15576

Effect of Certain Anti-anemic Substances upon Serum Cholinesterase Activity.*

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It has been shown that hyperchromic anemia can be produced in dogs by the daily administration of choline or acetylcholine, and that this anemia is abolished by the use of liver extract or pteroyl glutamic acid (folic acid).[1,2] It was further shown that an acetylcholine-like activity present in the serum of anemic dogs after the feeding of choline, was abolished or diminished after the onset of treatment with the antianemic substances. Certain evidence was presented to show that both liver extract and pteroyl glutamic acid probably acted by increasing the cholinesterase activity of the body.

The present article is concerned with a study of the effects of liver extract and folic acid in increasing the serum cholinesterase activity both *in vitro* and *in vivo*.

Procedure and Results. Serum cholinesterase activity was determined by the electrometric titration at a constant pH of 7.38, of the acetic acid liberated from a solution of 100 mg of acetylcholine bromide in 25 cc of water by 1 cc of blood serum in 10 minutes. The method is essentially that of Goodman, Carlson, and Gilman,[3] except that titrations were made at room temperature (28-31°C) and to a pH of 7.38. A Leeds and Northrup pH meter with a glass electrode was employed. Cholinesterase activity was estimated in terms of cc of 0.01 N sodium hydroxide required to neutralize the acetic acid liberated over a period of 10 minutes.

The *in vitro* experiments consisted of the incubation of 1 cc of dog's blood serum with various injectable liver extracts or with pteroyl glutamic acid† for 2½ hours at 37°C. It was necessary to add a little disodium phosphate to render the pteroyl glutamic acid soluble in water. The volume of solution employed was usually kept at 0.1 cc and maximal effect was usually obtained with 0.15 mg of folic acid per cc of serum. When liver extracts were used in the incubation tests, it was found impractical to use more than 0.1 unit (U.S.P.) of the crude liver extracts (*i.e.*, 1 or 2 units per cc), because the use of greater amounts caused inhibition of the cholinesterase in 1 cc of serum. The concentrated liver extracts (15 units per cc) were usually diluted 1 in 10 with distilled water and used in amounts rarely exceeding 0.3 U.S.P. unit, as this quantity seemed to give maximal effect if it gave any with a particular serum. Some dog sera do not show an increase of cholinesterase activity when incubated with either one or the other of these substances. We found, among our dogs, one whose serum showed a marked increase of the enzyme activity when incubated with either folic acid or liver injection. We created others by the experimental injection of di-isopropylfluorophosphate (D.F.P.)‡ which has been shown to inhibit irreversibly, or perhaps destroy cholinesterase.[4-6]

Fig. 1 shows the effects of incubation of 1 cc of serum with stated amounts of antianemic material upon its cholinesterase activity in terms of per cent increase. The

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1 Davis, J. E., *Science*, 1946, **104**, 37.

2 Davis, J. E., *Am. J. Physiol.*, 1946, **147**, 404.

3 Goodman, Carlson, and Gilman, *J. Pharmacol. and Exp. Therap.*, 1939, **66**, 15.

† Synthetic *L. casei* factor (Folvite) was kindly furnished by Lederle Laboratories, New York, New York.

‡ Furnished by the Medical Division of the Chemical Warfare Service, Edgewood Arsenal, Md., through the courtesy of Col. John R. Wood.

4 Bodansky, O., and Mazur, A., *Fed. Proc.*, 1946, **5**, 123.

5 Comroe, J. H., Todd, J., Koelle, G. B., and Gilman, A., *Fed. Proc.*, 1946, **5**, 172.

6 Mazur, A., and Bodansky, O., *J. Biol. Chem.*, 1946, **163**, 261.

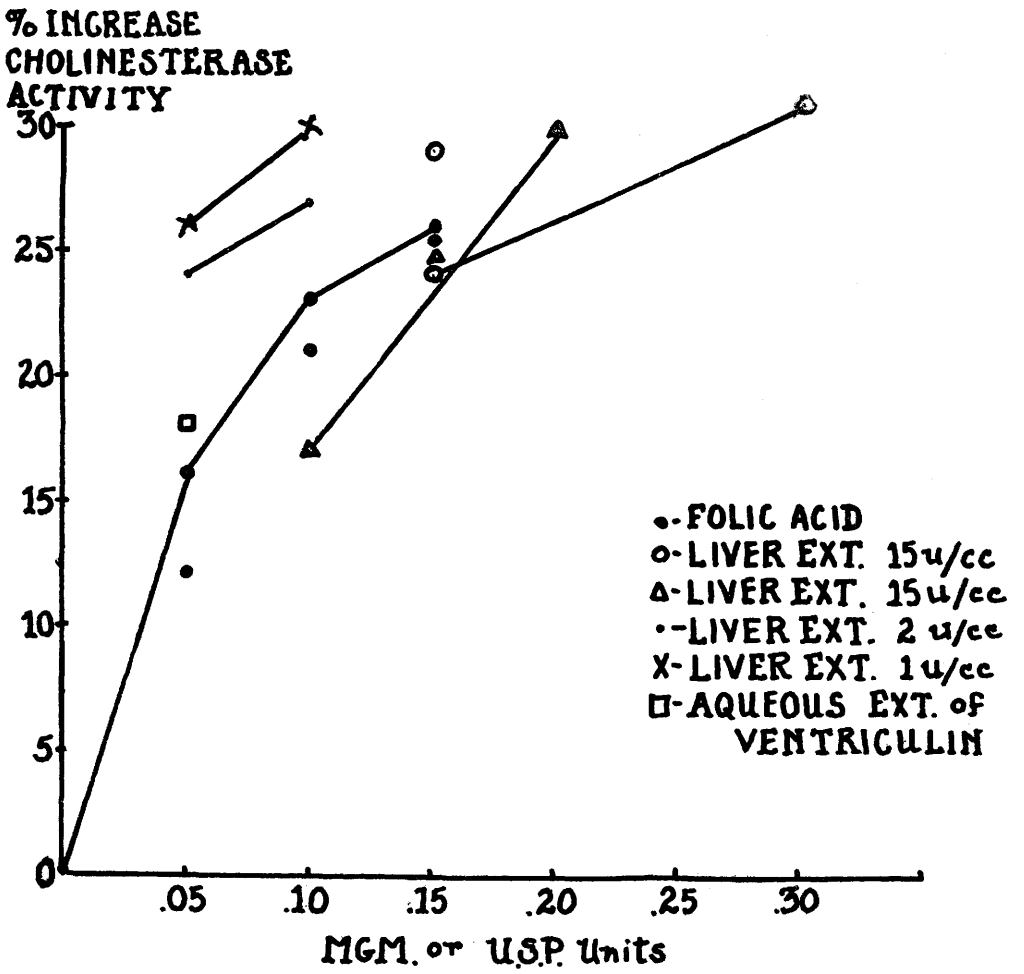

Fig. 1.

The effect of incubation of various amounts of antianemic preparations with dog serum upon its cholinesterase activity.

Amounts of folic acid used are given in terms of mg, while the amounts of other substances are given in terms of U.S.P. units according to their labelled potencies. All points connected by intact lines were obtained upon one sample of serum from one dog. All other points represent experiments on one sample of serum obtained from an animal treated with D.F.P. 2 days earlier.

data here shown were collected on the sera of 2 dogs that showed approximately the same capacity for increase of serum enzyme activity. All points connected by lines were obtained on tests of the serum of one dog obtained from one sample of blood. This dog had been untreated for 2 months prior to this experiment, but had previously received injections of acetylcholine twice daily for 2 months. All points not connected by lines, represent experiments made upon one serum sample from another dog which had received an injection of 1 mg of D.F.P. 2 days before the tests. It will be seen in Fig. 1 that the maximal increase in enzyme activity that was achieved under the conditions of this experiment was about 30%. The concentrated (high potency) liver extracts appear to be less effective (per U.S.P. unit as labelled) than the crude liver extracts investigated. Similar experiments have been performed on serum from several other dogs, but the sera used in the tests shown in Fig. 1 seem to be the most suitable for comparison of folic acid with different liver extracts. On the basis of other experiments as well as

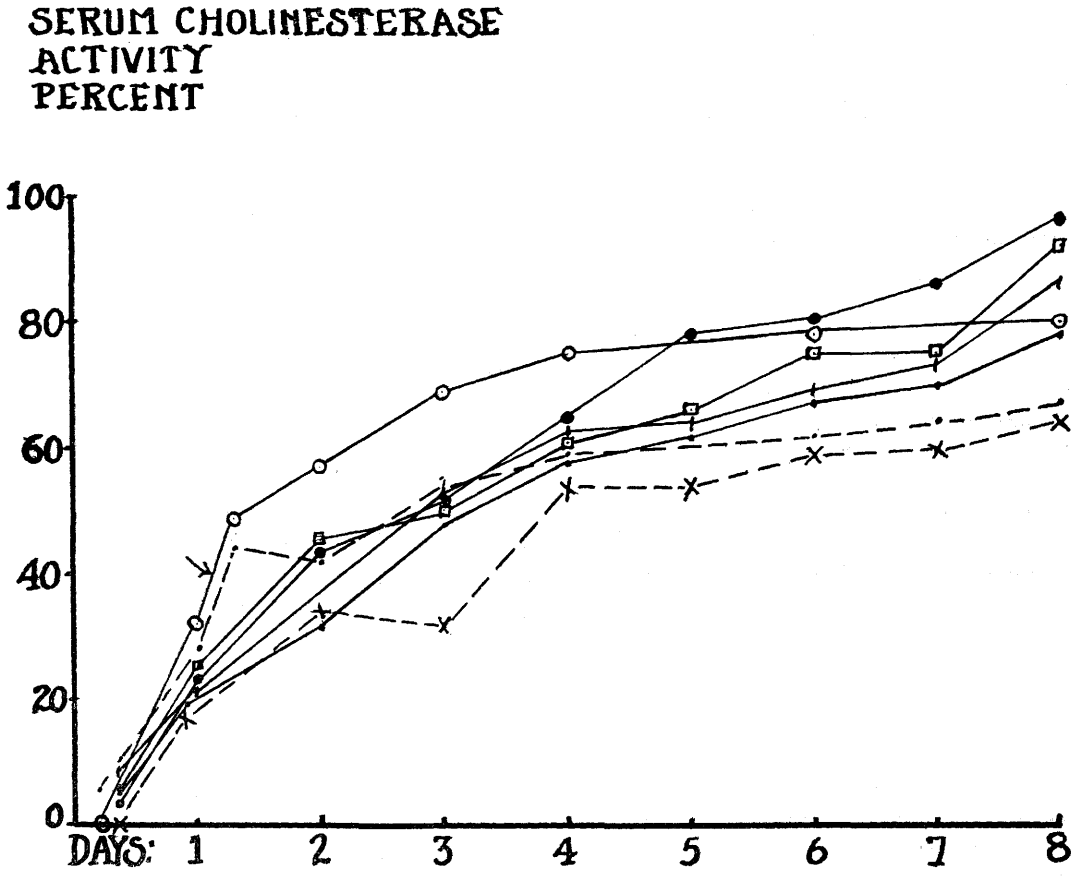

Fig. 2.

The effect of liver extract and pteroyl glutamic acid administration upon the regeneration of serum cholinesterase activity in dogs given D.F.P.

D.F.P. (diisopropyl-fluorophosphate) was injected intramuscularly into all dogs at zero time.

Liver extract and folic acid were administered from the 2nd to 5th days to all dogs represented by solid intact lines, except one (open circles) which received 2.4 mg of folic acid at the arrow.

these, it seems that 1 mg of pteroyl glutamic acid is roughly equal to 0.5 unit of the crude extracts or 1.5 units of the concentrated liver extracts used, in increasing cholinesterase activity. An aqueous extract of stomach U.S.P. (Ventriculin, Fig. 1) also appeared to be effective in increasing the activity of this enzyme in the serum.

The *in vivo* experiments consisted of injecting dogs with diisopropyl-fluorophosphate (D.F.P.), and studying the subsequent rate of regeneration of the cholinesterase activity of the serum. Fig. 2 shows the effect of the i.m. injection of 1 mg of D.F.P. upon the serum cholinesterase levels of 6 dogs. A 7th dog represented by open dots was given 0.6 mg (.05 mg per kg) because his body weight was markedly less than that of the others. It will be seen in Fig. 2 that the D.F.P. caused rapid and great reductions of the serum cholinesterase activities to rates ranging from 0 to 8% of the preexperimental levels. Beginning on the first day after the injections of D.F.P. 4 of the dogs were treated intensively with liver extracts and folic acid for 5 days, while 2 dogs (dashed lines) served as untreated control dogs. The other dog (open dots, Fig. 2) received only one injection of 2.4 mg of folic acid at 27 hours after the D.F.P. administration. It will be noted

in Fig. 2 that the 5 treated dogs all showed higher serum cholinesterase activities after the 5th day following D.F.P., than the 2 untreated control animals. The differences in serum cholinesterase prevailing between treated and untreated animals on the 8th day after D.F.P. ranged from 11 to 32%.

Discussion. The D.F.P. was administered intramuscularly in aqueous solution to 2 of the animals (open dots and top dashed line, Fig. 2). We found by experience that the D.F.P. lost potency rapidly when diluted in water, and for this reason we diluted our next sample in olive oil and injected the remainder of the animals intramuscularly with the oil solution. The 2 dogs injected with aqueous solution of D.F.P. showed recovery of their serum cholinesterase activities to normal in 15 and 21 days (for treated and untreated animals, respectively) but the other animals were not observed beyond the 8th day. From the 2nd to 4th days the smallest dog, which had received the low dose of 0.6 mg of D.F.P. showed the greatest regeneration of enzyme activity. It therefore seemed wise to give the other dogs a single dose of 1 mg irrespective of body weight. Variation in body weights do not account for any differences in rapidity of cholinesterase regeneration, as far as we can ascertain.

Liver extract or folic acid alone were given to particular animals at the start of the experiment shown in Fig. 2. Since no rapid changes were produced thereby, we commenced giving both preparations to all experimental dogs, except one, on the 3rd experimental day. The average amounts given each dog were 2 mg daily of folic acid (pteroyl glutamic acid) and 5 units of liver extract.

If proof should come forth that the cholinesterase potentiating actions of liver and folic acid are the important actions of these substances in the treatment of certain human anemias, then it would seem likely that liver preparations could be assayed by one of the methods reported in this paper.

Summary and Conclusions. The incubation of liver extracts or pteroyl glutamic acid with certain dog blood sera caused an increase in the cholinesterase activity of the sera. From such experiments it was estimated that 1 mg of pteroyl glutamic acid was roughly equal to 1 ± 0.5 U.S.P. unit of the liver extracts examined, in cholinesterase activity potentiating power.

The administration of liver extracts and pteroyl glutamic acid to dogs in which the serum cholinesterase had been greatly depleted by diisopropyl-fluorophosphate injections, caused more rapid regeneration of this enzyme activity than was observed in untreated control animals.

15577

Effect of Riboflavin and Other B Complex Vitamins on Work of Perfused Frog Muscles.

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Previous experiments have shown that when appropriate concentrations of thiamine,[1,2] cocarboxylase,[2] pantothenic acid,[3] or pyridoxine,[4] were added to the perfusion fluid; the work output of the frog gastrocnemius muscles was significantly increased. The fol-

* With the technical assistance of Frances S. Berg.

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