uptake and thereby prolonged action. There is a tendency, not statistically significant, for α-methyldopa, dopa and dopamine to be also potentiated by disulfiram. It is evident that the actions of disulfiram and diethyldithiocarbamic acid are much more complex than just an inhibition of dopamine-β-hydroxylase. In the case of these two inhibitors, especially diethyldithiocarbamic acid, their block of the norepinephrine reversal effect of the reserpine block of tyramine makes this test for dopamine-β-hydroxylase questionable.

Diethyldithiocarbamic acid is more effective than disulfiram in inhibiting α-methyldopa, α-methyldopamine, dopa, and dopamine reversal of the reserpine block of the pressor response of tyramine.

It is possible that diethyldithiocarbamic acid is also preventing decarboxylation as the 2 carboxylated agents, α-methyldopa and dopa, are inhibited more by diethyldithiocarbamic acid than by disulfiram.

Summary. In these experiments rats are reserpinized, and it is demonstrated that α-methyldopa reverses the tyramine pressor response blockade by reserpine. Diethyldithiocarbamic acid will inhibit the reversal process by α-methyldopa· α-methyldopamine, dopamine, and dopa. Disulfiram inhibits the reversal process of α-methyldopa and dopa. Disulfiram or diethyldithiocarbamic acid alone will not inhibit the tyramine response. Diethyldithiocarbamic acid is most potent of the two agents used in inhibiting the reversal process.

ADDENDUM: Musacchio, J. M., Bhagat, B., Jackson, C. J. and Kopin, I. J., J. Pharmacol., 1966 v152, 293, have used nearly the same technique to show inhibition by disulfiram of the restoration by dopamine and α-methyldopamine of the response to tyramine in reserpine-treated cats.

1. Burn, J. H., Rand, M. J., J. Physiol., 1958, v144, 314.

2. Potter, L. T., Axelrod, J., Kopin, J. J., Biochem. Pharmacol., 1962, v11, 254.

3. Bhagat, B., Arch, int. Pharmacodyn., 1964, v147, 26.

4. Trendelenburg, U., Gravenstein, J. S., Science, 1958, v128, 901.

5. Gillespie, J. S., Mackenna, B. R., J. Physiol., 1961, v156, 17.

6. Day, M. D., Rand, M. J., Brit. J. Pharmacol., 1964, v22, 72.

7. Conradi, E. C., Gaffney, T. E., Fink, D. A., Vangrow, J. S., J. Pharmacol., 1965, v150, 26.

8. Torchiana, M. L., Wenger, H. C., Stavoski, J., Ludden, C. T., Stone, C. A., ibid., 1966, v151, 242.

9. Kaufman, S., Friedman, S., Pharmacol. Rev., 1965, v17, 71.

10. Goldstein, M., Anagnoste, B., Lauber, E., McKereghan, M. R., Life Sci., 1964, v3, 763.

11. Musacchio, J. M., Kopin, I. J., Snyder, S., ibid., 1964, v3, 769.

12. Musacchio, J. M., Goldstein, M., Anagnoste, B., Poch, G., Kopin, J. J., J. Pharmacol., 1966, v152, 56.

Received May 31, 1966. P.S.E.B.M., 1966, v123.

In vitro Effects of Zinc on Insulin Activity in Adipose Tissue.* (31486)

AGNES M. HUBER AND STANLEY N. GERSHOFF (Introduced by Fredrick J. Stare)
Department of Nutrition, Harvard School of Public Health, Boston, Mass.

Since the isolation and crystallization of insulin by Scott in 1934(1), zinc has been associated with the insulin molecule. However, the role, if any, of zinc in insulin metabolism and whether it is required for the hormonal action of insulin has not yet been determined. Baker and Rutter(2) have commented that media containing more than 10^{-5} M zinc inhibited glucose uptake by isolated rat epididymal adipose tissue in the presence of insulin. Recently, in this laboratory we have been impressed both in *in vivo* and *in vitro* studies by the action of zinc in

* Supported by USPHS Grant AM03056
Fund for Research and Teaching, Dept. of Nutrition, Harvard School of Public Health.

suppressing insulin activity. In this report we shall present some *in vitro* observations of the effects of zinc and other divalent cations on insulin activity.

Experimental and results. Male Charles River CD rats were fed a complete semi-purified diet *ad libitum* consisting of in %: casein 25, sucrose 70.7, corn oil 9, cod liver oil 1, salts IV(3) 4 and choline 0.3. Four mg of thiamine hydrochloride, 8 mg of riboflavin, 4 mg of pyridoxine hydrochloride, 40 mg of niacin, 20 mg of calcium pantothenate, 1 mg of folic acid, 1 mg of menadione, 0.2 mg of biotin and 0.05 mg of vit. B_{12} were added to each kilogram of diet. This diet contained 1.2 mg of zinc per 100 g as determined by atomic absorption flame analysis.

Insulin activity was measured *in vitro* in isolated epididymal adipose tissue using a Warburg manometric technique(4). Pieces of epididymal adipose tissue weighing between 110 and 130 mg were excised from decapitated rats weighing approximately 200 to 220 g. The pieces of fat pads were weighed and placed immediately in Krebs Ringer bicarbonate, pH 7.4 buffer containing 3 mg glucose/ml, at room temperature. All tissues were transferred to Warburg vessels at essentially the same time. The Warburg flasks contained Krebs Ringer bicarbonate buffer, pH 7.4, to which 3 mg of glucose and 0.5 mg gelatin/ml were added. Crystalline insulin was added to give a final concentration of 0.5 mUnits per ml of medium. The Warburg flasks were incubated at 37°C after gassing with 5% CO_2, 95% oxygen for 10 minutes and equilibrated with open stop cocks for another 10 minutes. Net gas exchange was measured for a period of 3 hours, readings being taken every 30 minutes, and expressed as μl CO_2 exchanged/100 mg tissue. In one experiment the incorporation of C^{14} from uniformly labelled glucose into fat was determined. Glucose-U-C^{14} was added to give a final concentration of 0.8 μcuries/ml of medium. After incubation the fat was extracted with chloroform:methanol (2:1). Esterbonds in these extracts were determined by the method of Antonis(5) and C^{14} was estimated in a liquid scintillation counter. The specific activity of the fat was expressed as counts per minute per μ equivalent of esterbonds of fat.

TABLE I. Effect of $ZnCl_2$ on Crystalline Insulin Activity in Rat Epididymal Adipose Tissue.

mg $ZnCl_2$ per ml of medium	Net gas exchange,* μl CO_2/3 hr/100 mg fat pad
0	122 ± 8
.00001	126 ± 5
.0001	65 ± 3
.001	37 ± 2
.01	32 ± 8
.1	25 ± 4
1.0	26 ± 3

* Mean ± S.E. of 6 assays.

A. Effect of various zinc concentrations on the crystalline insulin activity: $ZnCl_2$ was added to the media in the Warburg flasks to give concentrations of 1, 0.1, 0.01, 0.001, 0.0001, 0.00001 mg per ml (one mg of $ZnCl_2$ is equivalent to 0.48 mg of Zn^{++} or 0.0073 mM). The zinc effect on net gas exchange is shown in Table I. Concentrations above 0.0001 mg of $ZnCl_2$ per ml medium inhibited insulin activity significantly. Addition of $ZnCl_2$ did not change the pH of the medium appreciably. The pH measured after incubation ranged between 7.5 and 7.65, being lowest with the highest amount of zinc. Addition of varying amounts of $ZnCl_2$ did not alter the blanks nor the thermobar readings. Table II shows how fat synthesis was affected by 2 concentrations of $ZnCl_2$: 0.01/ml which inhibited net CO_2 evolution and 0.00001 mg/ml which did not. The inhibition of the incorporation of labelled glucose into fat by the higher level of $ZnCl_2$ was in accord with the gas exchange experiment. To obtain the inhibitory effect of zinc ions, it was not necessary to add them to the insulin media prior

TABLE II. Effect of $ZnCl_2$ on Fat Synthesis by Epididymal Adipose Tissue in the Presence of Crystalline Insulin.

Contents of flask/ml	Fat synthesis* as cpm/mEq of esterbonds
Blank (no insulin)	271 ± 45
1 mU crystalline insulin	510 ± 41
1 mU crystalline insulin + .00001 mg $ZnCl_2$	473 ± 60
1 mU crystalline insulin + .01 mg $ZnCl_2$	272 ± 13

* Mean ± S.E. of 6 assays.

TABLE III. Effect of Various Divalent Ions on Insulin Activity in Epididymal Adipose Tissue.

Ion tested	Net gas exchange* as μl CO_2/3 hr/100 mg fat pad .0005 mMol/ml	.001 mMol/ml
None (control)	60 ± 3	72 ± 4
Ba	51 ± 4	64 ± 3
Sr	54 ± 1	66 ± 9
Hg	12 ± 4	16 ± 1
None	62 ± 5	79 ± 8
Cd	75 ± 4	72 ± 8
Ni	67 ± 4	53 ± 5
None	101 ± 7	79 ± 8
Zn	14 ± 2	19 ± 2
Co	75 ± 5	58 ± 6
None	60 ± 8	60 ± 8
Cr	53 ± 5	61 ± 6

* Mean ± S.E. of 5 assays.

to incubation with the adipose tissue. When 0.001 mM/ml of $ZnCl_2$ were added to incubating systems at half hour intervals from 0 to 150 minutes, the addition in each case resulted in inhibition of insulin activity as measured by net gas exchange.

B. Effects of other divalent ions on insulin activity: Table III shows the effect of a number of divalent ions on insulin activity. All salts were added as the chlorides in final concentrations of 0.0005 and 0.001 mM/ml of medium. Controls were included in each experiment for comparison. Barium, strontium, cadmium, and chromium ions did not interfere with the insulin activity. Highly significant inhibition occurred with both concentration levels of zinc and mercuric ions. To a lesser extent, cobalt and nickel also acted as inhibitors.

C. Effect of ethylenediaminetetraacetic acid (EDTA) on zinc inhibition of insulin activity: EDTA was added to the incubation mixture to give a final concentration of 0.002 mM/ml medium. Table IV shows that EDTA or zinc alone depressed the net gas exchange but when both $ZnCl_2$ and EDTA were added, no zinc inhibition occurred.

TABLE IV. Effect of EDTA on Zinc Inhibition of Crystalline Insulin Activity.

Contents of flasks/ml	No. of values	Net gas exchange,* μl CO_2/3 hr/100 mg fat pad
.5 mU insulin	7	71 ± 5
.5 mU insulin .001 mM $ZnCl_2$	8	.4 ± 5
.5 mU insulin .002 mM EDTA	8	20 ± 4
.5 mU insulin .001 mM $ZnCl_2$ .002 mM EDTA	8	70 ± 10

* Mean ± S.E.

D. Effect of phosphate on the zinc inhibition of insulin: Table V shows the effect of $ZnCl_2$ on net gas exchange in the presence of 2 different concentrations of phosphate, added in the form of the ternary sodium salt. A concentration of phosphate similar to that in serum (0.001 mM/ml) did not prevent zinc inhibition of insulin activity. However, a 10-fold increase in phosphate concentration prevented this effect of zinc.

E. Effect of dialysis at pH 7.6 and 3.75 on the biological activity of the zinc-insulin complex: Four ml of Krebs Ringer bicarbonate buffer containing 5 mUnits of insulin and 0.02 mM $ZnCl_2$ per ml were dialyzed overnight at 3°C against 300 ml of the buffer at pH 7.6 or 300 ml of buffer whose pH had been adjusted to 3.75 with HCl. Following dialysis the pH of the acid dialysate was adjusted to 7.6 with NaOH and both dialysates were diluted 10 times with buffer to give a concentration of 0.5 mUnits of insulin/ml. Table VI shows the insulin activity of the dialysates and appropriate controls on adipose tissue. Dialysis at pH 3.75 in great part prevented the zinc inhibition of insulin ac-

TABLE V. Effect of Phosphate on Zinc Inhibition of Crystalline Insulin Activity.

Contents of flasks/ml	Net gas exchange,* μl CO_2/3 hr/100 mg fat pad
.5 mU insulin	78 ± 9
.5 mU insulin .001 mM $ZnCl_2$	11 ± 3
.5 mU insulin .001 mM phosphate	83 ± 5
.5 mU insulin .001 mM $ZnCl_2$ .001 mM phosphate	17 ± .2
.5 mU insulin .01 mM phosphate	89 ± 13
.5 mU insulin .001 mM $ZnCl_2$ .01 mM phosphate	69 ± 12

* Mean ± S.E. of 8 assays.

TABLE VI. Effect of Dialysis at pH 3.75 and 7.6 on Zinc Inhibition of Crystalline Insulin Activity.

Content of flasks/ml	Treatment before incubation	Net gas exchange,* μl CO_2/3 hr/100 mg fat pad
.5 mU insulin	None	81 ±13
.5 mU insulin + .002 mM $ZnCl_2$	"	1 ± 8
Insulin + $ZnCl_2$†	Dialysis at pH 3.75	49 ± 7
Insulin + $ZnCl_2$†	Dialysis at pH 7.6	10 ± 1

* Mean ± S.E. of 6 assays.
† 5 mU insulin and .02 mM $ZnCl_2$/ml before dialysis. The dialysates were diluted 10× before incubation.

tivity while dialysis at pH 7.6 was not effective, presumably because it did not remove the zinc from the insulin molecule. Dialysis of insulin alone at either pH does not significantly alter its biologic activity.

Discussion. It has been known for many years that interactions occur between insulin and zinc or other divalent ions. Such complexes have been subjected to extensive physical chemical studies to characterize their molecular weights, sedimentation constants, electrophoretic properties, and crystal structures but few data exist concerning their biologic activities.

From sedimentation constant studies Cunningham *et al*(6) have concluded that the binding of zinc to insulin is accompanied by aggregation of the protein components of amorphorus insulin. In the current studies insulin activity as measured on isolated adipose tissue decreased sharply in the presence of increasing amounts of zinc.

Cohn *et al*(7) reported that the zinc content of crystalline insulin increased as the pH of crystallization increased from 5 to 6.5. Cunningham *et al*(6) found that zinc binding by insulin did not occur at pH 3.75. In the present work a solution containing 5 mUnits of insulin per ml, made almost biologically inactive to adipose tissue by addition of 0.02 mM of $ZnCl_2$/ml was reactivated by dialysis overnight at pH 3.75 but not at pH 7.6. Cunningham *et al*(6) also showed that phosphate prevented the formation of zinc insulin aggregates with sedimentation constants above 3.5. In the present experiments phosphate in concentrations equal to that of the zinc in the incubation media did not alter the effect of zinc on insulin activity, but concentrations 10 × higher prevented the zinc inhibition. This suggests that zinc insulin aggregates with sedimentation constants greater than 3.5 have diminished biological activity for adipose tissue.

Since EDTA completely prevented zinc inhibition of insulin activity, zinc insulin complexes must be less stable than zinc-EDTA. Vohra and Kratzer(8) estimated a stability constant for zinc and EDTA of 16.5. Thus the stability constant for zinc insulin must be smaller. It is well known that histidine complexes with zinc, and the suggestion has been made(9) that zinc binds to insulin at histidine sites. The addition of 0.002 mM of L histidine/ml of media did not affect insulin activity in the absence of zinc in the system used in these studies and did not affect inhibition of insulin activity caused by 0.001 mM of $ZnCl_2$/ml of media. Thus it appears that zinc insulin may be a more stable complex than zinc histidine.

When divalent ions other than zinc were added to the incubation mixture inhibition of insulin activity occurred only with mercuric and to a lesser extent with cobalt and nickel ions. Since all additions were made in equimolecular amounts as chloride salts, it appears that chloride is probably not involved in the inhibition of insulin activity as studied here. It is not certain whether mercuric ions form insulin aggregates or act as a tissue poison. The significant but less pronounced inhibition with cobalt may be brought about by a direct interaction with insulin, since the sedimentation data of Cunningham *et al*(6) show that insulin complexes with cobalt, although to a lesser extent than with zinc.

In vivo conditions are certainly more complicated than the relatively simple buffer system used in these studies. There are many metabolites which may complex with insulin or zinc *in vivo* and which may affect the binding of zinc to insulin. Since the lower inhibiting levels of zinc employed in these studies fall within the normal zinc levels of human plasma(10), it would be of interest in

future work to study effects of plasma and tissue levels of zinc on insulin activity.

Summary. Zinc in concentrations in the range of those observed in human plasma and higher inhibited the activity of isolated rat epididymal adipose tissue in the presence of insulin and glucose. Of the other divalent ions studied only mercuric and to a lesser extent cobalt and nickel ions inhibited insulin activity. Zinc inhibition could be prevented in part by dialysis at pH 3.75 but not 7.6. The inhibition by zinc could be prevented by EDTA or large amounts of phosphate.

The authors wish to thank Mrs. Zalfa Sayegh and Miss Eileen Foster for their technical assistance.

1. Scott, D. A., Biochem. J., 1934, v28, 1592.
2. Baker, W. B., Rutter, W. J., Arch. Biochem. Biophys., 1964, v105, 68.
3. Hegsted, D. M., Mills, C. R., Elvehjem, C. A., Hart, E. B., J. Biol. Chem., 1941, v138, 459.
4. Ball, E. G., Martin, D. B., Cooper, O., ibid., 1959, v234, 774.
5. Antonis, A., J. Lipid. Res., 1960, v1, 485.
6. Cunningham, L. W., Fischer, R. L., Vestling, C. S., J. Am. Chem. Soc., 1955, v77, 5703.
7. Cohn, E. J., Ferry, J. D., Livingwood, J. J., Blanchard, M. H., ibid., 1941, v63, 17.
8. Vohra, P., Kratzer, F. H., J. Nutrition, 1964, v82, 249.
9. Tanford, C., Epstein, J., J. Am. Chem. Soc., 1953, v76, 2170.
10. Vallee, B. L., Physiol. Rev., 1959, v39, 443.

Received June 9, 1966. P.S.E.B.M., 1966, v123.

Experimental Specific Serum Therapy of Staphylococcus.* (31487)

BERRY CAMPBELL AND ELTON L. LASSILA
Department of Neurosurgery, Loma Linda University Medical School, Los Angeles and Los Angeles County General Hospital

The recent history of the Staphylococcus problem is replete with quotations such as the following: "Among the more chastening chapters in the annals of microbiological research is the story of our apparently dismal failure to control the depredations of the Staphylococcus"(1). The seriousness of the problem has been emphasized by Rogers(3) who proposes the view that antibiotics are probably the cause of "the emergence of Staphylococci as a troublesome hospital problem." A possibility of immune therapy has been raised by Fisher and Manning(4) as they report the high content of natural antibodies in pooled human serum (See also Fisher, 5). Active immunization, with the use of improved vaccines, is shown to be feasible by McCoy and Kennedy(2). In the experiments reported below, specific immunotherapy is examined with the use of the mouse protection test.

Materials and methods. Experimental animals. Mice from the Charles River Colony were used in these experiments. The rabbits were albinos purchased from the Mission Laboratories in Los Angeles.

Vaccines. An isolate of *Staphylococcus aureus* (Ha #1) from a fulminating and fatal infection in the Los Angeles County General Hospital was obtained and the method of McCoy and Kennedy(2) was used for preparation of the vaccine. We had attempted earlier to evoke antibodies in goats with vaccine prepared by the usual pasteurization procedures, but found that even when the heat treatment was carried to the point where no growth in standard media could be detected, the vaccine still showed viable organisms upon injection into the animals. In the present series of experiments the organisms were grown for 18 hours on blood agar plates and washed off with sterile saline. After 3 washings and resuspensions of the organisms, the final suspension was adjusted to MacFarland standard tube #2 and 1 part in 7 of benzalkonium chloride (1-1,000) added. The vaccine was agitated and incubated at 37°C for 30 minutes. Following this step,

* Supported by the John A. Hartford Foundation.