TABLE I. Hexosamine and Hydroxyproline Content in Fracture Callus, γ/mg Acetonic Powder Dried at 104°C.

Days after fracture	Hexosamine		Hydroxyproline	
	Exp.	Controls	Exp.	Control
5	20.5	18	30.4	25.3
10	23.4	36.2	24.2	22.9
15	23.8	55.3	27.2	33.4
20	9.3	27.0	16.5	17.5

sharply in the 20-day-old callus. A similar decrease in hexosamine was found in the epiphyseal plates of AAN-treated rabbits by Castellani and Castellani-Bisi(7). Pedrini and Pedrini-Mille also demonstrated that the synthesis of hexosamine in the epiphyseal plates of these animals was greatly decreased in comparison with the controls(8).

On the other hand, no significant differences were found in the hydroxyproline content of the fracture callus of normal and AAN-treated rats.

The values of hexosamine and hydroxyproline in the femoral shafts of normal and AAN-treated rats were similar throughout the experiment.

Summary. The content of hexosamine in the fracture callus of normal rats increased sharply during the first 2 weeks. Much less hexosamine was found in the fracture callus of AAN-treated rats. No significant difference was found in the content of hydroxyproline of the callus of normal and AAN-treated rats.

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Cholinesterases in Serum as Demonstrated by Starch Gel Electrophoresis.* (26848)

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The demonstration by Alles and Hawes(1) that cholinesterase of red blood cells differs from that of serum has led to our present understanding that 2 types of these enzymes exist, one possessing maximum hydrolytic activity for the 2-carbon acyl group and one for the 4-carbon chain. While other esterases can split some choline esters(2), the property of physostigmine in low concentration to inhibit cholinesterases in a reversible manner(3,4) distinguishes these enzymes from other esterases. In the serum of various species the 2 types of cholinesterases exist in varying proportions(5), predominantly the butyrylcholine-splitting enzyme in the human and the acetylcholine-splitting enzyme in the rabbit. Other authors(6,7) have reported the presence in serum of various butyrylcholine esterases.

In a study of serum esterases by starch gel electrophoresis, a number of bands were obtained, in addition to the expected cholinesterase band, which were active against choline esters and which possessed the property of being inhibited by physostigmine. The number of these bands varied with the species.

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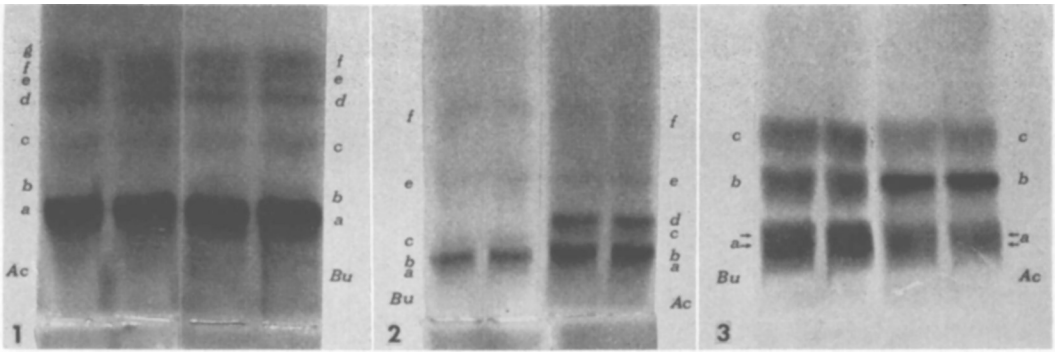

FIG. 1. "Zymograms" of serum cholinesterase. 1. Human. 2. Rat. 3. Cat. (Ac = acetylthiocholine iodide as substrate; Bu = butyrylthiocholine iodide.) Individual bands are lettered.

Methods. Blood was drawn from the human, the cat and the rat by venous or cardiac puncture and after clotting had occurred the serum was obtained by centrifugation. Samples showing hemolysis were rejected. The starch gel was prepared according to Smithies (8) and, after introduction of 50 lambda of serum in each sample slot, the separation was performed by the vertical technic. The cell was placed in a constant temperature incubator at 13°C for 15-18 hours with an applied e.m.f. of 4.5 volts/cm. At completion of the run the block was cut into halves and then into strips along the plane of migration. Each strip embraced 2 sample slots and was placed in a glass tray and immersed in the appropriate incubating medium. In inhibition experiments the gels were pre-treated with the inhibitor at room temperature for 30-60 minutes in a solution identical to the final incubating medium save that the substrate was lacking. This pre-treatment was followed by incubation in the full substrate plus inhibitor. All incubations, with or without the presence of inhibitors, were for 4 hours at 37°C.

The substrate solutions contained acetylthiocholine iodide or butyrylthiocholine iodide and were identical to those devised by Gomori(9) except that 7.5 g % polyvinylpyrrolidone was substituted for saturated sodium sulfate. Inhibitors used were physostigmine sulfate (1×10^{-5} to 1×10^{-4} M), DFP (di*iso*propyl fluorophosphonate) (1×10^{-5} M), 62C47 [1:5-(4-trimethylammoniumphenyl) pentan-3-one diiodide] (5×10^{-5} to 1×10^{-4} M), and mytelase [N', N', -bis-(2-diethylaminoethyl) oxamide bis-2-chlorobenzyl chloride] (10^{-6} M). The bands of enzyme reaction product were visualized by immersion of the gel strips in 5% $(NH_4)_2$ S. A dark brown or black zone of copper sulfide represented the site of cholinesterase activity.

Results. The results obtained with serum of human, rat and cat are shown in Fig. 1. These are unretouched photographs. It can be seen readily that the sera of the 3 species show the presence of a number of stained areas with both acetylthiocholine iodide (AcThCh) and butyrylthiocholine iodide (BuThCh). In human serum there is a prominent band of activity (band A) against both substrates near the origin and, additionally, 5 lighter staining zones (B to F) can be discerned. Against AcThCh another discrete area of staining is demonstrable at G. In addition to these well-defined zones of staining, a rather diffuse pale area of coloration occurs proximal to A and merging with it. Employment of physostigmine (1×10^{-5} M) as an inhibitor results in complete disappearance of bands B to G, but band A is only partially inhibited (about 50% as judged visually). With 10^{-4} M physostigmine inhibition of band A is almost, but not quite, complete. Use of DFP 10^{-5} M results in complete abolition of all enzymatic activity. Thus it would seem that part of the hydrolysis of AcThCh and BuThCh is accomplished by an esterase similar to Aldridge's B- esterase(10) in respect to its relative insensitivity to eserine and its sensitivity to DFP. Yet B- esterase is believed to be present only in erythrocytes in the human, being absent from plasma(11).

Neither 62C47 nor mytelase, both acetylcholinesterase inhibitors, caused any inhibition and this suggests that none of the activity observed was due to this enzyme.

In rat serum, hydrolysis of AcThCh resulted in 6 bands, 2 of them prominent, while there were only 5 bands of activity against BuThCh, the prominent band D being absent with this substrate. A number of further distinctions between human and rat serum appear when enzymatic activity is studied with the aid of inhibitors. In the rat all activity against both substrates is abolished by physostigmine 10^{-5} M, while 62C47 10^{-4} M abolishes band D. No inhibition occurs with mytelase. In the case of cat serum, both substrates yield 3 rather diffuse zones of staining. In the zone nearest the origin 2 distinct dark bands may be discerned (arrows) against the diffuse background coloration. BuThCh gives approximately equal staining in zones A, B and C but, in the case of AcThCh, a discrete dark band appears in zone B. All the enzyme activity of cat serum was eliminated by physostigmine 10^{-5} M. DFP 10^{-5} M completely inhibited zones A and C only.

Discussion. The results described indicate that the hydrolysis of choline esters in serum is more complex than has been realized. Thus, in the rat, 6 eserine-sensitive enzymatically active zones can be visualized with AcThCh as substrate. In the human, an even larger number of cholinesterases, but with different sensitivities to inhibitors, is demonstrable. Whether the various zones delineated represent specific molecular species of enzymes, or whether they represent one or several enzymes adsorbed onto inert proteins and migrating with the latter is the object of further study by quantitative methods in this laboratory. Others have adduced evidence, admittedly not final, in favor of the specific identity of the enzymatic activities demonstrated by "zymogram" technics(12), and the results described here (variations of staining pattern with each substrate and variations in sensitivity to the different inhibitors employed) would uphold such a view.

Summary. As many as 7 bands of enzymatic activity against choline esters are demonstrable in human, rat and cat sera by starch gel electrophoresis. The evidence suggests that these bands derive from distinct molecular species of enzyme. However, possible influences on "zymogram" patterns of enzyme adsorption by inert protein require investigation.

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