≧1:4 to *M. pharyngis*, but the importance of this finding is not clear. Continued elevation of titers in convalescence and insignificant alteration of MI titers by absorption with bovine red-cell antigens served to differentiate antibody to *M. pharyngis* from the heterophil antibody. The finding that titers to *M. pharyngis* in serum pairs were stable and that only 40% of patients had antibody titers of ≧1:4 suggests that the observed difference is unrelated to the pathogenesis of infectious mononucleosis. Conversely, the sensitivity of the MI test for *M. pharyngis* antibody and the importance of antigenic differences among strains, as found with *M. hominis* (8), are not known. It might be hypothesized that antibody to *M. pharyngis* represents yet another early abnormal immunoglobulin response in some patients with infectious mononucleosis. Further work is needed for clarification.

Summary. Mycoplasma flora of the pharynx and anterior oral pool of 16 patients with early infectious mononucleosis did not differ significantly from that of 12 controls. Patient-derived strains of *M. pharyngis, M. salivarium, M. hominis,* and T-strain mycoplasma were employed in metabolic inhibition antibody tests of paired and single sera from patients and controls. The only difference observed was the presence of MI antibody titers of ≧1:4 to *M. pharyngis* in 40% of 42 patients' sera compared to 14% to 22 sera from controls. Antibody responses to mycoplasmas were not observed during the course of illness.

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A Direct Radioisotopic Microassay for Cholinesterase (33325)

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Radioisotopic methods for the determination of cholinesterase[1] (1) activity in microquantities of biological tissue and fluids are undoubtedly advantageous over manometric (2), polarographic (3) or microchemical methods (4). Winteringham and Disney (5, 6) were first to use acetate ^{14}C labeled acetylcholine as a substrate for the enzyme. They incubated blood with acetylcholine-^{14}C and measured the amount of the remaining unhydrolyzed substrate after careful removal of the volatile acetate under vacuum. Reed *et al.* (7) also used the labeled substrate for incubation with enzyme but removed the unhydrolyzed portion with an ion-exchange resin batch treatment method.

A simple and sensitive isotopic method will be described for repeated routine microdetermination of cholinesterase activity in a large number of samples from biological material utilizing acetylcholine as a substrate.

Method and Procedure. Rat brain was used for tissue material. A 10% rat brain

[1] Cholinesterase is used in this manuscript to designate enzymatic hydrolysis of acetylcholine by eserine-sensitive esterases.

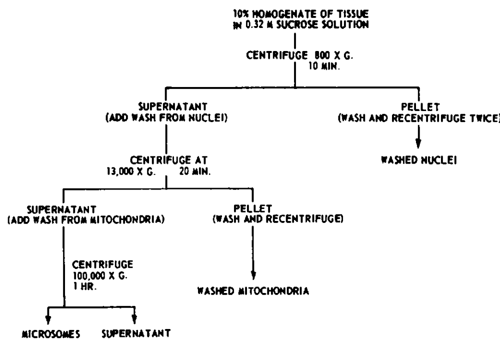

FIG. 1. Flow diagram of a fractionation procedure as applied to rat brain to separate the main subcellular constituents.

homogenate in 0.32 M sucrose solution was prepared at 2–4°. In order to emphasize the applicability of this method and to compare these values with published work, further subcellular fractionation was carried out according to a procedure adapted from Whittaker (8) and DeRobertis (9). The fractionation procedure that was followed in this investigation is summarized and shown in Fig. 1.

Whole blood was diluted with phosphate buffer containing 0.05% (w/v) saponin. A volume of 20 μl of blood was diluted in 2 ml and after 1-hr hemolysis period at room temperature 10–20 μl were used for cholinesterase activity measurement. Labeled free acetate was removed from the acetylcholine-^{14}C iodide substrate before making a stock solution. Each lot, approximately 10 mg of acetylcholine iodide 2.5 mCi/mmole, was dissolved in 2 ml of 0.1 N HCl and extracted with three successive 10-ml aliquots of a mixture of toluene isoamyl alcohol (9:2 v/v). An 0.08 M aqueous stock solution of 0.2 mCi/mmole of acetylcholine-^{14}C iodide was found to be stable at —20° for a period of at least 4 weeks. A buffer substrate solution was made up of 50–100 μl of acetylcholine stock solution in 0.05 M phosphate buffer, pH 7.0; 0.04 M $MgCl_2$; and 0.05 M NaCl for daily routine determinations. A 10 to 20 μl sample of hemolyzed blood was incubated with 50–100 μl of buffer substrate solution, shaking at 37° for 5 min in 10-ml glass stoppered test tubes. The concentration of buffer substrate solution used was such that on subsequent addition of enzyme preparation, the correct initial substrate concentration was obtained. The initial count rate used in this study lay within the range of 13–45 thousand cpm. The reaction mixture was chilled on ice and was stopped by the addition of 100 μl of 0.2 N HCl to terminate all cholinesterase activity and suppress ionization of enzymatically liberated acetic acid without significantly hydrolyzing the remaining substrate. Enzymatically liberated acetate-^{14}C was then extracted with a 2-ml mixture of toluene isoamyl alcohol (9:2 v/v), centrifuged for 15 min and 1-ml aliquot of the organic phase was transferred to counting vials in 15-ml scintillator solution. Control determinations were incubated at 4° for 1 hr with 10^{-4} M physostigmine sulfate to inhibit nonspecific cholinesterase (2). Blank values for each set of determination in which the enzyme was absent were also obtained to check nonenzymatic hydrolysis of substrate which was usually negligible under these conditions. The partition coefficient for acetate after one extraction was 92–94% in the organic phase and did not change materially with a second extraction. Protein was determined according to the Lowry (10) method. The time required to handle 20–30 samples is approximately 2 hr.

The number of moles of acetate hydrolyzed was calculated from the concentration and counts per minute of the initial substrate used and the counts per minute that were determined after incubation.

Acetyl-1-^{14}C choline iodide was purchased from New England Nuclear Corp., acetylcholine iodide was purchased from Eastman Kodak, Rochester, New York and physostigmine sulfate was a Mallinckrodt product. Precalibrated micropipettes from H. E. Pedersen of Denmark were used exclusively. The Beckman model LS 200 liquid scintillation counter with an efficiency of 65% was used for ^{14}C determinations and the Liquifluor which was used was a product of Nuclear Chicago Corp. The ultracentrifugation was carried out with a Beckman model L2 preparative ultracentrifuge.

Results and Discussion. The rate of hydrolysis of acetylcholine-^{14}C by brain cholines-

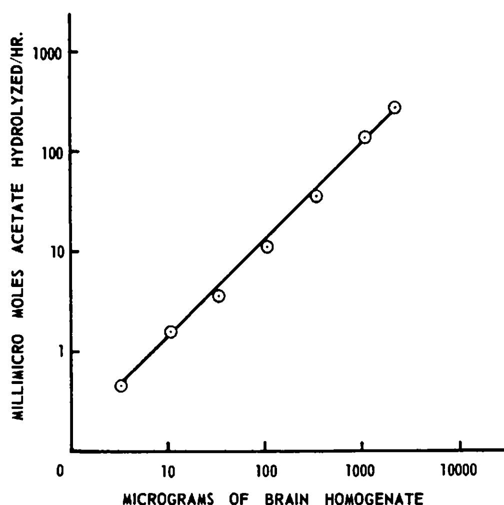

FIG. 2. Linear rate of hydrolysis of acetylcholine as a function of enzyme concentration. Incubation was carried out at 37° for 5 min with 100 μl of buffer substrate solution at pH 7.0, $MgCl_2$, 0.04 *M*; NaCl, 0.05 *M*, containing 0.27 μmoles of acetylcholine containing 36,000 cpm. Bovine serum albumin was added to samples of low tissue concentration. Data are expressed as individual values of duplicate or triplicate experiments and corrected for eserine-inhibited controls.

terase as a function of enzyme concentration is shown in Fig. 2. The observed rate was a linear function of tissue concentration. Enzyme activity of brain homogenate is expressed in mμmoles of acetylcholine hydrolyzed per hour and enzyme concentration as micrograms of tissue. Dilution was carried out with phosphate buffer or sucrose solution containing 0.05% serum albumin. Lower activities were obtained without the addition of albumin to greatly diluted homogenates.

Characteristic kinetics of enzyme substrate interaction can be seen in Fig. 3. When acetylcholine was varied in concentration in the assay from 10^{-2} to 10^{-5} *M*, optimal substrate concentration was 2.7×10^{-3} *M*. The optimal substrate concentration was used for 24 determinations of enzyme activity showing that brain homogenates hydrolyzed 241 ± 12% mmole/hr/g of tissue. Lapetina (11) using the chemical method of enzyme determination found a value of 219 mmole/hr/g of brain cortex.

It is evident from Fig. 3 that characteristic substrate inhibition, as expected from true cholinesterase, was observed in this investigation. Substrate inhibition for true cholinesterases was also reported by other authors (6). It should be pointed out that satisfactory results were obtained with lower initial substrate concentration in agreement with similar experiments reported by Reed *et al.* (7) and by Winteringham (6) and discussed by the latter. An increase in enzyme activity was observed with the addition of Mg^{2+} and Na^{+} ions. Table I shows that the addition of

TABLE I. Optimal Ion Concentrations for Cholinesterase.[a]

Salt	Concentration (*M*)	Hydrolysis of acetate[b]
Na_2HPO_4	0.01	82
	0.05	100
	0.1	97
$MgCl_2$	None	79
	0.04	100
	0.1	100
	0.2	93
NaCl	None	88
	0.05	102
	0.1	100

[a] Fresh brain homogenates were used.
[b] Standard assay = 100%.

Mg^{2+} ion increased the activity by 21% on 5-min incubation of standard media while Na^{+} shows a 12% increase.

Table II summarizes the distribution and recovery of the enzyme in the main subcellu-

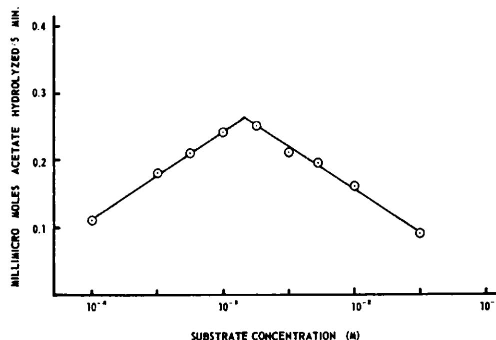

FIG. 3. Rate of acetate production as a function of substrate concentration. Medium composition and incubation conditions as described for Fig. 2.

TABLE II. Comparative Distribution of Protein and Cholinesterase in Main Cellular Fractions.

Fraction	Protein (mg/g of tissue ± SD)	Cholinesterase activity (μmole/mg of protein/hr)	Relative specific activity[a]
Homogenate	88.5 ± 3.63	164.5 ± 2.08	1.0
Nuclear	7.5 ± 0.28	11.5 ± 1.45	0.8
Mitochondrial	29.9 ± 1.07	55.7 ± 1.07	1.1
Microsomal	24.7 ± 1.09	52.3 ± 1.70	1.2
Supernatant	19.3 ± 1.02	21.8 ± 2.86	0.6
Recovery (%)	91.9	85.9	—

[a] Relative specific activity = (% recovered activity in fraction)/(% recovered protein in fraction).

lar fractions relative to protein content. It will be noted that protein content and enzyme activity was highest in both the mitochondrial and microsomal fractions. The finding that cholinesterase is a cellular membraneous enzyme is substantiated in reports by Homstedt and Toschi (12), Nathan and Aprison (13), and Aldridge and Johnson (14). Although experimental conditions and assay methods were different in these investigations, nevertheless, the different cellular membranes are clearly localized in the two fractions. The relatively high enzyme content in the supernatant fraction could possibly be attributed to hydrolysis of acetylcholine by other esterases and use of a more selective inhibitor would clarify this point. To compare the enzyme content of all fractions with the total homogenate on a similar basis the relative specific activity expression is used (Table II). Here again the relative specific activity was highest in the two mentioned fractions. Further purification of the main fractions and their enzyme content will be reported elsewhere.

In Fig. 4 the percentage enzyme distribution relative to protein content per fraction is shown. As shown, the nuclear fraction had a negligible level (7.5%) of the percentage activity, and as anticipated, the highest percentage was localized in mitochondrial (33.8) and microsomal (31.7) fractions. A similar pattern of percentage distribution relative to protein content was found in rat brain (14) using manometric methods for enzyme assay.

Whole human blood samples with optimal substrate concentration gave a value of 4.2 ± 0.2 μmoles/ml/min. This value was obtained from 13 experiments. The characteristic substrate inhibition curve obtained with whole blood was similar to that of brain tissue homogenate, indicating that activities measured in the present experiment for whole blood were largely due to erythrocyte enzyme (6, 15).

Summary. A sensitive and rapid isotopic microassay method for cholinesterase determination in biological fluid and tissue has been described. The method employed acetyl-1-^{14}C choline as a specific substrate. Free acetic acid labeled with ^{14}C liberated enzymatically is selectively extracted in toluene isoamyl alcohol and measured by simple counting technique. The potential of the method is illustrated by determination of the level of the enzyme in brain homogenates and its subcellular fractions as well as in human whole blood. The method has the advantage that it can be applied to samples

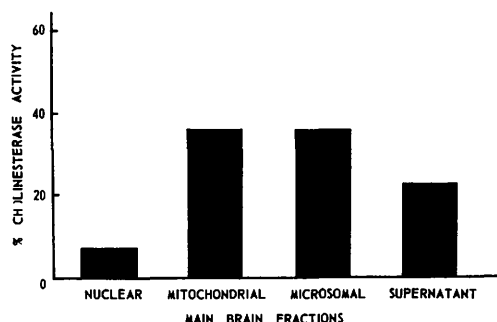

FIG. 4. Histogram of percentage distribution of cholinesterase activity in main brain fractions of adult rats. The values are means of 4–6 experiments and percentage activity is expressed relative to protein content per fraction and homogenate.

from 10 μg to 2 mg at constant pH. A wide range of technique is demonstrated.

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Accumulation of Endogenous Protein in the Cecum of the Germfree Rat* (33326)

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The enlarged cecum of germfree rats and mice contains considerable amounts of protein and carbohydrate (1). The endogenous origin of some of this material is indicated by the demonstration of high molecular weight mucins (1–3) and of elevated levels of trypsin and chymotrypsin in cecal contents (1). The contribution of dietary protein to the material found in cecal contents is not known. The aim of the present investigation was to obtain information concerning the origin of the soluble cecal protein. For this purpose germfree rats were placed on either 0% casein or 10% casein diets. After several days on these regimens, the animals were sacrificed and the cecal protein was measured. Additional studies were performed to determine the efficiency of the terminal half of the small intestine in reabsorbing nitrogen.

Materials and Methods. Gnotobiotic CDF rats were obtained from the Charles River Breeding Laboratories (North Wilmington, Mass.) and were housed in plastic germfree isolators or in a Reyniers stainless steel unit. Conventional animals of the same strain were housed in the animal room. During the experimental periods animals were fed diet 585 (4) modified as described below and water *ad libitum.* In the experiment involving protein restriction, germfree rats had been maintained for a period of 10 days postweaning on a diet containing 20% casein. Three animals were then sacrificed and the cecal contents were analyzed to obtain zero time values. The remaining animals were placed on diets in which the casein content was reduced to 10% (10% protein group) or completely

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