Precipitation of Insulin by Lysozyme and Effect of the Complex on the Blood Sugar Level. (17469)

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Because of the possible therapeutic application of a lysozyme-insulin complex, the properties of which might be expected to resemble those of protamine-insulin, experiments have been done on the precipitation of insulin by crystalline egg lysozyme (Armour) isoelectric point 10.5 to 11[1,2] and lysozyme which was treated in several different ways intended to destroy its lytic power as tested on *M. lysodeikticus*. The effect of the resuspended precipitates on the blood-sugar level of rabbits was also tested.

Table I shows the data obtained in measuring the precipitation of zinc-containing crystalline insulin dissolved in a minimal volume of N/100 HCl, adjusted to pH 7.3 with N/100 NaOH and made up to 0.10% concentration. The indicated quantities of a 0.10% (uncorrected for 9.56% moisture) solution of lysozyme were added to 1 ml of the insulin solution and the volume made up to 2 ml with water. After standing over night the tubes were centrifuged and the supernatant fluids tested for insulin and lysozyme by adding more of one or the other reagent. The precipitates were washed twice with 2 ml of water, centrifuging both times, and analyzed for nitrogen by the Kjeldahl method using H_2SO_4 and H_2O_2 for digestion, the color being developed with Nessler's reagent and measured in a photoelectric colorimeter. All the mixtures were set up and analyzed in duplicate.

While 0.60 ml of a 0.10% solution of untreated lysozyme precipitated almost all the insulin in 1 ml of the insulin solution,* 96% of the total nitrogen present in the 2 reactants being found in the precipitate, 0.90 ml of lysozyme solution which had been a) dissolved in 75% ethanol and heated in a boiling water bath for 2 hours,† b) dissolved in water and heated in the autoclave at 123°C for ½ hour,† c) dissolved in water and photodynamically treated (1-250,000 methylene blue and exposure of a thin layer of solution in a Petri dish to daylight for 6 days‡) precipitated respectively 64%, 80% and 75% of the insulin. Untreated lysozyme added in excess of that required to precipitate all the insulin (at least up to the addition of an excess of 50%) did not change the quantity of nitrogen in the precipitate, but remained in the supernatant fluid. It was also found (not recorded in the table) that the addition of twice as much insulin as was required to combine with a given quantity of lysozyme resulted in the excess remaining in solution.

Rabbits weighing 2.5 to 2.8 kg were injected subcutaneously with 0.086 ml of lysozyme-insulin suspensions containing 20 units of insulin per ml and prepared with (a) untreated lysozyme, (b) untreated lysozyme with added zinc, (c) lysozyme in 75% ethanol solution and heated as described, (d) lysozyme in water solution and autoclaved as described, (e) lysozyme photodynamically treated as described, followed by additional exposure for 18 hours to a 200 watt Mazda lamp at a dis-

[1] Meyer, K., Thompson, R., Palmer, J. W., and Khorazo, D., *J.B.C.*, 1936, **113**, 303.

[2] Alderton, G., and Fevold, H. L., *J.B.C.*, 1945, **157**, 43; *ibid.*, 1946, **164**, 1.

* Taking the molecular weight of lysozyme as 13,900[3] and that of insulin as 32,000[4] (as found in 0.25% solution) it appears that 5 molecules of the former combine with 4 of insulin. Moisture content of the insulin was disregarded. The small quantity of zinc in the insulin (*ca.* 0.28 γ per unit) may have an influence on this ratio.

[3] Fromageot, C., and Privat de Garilhe, M., *Biochim. et Biophys. Acta*, 1949, **3**, 82; Palmer, K. J., Ballantyne, M., and Galvin, J. A., *J.A.C.S.*, 1948, **70**, 906.

[4] Gutfreund, H., *Biochem. J.*, 1948, **42**, 156, 544.

† Lytic power for *M. lysodeikticus* was destroyed by this treatment.

‡ Lytic power for *M. lysodeikticus* was about 10% of the original value after this treatment.

TABLE I.

Precipitation of Insulin by Untreated and Treated Lysozyme. (1.0 ml of 0.10% solution of insulin was used in all the tests).

Ml of 0.10%-lysozyme (uncorrected for 9.54% moisture)	0.30	0.40	0.50	0.60	0.70	0.80	0.90	Treatment of lysozyme prior to its addition to the insulin
Insulin in supernate	+	+	+	+	—	—	—	Untreated (N = 17.0% on wet basis). The insulin contained 14.5% N.
Lysozyme in supernate	—	—	—	+	+	+	+	
mg N in ppt.	0.138	0.185	0.216	0.236	0.237	0.236	0.241	
% of insulin precipitated	60	80	90	?	100	100	100	
Insulin in supernate	+	+	+	+	—	—	—	Methylene blue added, used immediately after mixing.
Lysozyme in supernate	—	—	—	+	+	+	+	
mg N in ppt.	0.138	0.180	0.215	0.235	0.238	0.244	0.242	
% of insulin precipitated	60	77	90	?	100	100	100	
Insulin in supernate	+	+	+	+	+	+?	+?	Methylene blue addition + exposure to daylight for 6 days.
Lysozyme in supernate	—	—	—	—	—	—	—	
mg N in ppt.	0.112	0.145	0.174	0.206	0.220	0.242	0.261	
% of insulin precipitated	42	53	61	72	70	73?	75?	
Insulin in supernate	+	+	+	+	+	+	+	Dissolved in 75% ethanol, heated for 2 hr in boiling water bath.
Lysozyme in supernate	*	*	*	*	—	—	—	
mg N in ppt.	0.019†	0.069†	0.090	0.156	0.191	0.219	0.250	
% of insulin precipitated	?	?	?	?	50	57	64	
Insulin in supernate	+	+	+	+	+	+	+	Dissolved in water and autoclaved for ½ hr at 123°C.
Lysozyme in supernate	*	*	*	*	—	—	—	
mg N in ppt.	0.021†	0.023†	0.063†	0.167	0.206	0.256	0.269	
% of insulin precipitated	?	?	?	?	60	83	80	

* Supernate, after centrifuging, is opalescent.

† Nitrogen in precipitate is less than in added lysozyme alone, although test for latter in supernatant fluid was negative. This is possibly owing to incomplete sedimentation of the lysozyme-insulin by centrifuging; the opalescence of the supernate suggested that the complex was present in the form of particles too small to be thrown down by the centrifugal force employed.

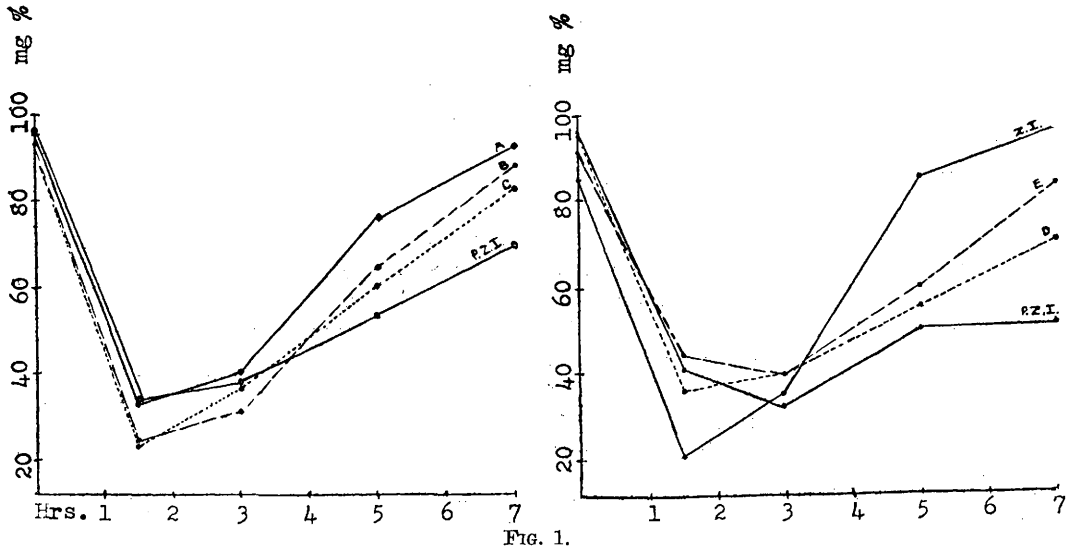

FIG. 1.

Blood-sugar curves following injection of lysozyme-zinc-insulin, protamine-zinc-insulin and zinc-insulin into rabbits. See text for description of the preparations injected.

tance of 8 inches.§ Three γ of zinc‖ per unit of insulin were added to samples B, C, D and E.

Samples A, B and C were tested simultaneously, along with a protamine-zinc-insulin reference standard containing 40 units of U.S.P. zinc-insulin and 91.5 γ of zinc per ml,¶ of which 0.043 ml were injected. On the second day, the remaining 2 samples of lysozyme-in-insulin were tested, along with the reference standard of protamine-zinc-insulin and a solution of U.S.P. zinc-insulin crystals containing 40 units per ml, of which 0.043 ml were administered. Blood was drawn from the marginal ear vein before, and 1½, 3, 5 and 7 hours after administering each of the insulin preparations to 6 rabbits, and was analyzed by the U.S.P. XIII method.

The average blood-sugar values found in the fasting state and 1½, 3, 5 and 7 hours after the injections for the 6 animals** in each group are shown graphically in Fig. 1.

In both days' experiments the values, 1½ hours after administering lysozyme-insulin, were either approximately the same, or somewhat lower than following protamine-zinc-insulin. Between 1½ and 3 hours, 4 of the 5 curves for lysozyme-insulin and 1 for protamine-zinc-insulin were slowly rising. Five hours after the injections the blood-sugar values were, respectively, 80%, 70%, 66%, 58% and 66% of the fasting levels for samples A, B, C, D and E and 56% and 52% for the 2 protamine-zinc-insulin samples. Two hours later the corresponding figures were 98, 92, 89, 74 and 91 and 73 and 53. In the case of insulin in solution the blood-sugar was back to the fasting level in 5 hours.

If any therapeutic application is to be made of lysozyme-insulin it will have to be shown that repeated injections do not sensitize to lysozyme. Crystalline lysozyme has been reported to be mildly antigenic.[5] Less pure preparations are reported to be non-antigenic.[6] Upon gently evaporating the heated, alcoholic

§ Lytic power for *M. lysodeikticus* was about 1% of the original value after this treatment.

‖ This, and the use of 50% more lysozyme than was required to precipitate all the insulin when using untreated lysozyme, resulted in complete precipitation of insulin and the presence of lysozyme in the supernatant fluid of all the mixtures.

¶ This is somewhat less than the 3 γ zinc per unit of insulin in samples B, C, D, and E.

** The results for one rabbit injected with protamine-zinc-insulin in the second group of experiments who had convulsions and died are not included in the curve.

[5] Smollens, J., and Charney, J., *J. Bact.*, 1947, **54**, 101.

[6] Kigasawa, T., *Ztschr. f. Immunitätsforsch.*, 1928, **57**, 146; Wolf, L. K., *ibid.*, 1927, **50**, 88.

solution described above to dryness the lysozyme was found to be insoluble in water. This may be taken as an indication of denaturation. While a marked serological change follows denaturation it does not necessarily destroy antigenicity. More study of the antigenic properties of lysozyme seems indicated.

The presence of lysozyme in secretions and tissues[7] suggests a possible precipitating effect on insulin *in vivo*. The solubility of the complex in serum is sufficiently great, however, to make this unlikely.

Summary. Crystalline egg lysozyme precipitates insulin. The complex produces a prompt lowering of the blood-sugar level in rabbits. Precipitates formed by mixing insulin with lysozyme which has been heated, or treated photodynamically so that 90 to 100% of its lytic activity on *M. lysodeikticus* is destroyed, but about 60% of its precipitating power for insulin is left, produce a hypoglycemic response which, up to 7 hours after injection, is intermediate between that resulting from the injection of insulin in solution and that following administration of protamine-zinc-insulin.

I wish to record my appreciation to Mr. E. K. Wolfe of the Biochemical Control Department of Sharp and Dohme, Inc., Glenolden, Pa., for performing the animal experiments.

[7] Fleming, A., and Allison, V. D., *Brit. J. Exp. Path.*, 1922, **3**, 252.

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Renal Excretion of Digitoxin in Man Following Oral Administration.* (17470)

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The amount of digitoxin excreted in the urine of human subjects receiving the drug has not been determined, primarily because of previous inability to measure minute amounts of any type of digitalis glycoside in biological media. However, the discovered sensitivity of the embryonic duck heart preparation[1] to minute amounts of digitoxin when present in Tyrode's solution and human serum offered the possibility that this method also could be used for the quantitative detection of digitoxin excreted in the urine of patients receiving the drug. The preliminary results of this study are reported herein.

Methods. A human urine sample of 200 ml was boiled down to approximately 50 ml on a hot plate and thereafter more slowly evaporated until the volume was reduced to

TABLE I.
Relationship Between Initial Concentration of Digitoxin in Human Urine (200 ml) and Time of Occurrence of "Digitalis Effect" in Final Extract.

Amt. dig. added (μg)	No. of extracts assayed	Avg time, "dig. effect" (min.)	S.E. of mean (min.)
1	5	—	—
2	12	47	2.28
3	10	35	1.82
4	11	27	1.45
6	9	22	1.14
8	10	17	0.73
10	5	14	0.35
12	6	11	0.20

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