TABLE IV. Lipid Composition of Chylomicron and Subnatant Fractions.

Group and emulsion	2	3	4	5	6	7	8	9	10
	%								
Chylomicron fractions									
Neutral fat	74.4	89.1	86.9	57.9	80.9	91.1	85.2	82.6	93.0
Phospholipide	19.5	6.3	4.2	Trace	Trace	5.6	8.2	8.6	4.6
Free cholesterol	2.5	1.2	1.3	10.5	2.8	.8	1.0	.9	1.5
Cholesterol esters	3.6	3.4	7.6	31.6	16.7	2.5	5.6	7.9	.9
Subnatant fractions									
Neutral fat	75.5	81.8	70.2	61.7	32.5	80.4	70.6	78.9	86.6
Phospholipide	8.8	11.0	11.2	16.5	24.9	11.4	12.1	6.8	6.6
Free cholesterol	3.9	1.5	3.0	4.2	5.6	1.5	2.3	1.7	1.5
Cholesterol esters	11.8	5.7	15.6	17.6	37.0	6.7	15.0	12.6	5.3

Summary. Changes in distribution of neutral fat, phospholipide, and cholesterol between chylomicron and subnatant fractions of rat lymph have been produced by feeding emulsions containing cholesterol, fatty acid, triglyceride, and taurocholate in various combinations. Increases in neutral fat were carried largely in the chylomicron fraction while increases in cholesterol were carried largely in the subnatant fraction. The emulsions also produced changes in percentage composition of the lipide mixture in each fraction.

1. Vahouny, G. V., Fawal, I., Treadwell, C. R., *Am. J. Physiol.*, 1957, v188, 342.

2. Vahouny, G. V., Treadwell, C. R., *ibid.*, 1957, v191, 179.

3. Vahouny, G. V., Woo, C. H., Treadwell, C. R., *ibid.*, 1958, v193, 41.

4. Laurell, C. B., *Acta Physiol. Scand.*, 1954, v30, 289.

5. Byers, S .O., Friedman, M., *Am. J. Physiol.*, 1954, v179, 79.

6. Jobst. H., Schetter, G., 3rd International Conf. on Biochem. Problems of Lipids, 1956, 136.

7. Lindgren, F. T., Elliot, H. A., Gofman, J. W., *J. Phys. Colloid Chem.*, 1951, v55, 80.

8. Bragdon, J. H., *J. Biol. Chem.*, 1951, v190, 513.

9. Sperry, W. M., Webb, M., *ibid.*, 1950, v187, 97.

Received July 23, 1958. P.S.E.B.M., 1958, v99.

Immunological and Enzymological Specificity of Antibodies Produced by Injection of Purified Acetylcholinesterase. (24474)

M. KATHLEEN CARTER* AND GILBERT L. MARX† (Introduced by F. W. Schueler)

Dept. of Pharmacology, Tulane University School of Medicine, New Orleans, La.

This paper is concerned with results of experiments in which an attempt was made to produce specific inhibitory antibodies against a particular type of cholinesterase enzyme. Reports have been published in which specific inhibitory antibodies have been obtained against certain enzymes. Mansour *et al.*(1) immunized roosters with rabbit muscle lactic dehydrogenase and obtained sera which markedly inhibited the activity of this enzyme but had no effect on lactic dehydrogenase of *Schistosoma mansoni* or *Schistosoma japonicum*. Conversely, it was shown(2) that antisera to the lactic dehydrogenase of *Schistosoma mansoni* markedly inhibited the activity of this enzyme and that from *Schistosoma japonicum* and *haematobium* but not the lactic dehydrogenase from rabbit muscle. The antigenicity of a cholinesterase enzyme had been suggested by Holland(3). He reported that sera from rabbits immunized with purified ox erythrocyte cholinesterase inhibited

* This investigation supported by Senior Research Fellowship from Public Health Service.

† Support received from Medical Student Part-Time Research Fellowship of P.H.S.

hydrolysis of acetylcholine by fresh ox erythrocytes about 50% and caused hemolysis of erythrocytes in the complement hemolysis test. Hydrolysis of acetylcholine by the purified enzyme was inhibited about 25% with antisera. Therefore, since it was anticipated that animals immunized with a cholinesterase enzyme would produce antibodies that would be specifically inhibitory for a certain species or particular type of cholinesterase, rabbits and dogs were injected intravenously with purified bovine erythrocyte acetylcholinesterase.

Materials and methods. Five male rabbits, weighing about 3 kg each, and 3 female dogs, weighing about 10 kg each, were injected intravenously every other day total 10 injections with purified acetylcholinesterase or gelatin used to stabilize the enzyme. The purified bovine erythrocyte acetylcholinesterase and the gelatin were obtained from Winthrop Laboratories. Three rabbits each received 20 mg of acetylcholinesterase in 1 ml of 0.9% sodium chloride solution (saline) and 2 rabbits each received equivalent amounts of gelatin (6.7 mg in 1 ml saline). This was the amount of gelatin present in the injection of acetylcholinesterase. Two dogs received 30 mg of acetylcholinesterase in 1.5 ml of saline and one dog received 10 mg of gelatin in 1.5 ml of saline. Sterile technic was used whenever possible. All animals received intermittent intramuscular injections of 50,000-100,000 units of penicillin. Forty days after last injection, an extra injection of 50 mg of acetylcholinesterase or 20 mg of gelatin was administered/rabbit. One rabbit (#4) that received acetylcholinesterase died within 24 hours after this injection. Blood, 10-20 ml, was drawn aseptically from 12 hour fasted animals before, during and after the series of injections. Heparin prevented clotting. Blood samples were centrifuged 15 minutes at 3,000 rpm. (International Centrifuge) and the plasmas removed. Plasma samples were tested for their effect on purified acetylcholinesterase activity using the manometric technic, and for presence of circulating antibodies using the ring precipitin test. Activity of acetylcholinesterase was determined in the presence and absence of plasma samples. Acetylcholine and acetyl-beta-methylcholine (initial concentrations of 0.01 M) were used as substrates. The purified acetylcholinesterase, diluted in 0.1% gelatin solution for stabilization purposes, could be stored in refrigerator for several weeks without appreciable loss in activity. The quantity of enzyme used/flask was 0.05 mg contained in 0.5 ml of gelatin solution. Augustinsson's buffer (0.015 M NaCl, 0.04 M $MgCl_2$, 0.025 M $NaHCO_3$, final concentrations) was used throughout manometric assays(4). When testing for inhibitor activity 0.1 ml of plasma was used/flask. An amount of plasma greater than 0.1 ml added to purified enzyme resulted in total amount of cholinesterase which was too great to maintain an optimum substrate concentration for any length of time. It was necessary to use plasma control flasks since there was appreciable activity in the plasma using either acetylcholine or acetyl-beta-methylcholine. The cholinesterase in rabbit plasma corresponds to the "true" cholinesterase(4). The final volume of solution in each flask was 3 ml. The flasks were equilibrated with 5% CO_2 and 95% N_2 at 37°C. Activity of purified acetylcholinesterase is expressed as number of microliters of CO_2 evolved from the bicarbonate buffer/hour/0.05 mg of purified enzyme. The effect of plasma on purified acetylcholinesterase activity was calculated from the resultant activity obtained by subtracting activity in control flasks containing only plasma from activity obtained in flasks containing both plasma and purified enzyme. Using the standard ring technic(5), the precipitin test was usually carried out concomitantly with cholinesterase assays. The ring test consisted in layering 0.2 ml of antigen, the purified acetylcholinesterase in 0.9% sodium chloride solution, over 0.2 ml of clear plasma. Formation of a just detectable cloudy ring at the interface of the 2 solutions within ½ hour was considered a positive reaction. Dilutions of the antigen ranged from 1:1,000 (1 mg/ml saline) to 1:50,000. In an identical manner gelatin was layered over the plasma. The 1:1,000 dilution of gelatin contained 0.33 mg of gelatin in 1 ml of saline. This was the amount con-

TABLE I. Effect of Plasma from Rabbits and Dogs Injected with Purified Acetylcholinesterase and/or Gelatin on *In Vitro* Activity of Purified Acetylcholinesterase (AChE).

Animal No.	Activity of acetylcholinesterase (mm^3 CO_2/hr/0.05 mg enzyme)					
	Acetylcholine (.01 M)			Acetyl-beta-methylcholine (.01 M)		
	AChE alone	AChE in presence of plasma*	% inhibition	AChE alone	AChE in presence of plasma*	% inhibition
Rabbits						
1† B‡						
A (10)	168	173	−3	55	46	16
2 B						
A (9)	176	172	2	60	49	18
3 B	182	172	6	66	54	18
A (0)	162	162	0	54	52	4
A (7)	178	180	−1	62	51	18
4 B	175	173	1	67	50	25
A (9)	175	175	0	56	46	18
A (18)	172	171	<1	53	42	21
5 B						
A (8)	170	163	4	57	42	26
A (17)	176	170	2	57	48	16
Dogs						
1 B	168	162	4	59	48	19
A						
2 B	173	156	10	63	57	10
A (7)	172	163	5	64	52	19
3 B	172	169	2	50	46	8
A (3)	176	161	9	58	49	16
A (12)	162	157	3	54	49	9

* AChE in presence of plasma is the resultant activity of AChE plus plasma minus activity of plasma alone.

† Rabbits 1 and 2, and Dog 1 received gelatin. Rabbits 3, 4, 5 and Dogs 2 and 3 received acetylcholinesterase.

‡ B = Activity before, and A = Activity after series of inj. of AChE or gelatin.

Numbers in parentheses are days after last inj. of antigen on which blood was drawn. (0) refers to a sample drawn during series of injections.

tained in the 1:1,000 dilution of purified acetylcholinesterase.

Results. The effect of pre- and post-injection plasmas from rabbits and dogs on activity of purified acetylcholinesterase are given in Table I. Plasmas from animals injected with acetylcholinesterase, when compared with plasmas from control animals or with plasmas from the same animals before injection with the enzyme, showed no significant increase in inhibition of acetylcholinesterase. Plasmas before the series of injections and control animal plasmas as well as post injection plasmas, all exhibited a "natural" inhibition of the enzyme ranging from 10 to 25% using acetyl-beta-methylcholine as substrate for the purified acetylcholinesterase. The "natural" inhibition exhibited by plasmas when acetylcholine was used as substrate was less. There was no greater inhibitory activity in plasmas of rabbits tested 13 days after the booster injections than before this injection.

Plasmas from rabbits injected with acetylcholinesterase exhibited a positive ring precipitin reaction with a titer up to 1:10,000 using acetylcholinesterase as the antigen. There was no precipitin reaction using gelatin as the antigen in the precipitin test. Plasmas of all dogs and the rabbits injected with gelatin exhibited negative precipitin reactions against enzyme and gelatin. A 1:20,000 titer was obtained with plasmas of rabbits 13 days after the booster injection of acetylcholinesterase.

Discussion. Several explanations can be offered for the presence in plasmas of antibodies which caused a positive ring test and

absence of inhibitory activity toward the purified enzyme. First, one could assume that purified acetylcholinesterase was not antigenic. Antibodies produced from injection of purified acetylcholinesterase could have resulted from foreign proteins present in the preparation. The activity/unit weight of purified enzyme preparation varies from batch to batch; therefore more or less extraneous proteins could have been present. Secondly, one could assume that the purified acetylcholinesterase was antigenic. Studies have indicated that the molecular weight of acetylcholinesterase of electric eel is around 3,000,000(6). However, it has been proposed, based on irradiation data(7), that this macromolecule is composed of 30-50 enzymatically active units, each with a molecular weight around 105,000. These should be large enough to be antigenic. On this basis, it could be postulated that there might be a further dissociation of this subunit into an antigenic non-enzymatically active part and an enzymatically active non-antigenic part. We found that incubation for one hour at 37°C or refrigeration overnight of the purified enzyme with a rabbit's plasma, which had a ring precipitin titer of 1:10,000, produced a flocculent precipitate. In either case the activity of the purified acetylcholinesterase in the supernatant fluid remaining after such incubation was unchanged from that in the original enzyme solution when corrected for activity of added plasma. Therefore, no active enzyme was removed from solution in the precipitate. Antibodies produced under experimental conditions reported here must either have resulted from contaminating proteins in the acetylcholinesterase preparation or else antibodies produced against the acetylcholinesterase enzyme did not inhibit its enzymatic activity. On the basis of the assumption that this cholinesterase enzyme is not antigenic, experiments are presently being conducted to test this procedure as a method for further purification of this enzyme; *i.e.*, precipitation of extraneous proteins in the purified acetylcholinesterase preparation using the gamma globulin fraction of immunized rabbit's sera.

Lack of agreement between results presented here and those of Holland(3) is difficult to explain. In the report by Holland, data using purified ox erythrocyte cholinesterase as the enzyme in the manometric studies were not given. However, it was stated that antisera from the immunized rabbits inhibited the purified cholinesterase preparations 25 to 30%. This degree of inhibition was approximately the same as that obtained in experiments reported here using normal plasma and purified enzyme with acetyl-beta-methylcholine as substrate. The presence of "natural" inhibitors of cholinesterase enzymes in serum has been demonstrated by Karczmar and Koppanyi(8). These workers reported that serum, diluted 1-4, inhibited acetylcholinesterase and cholinesterase in various tissue mixtures as much as 60%. It should be pointed out that in the present experiments there were no detectable antibodies in any of the dog plasmas. However, up to 20% inhibition of the purified enzyme preparation was obtained in several cases using either pre- or post-injection plasmas when acetyl-beta-methylcholine was the substrate, and up to 10% inhibition was obtained using acetylcholine as the substrate.

Summary. 1) Intravenous injection of purified bovine erythrocyte acetylcholinesterase caused production of circulating antibodies in rabbits but not dogs. 2) Pre- and post-injection plasmas of all animals caused up to 25% inhibition of the purified acetylcholinesterase using acetyl-beta-methylcholine and up to 10% inhibition using acetylcholine as substrate.

1. Mansour, T. E., Bueding, E., Stavitsky, A. B., *Brit. J. Pharmacol.*, 1954, v9, 182.

2. Henion, W. F., Mansour, T. E., Bueding, E., *Exp. Parasitol.*, 1955, v4, 40.

3. Holland, W. C., *Brit. J. Exp. Pathol.*, 1951, v32, 383.

4. Augustinsson, K. B., *The Enzymes*, v1, Part 1, pp454-456. Ed. by Sumner, J. B., and Myrbaäck, K., Academic Press, N. Y., 1950.

5. Sherwood, N. P., *Immunology*, 3rd Ed., 294, C. V. Mosby Co., St. Louis, 1951.

6. Nachmansohn, D., Wilson, I. B., *Advances in Enzymology*, 1951, v12, 259.

7. Serlin, I., Fluke, D. J., *J. Biol. Chem.*, 1956, v223, 727.

8. Karczmar, A. G., Koppanyi, T., *Fed. Proc.*, 1954, v13, 372.

Received July 29, 1958. P.S.E.B.M., 1958, v99.