophoresis suggest that there may be a very small amount of normal serum cholinesterase present in her serum. The failure to demonstrate normal serum cholinesterase immunologically in her serum by the Ouchterlony technique indicates that the amount present must be under 0.25–0.50% of normal values, the lowest amount detectable by the method employed. It is possible that she is heterozygous for both the S_1 and S_2 genes, but it is also possible that she possesses yet another "silent" cholinesterase gene. It is conceivable that there exists a family of closely related silent cholinesterase genes each differing somewhat in the amount of gene product synthesized.

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