EDTA (Ethylene Diamine Tetraacetic Acid) Potentiation of Insulin Activity as Assayed by the Epididymal Adipose Tissue Technique.* (29845)

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We initiated these studies of the effect of EDTA, (ethylene diamine tetraacetic acid) on insulin activity(1) because EDTA chelates zinc and other metal ions. Crystalline insulin contains between 0.3 and 0.6% zinc(2), and zinc addition, as well as certain other divalent metal ions (cobalt, nickel and cadmium), facilitates the crystallization of insulin.

Histochemical studies of mammalian islets (3) and microchemical studies of fish islets both indicate a high zinc content(4,5). In the toadfish (*Opsanus tau*) the zinc content is about 112 μg per gram (wet weight) of islet tissue(4). Maske and coworkers(5) observing a close parallelism between the zinc and insulin contents of the islet, have suggested that insulin may be combined with zinc within the beta cells.

Methods and materials. Epididymal fat pads were removed from rats of the Holtzman strain, weighing about 250 g. Fat pads from each rat were cut into 6 pieces and placed individually in 1 ml medium containing 2 mg gelatin, 1 mg glucose and 0.2 μc glucose 1-C^{14}. In studying the effect of EDTA alone, one piece of the fat pad was used for the control, a second piece was incubated with insulin (500 μunits per ml) and the remaining 4 pieces were incubated respectively in 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} M concentrations of EDTA. In any given experiment, 6 rats (*i.e.*, 36 pieces of epididymal fat pads) were used. The vials were stoppered, gassed with a mixture of 95% O_2 and 5% CO_2 and then incubated for 3 hours in the manner described previously(6). The $C^{14}O_2$ evolved was collected in 0.5 ml hyamine and carbon-14 was counted in a tricarb liquid scintillation counter at an efficiency of 60%. The amount of glucose oxidized per gram tissue per 3 hours of incubation period was calculated from the amount of labelled CO_2 evolved.

After completion of the incubation, the fat pads were removed, lipids were extracted, using a chloroform-methanol mixture (2:1 by volume), and the carbon-14 incorported into the lipid was determined(7).

In studying the effect of EDTA on crystalline beef zinc insulin† or low-zinc amorphous insulin,‡ two hundred milliunits of insulin (in 20 μl of 0.005 N HCl) were diluted to 20 ml, using a medium containing both gelatin and glucose. This insulin solution was further diluted to give a final concentration of 100 or 250 μunits per ml of medium using a buffer medium containing either no EDTA or 10^{-3} M EDTA.

In studying the insulin activity of microdissected rat islet tissue, the islets were removed from lyophilized sections of pancreas and weighed(6,8). About 30 to 50 islets (weighing between 1.0 to 2.0 μg) were placed in a vial containing 20 to 25 ml of the medium and shaken intermittently for ½ hour. The solution was centrifuged (5 to 10 minutes at 2000 rpm) and nine-tenths ml of the supernatant was placed in each incubation vial; 0.1 ml of the medium containing 10^{-2} M EDTA was added. The controls contained identical amounts of media (with or without EDTA), but no insulin.

Goosefish insulin was prepared from islet tissue and purified by precipitation as a

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† Crystalline beef zinc insulin Lot No. 693502, was obtained through the courtesy of Lilly Research Laboratories.

‡ Low zinc amorphous insulin preparation Lot No. W-3255 had an activity of 19.8 units/mg, and a zinc content of 0.041%. It was supplied through the courtesy of Dr. Mary A. Root, Lilly Research Laboratories.

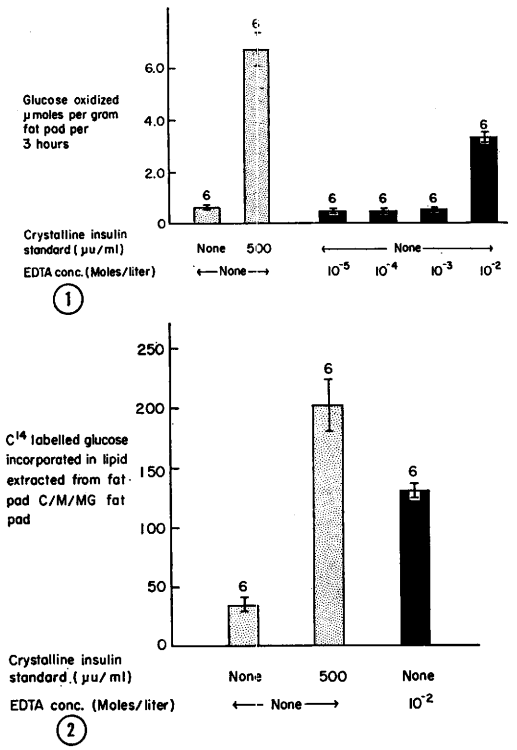


FIG. 1. *Effect of varying concentrations of EDTA on glucose oxidation by epididymal fat pad tissue.* Number of animals used shown at top of each bar. Vertical line indicates standard error of mean. The increased glucose oxidation produced by 10^{-2} M EDTA was highly significant ($p = <0.0001$).

FIG. 2. *Effect of EDTA on glucose incorporation into lipids by epididymal fat pad tissue.* Number of animals and standard error of mean as in Fig. 1. The increased incorporation of labeled glucose into lipids in presence of 10^{-2} M EDTA was highly significant ($p = <0.0001$).

zinc salt.§ Two 100 and two 200 μl aliquots of the insulin solution (in acid-alcohol) were placed in test tubes and the alcohol was removed under a stream of nitrogen. The residues were dissolved in 10 ml media (with and without 10^{-3} M EDTA) and one ml amounts were placed in the incubation vessels.

Rat plasma (50 ml) was extracted with acid alcohol and the extract was lyophilized according to the procedure described by Steinke *et al*(10). The lyophilized material was dissolved in buffer and assayed at 2 concentrations in the presence or absence of EDTA (10^{-3} M).

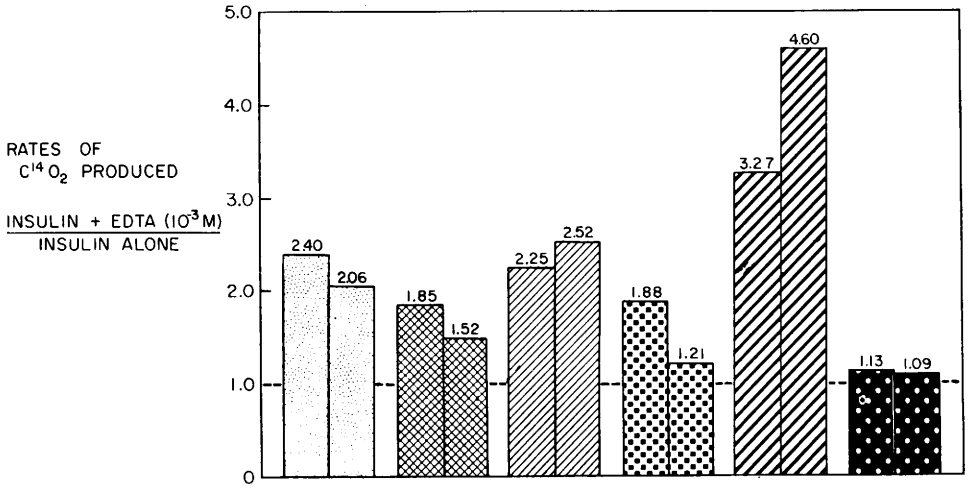
Results. Effect of EDTA alone on epididymal fat pad tissue. When high concentrations of EDTA (10^{-2} M) were present in the incubation medium, glucose oxidation was increased more than 4-fold (from 0.65 μM per gram tissue in the control, to 3.29)(Fig. 1), and C^{14} incorporation into lipids was increased correspondingly (Fig. 2). On the other hand, lower concentration of EDTA, *viz.*, 10^{-3} to 10^{-5} M, did not significantly affect glucose oxidation.

Potentiation of crystalline beef zinc insulin. In the absence of insulin, EDTA (10^{-3} M) did not significantly affect glucose oxidation by the adipose tissue, (Fig. 1). Whereas insulin alone (100 μunits per ml) in the absence of EDTA produced a 3.3-fold increase in glucose oxidation (from 0.75 to 2.51 μM); insulin in the presence of EDTA (10^{-3} M) produced a 7.9-fold increase (from 0.60 to 4.74 μM). Thus EDTA potentiates the effect of 100 μunits of insulin by a factor of 2.40 (Fig. 3). When 250 μunits of insulin were used, there was a 6.4-fold increase in glucose oxidation in the absence of EDTA, and a 13.2-fold increase in the presence of EDTA (10^{-3} M); this is a 2.06-fold potentiation of glucose oxidation.

EDTA likewise potentiated the activity of low-zinc bovine insulin. Thus when 100 μunits of the amorphous insulin were present in the incubation medium, this addition of 10^{-3} M EDTA, produced a 1.85-fold increase in glucose oxidation. With 250 μunits of insulin, EDTA produced a 1.52-fold increase in glucose oxidation, but this increase was not statistically significant (Fig. 3).

EDTA potentiation of insulin extracted from microdissected islets and pancreatic tissue. In 2 experiments there was a 1.85- and 3.03-fold increase in glucose oxidation (over the corresponding controls) when extracts of the microdissected rat islets were added to the medium. However, in the presence of EDTA (10^{-3} M) the corresponding increase was 4.17- and 7.62-fold respectively. Thus, EDTA produced more than a 2-fold potentiation of the glucose oxidation (2.25- and 2.52-fold) (Fig. 3).

§ The purified goosefish insulin samples were supplied by Dr. G. Eric Bauer and prepared by the method described by Bauer, Lindall, and Lazarow(9).



INSULIN PREPARATION	CRYSTALLINE BEEF-ZINC	AMORPHOUS ZINC-FREE	MICRO-DISSECTED ISLETS (RAT)	PANCREATIC EXTRACT (RAT)	GOOSEFISH ISLET EXTRACT	PLASMA EXTRACT (RAT)
"INSULIN" ADDITION PER ML MEDIUM	100* 250*	100* 250*	0.05†	5§ 10§	10§ 20§	0.45◒ 0.90◒
BASAL GLUCOSE OXIDIZED (μM/GM / 3 HR)						
WITHOUT EDTA	0.75	0.82	0.87 0.77	0.37	0.66	1.03
WITH EDTA	0.60	0.81	0.72 0.69	-	0.64	0.98

FIG. 3. *Potentiating effect of EDTA on various insulin preparations.*
* μunits insulin per ml medium.
† μg dry tissue per ml medium.
§ μl acid-alcohol extract.
◒ Ml. of plasma equivalent.

Similarly a 2-fold potentiation of glucose oxidation was observed with EDTA (10^{-3} M) and 5 μl acid-alcohol extracts of rat pancreas (Fig. 3). However, when larger amounts (10 μl) of pancreatic extracts were used the effect on glucose oxidation was considerably greater than that observed with 500 μunits insulin and hence EDTA potentiation effect was small and not statistically significant.

Potentiation of goosefish insulin. A similar but more pronounced effect of EDTA was observed with purified insulin prepared from goosefish islet tissue. As shown in Fig. 3, the addition of 10 μl of a goosefish insulin solution produced a 3.53-fold increased glucose oxidation in the absence of EDTA and 11.53-fold increase in the presence of EDTA (10^{-3} M); this is a 3.27-fold potentiation of the goosefish insulin effect. With 20 μl of the goosefish extract, EDTA produced greater potentiation namely 4.60-fold.

Action of EDTA on acid-alcohol extracts of plasma. In contrast to the previous results the "insulin-like activity" extracted from rat plasma (by acid alcohol) was not enhanced by EDTA (Fig. 3).

Discussion. The data presented clearly demonstrate that whereas high concentrations of EDTA alone (10^{-2} M) exhibit an insulin-like activity, lower concentrations of EDTA (10^{-3}, 10^{-4} and 10^{-5} M) do not significantly affect glucose oxidation (Fig. 1). This confirms the studies of Leonards and coworkers(7) who have reported that versene (EDTA, 10^{-2} M) increased the oxidation of both C-1 and C-6 labelled glucose into both lipid and glycogen. They suggest that "versene complexes with an inhibitory substance and as a consequence the fat pad is more active."

Although 10^{-3} M EDTA by itself does not activate glucose oxidation by the fat pad, we have found that this concentration of

EDTA produces a more than 2-fold potentiation of the action of insulin; potentiation was observed using preparations of crystalline beef insulin, purified goosefish islet insulin, pancreatic extracts (rats) and microdissected islets. The fat pad apparently differs from the muscle in its response to EDTA; the effect of insulin on glucose uptake by the diaphragm is markedly suppressed by EDTA (10^{-4} and 10^{-5} M) (11).

In explaining the action of the EDTA, the following possibilities were considered.

(a) EDTA, being a chelating agent, removed a metal ion from the cell membrane, which controlled the entry of glucose. It has been suggested that a metal-lipoprotein complex(12) may play a role in the cell membrane response of muscle (or adipose tissue) to insulin(11).

(b) EDTA protected insulin (in the medium) from being destroyed or inactivated (during incubation).

(c) EDTA depolymerized insulin complexes by chelating with zinc.

(d) EDTA (10^{-3} M) altered the tertiary structure of insulin and unmasked "reactive centers."

Tanford and Epstein(13) have postulated that 2 molecules of insulin of 6,000 molecular weight combine with zinc to form a 12,000 molecular weight dimer; one zinc atom presumably links the two B chains together by interacting with the histidine at the tenth position in the polypeptide chain. The removal of zinc (by chelation), would release two 6,000 M.W. units from the dimer and thus double the number of free insulin molecules.

It should be noted that with most of the insulin preparations used (microdissected rat islet, pancreas extract, and crystalline beef zinc), EDTA causes a 2-fold potentiation of the effect of insulin on glucose oxidation. With the goosefish insulin sample, however, EDTA produced more than a 3-fold potentiation of insulin; this could be explained by the presence of polymers of molecular weight greater than 12,000.

However, the observed potentiation of low zinc bovine insulin (Fig. 3) by 10^{-3} M EDTA, though not as great as that observed with crystalline zinc insulin cannot readily be explained on this basis. The low zinc insulin preparation contains less than 0.05 mole of zinc per mole of insulin. It would be difficult to attribute the observed potentiating effect of EDTA to depolymerization of a Zn insulin complex, unless EDTA prevented secondary dimer formation when the zinc free insulin preparation was added to the medium.

It is of interest that EDTA did not potentiate the "insulin-like" activity of plasma acid-alcohol extracts. This could be explained if the plasma insulin was present as a monomeric form or, alternatively if the plasma "insulin-like" activity was due to factors closely resembling insulin(10).

Summary. High concentrations of EDTA (10^{-2} M) when added to the incubation medium produced a several-fold increase in glucose oxidation, by epididymal fat pads. Thus 10^{-2} M EDTA has an "insulin-like" activity. By contrast lower concentrations of EDTA (10^{-3} to 10^{-5} M) did not significantly affect glucose oxidation. However, lower concentrations of EDTA (10^{-3} M) produced a 2-fold potentiation of the effect of insulin on the epididymal fat pad; this potentiation was observed with crystalline bovine insulin, low zinc amorphous insulin, acid-alcohol pancreatic extracts (rat), and preparations of microdissected rat islet tissue. With a goosefish islet insulin preparation the EDTA potentiation was over 3-fold. By contrast EDTA did not potentiate the "insulin-like" activity of rat plasma acid-alcohol extracts.

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Effect of Furosemide on Renal Medullary Sodium Gradient.* (29846)

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Furosemide (4-chloro-N-(furylmethyl)-5-sulfamoylanthranilic acid) has been shown to be an effective diuretic-saluretic agent in both man and laboratory animals(1,2,3). Though similar in chemical structure to the thiazide diuretics(1), furosemide is capable of producing a greater maximal response than hydrochlorothiazide(1). Suki *et al*(4) have reported that furosemide impairs the concentrating ability of the kidney. The process of urinary concentration is dependent upon the renal medullary sodium concentration which increases toward the papilla. This gradient is believed to result from the reabsorption of sodium from the ascending limb of the loop of Henle(5) and Suki *et al*(4) have proposed that furosemide acts in this area of the nephron. An agent that inhibits sodium reabsorption in the ascending limb of the loop of Henle would be expected to abolish the medullary sodium gradient. Thus, the purpose of this investigation was to evaluate the effect of furosemide on the renal medullary sodium gradient in the dog.

Methods. Seven female mongrel dogs weighing 6.5 to 12 kg were used. Food and water were withheld 18 hours prior to the experiments. The animals were anesthetized with pentobarbital sodium (30 mg/kg, intravenously) and tracheotomized. Carotid blood pressure was monitored by means of a Statham arterial pressure transducer and a Gilson direct-writing oscillograph. Isotonic sodium chloride was infused intravenously at a rate of 0.5 ml/kg/minute. The left kidney was exposed by means of a flank incision and the ureter was cannulated. When a quantity of urine sufficient for analysis (2 ml) had been collected, the renal pedicle was ligated and the kidney removed for analysis. Furosemide (25 mg/kg) was then administered intravenously and the saline infusion was replaced with a saline solution containing furosemide (25 mg/kg/hr). The right kidney was then exposed through a flank incision and the ureter was cannulated. Thirty to forty minutes after furosemide infusion was begun, a sample of urine was collected and the kidney was removed. To monitor changes that might occur with time, 4 animals did not receive furosemide but were infused only with saline after removal of the left kidney.

Immediately upon removal of a kidney from an animal, a cross-sectional slice was taken from the organ. From this slice, duplicate samples (50-100 mg) of tissue were obtained from the papillary, middle and basal regions of the medulla and from the cortex.

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