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1. Feller, D. D., Neville, E. D., Am. J. Physiol., 1965, v208, 892.
2. Somogyi, M., J. Biol. Chem., 1952, v195, 19.
3. Peterson, R., ibid., 1957, v225, 25.
4. McIlwain, H., Buddle, H. L., Biochem. J., 1953, v53, 412.
5. Neville, E. D., Feller, D. D., Anal. Biochem., 1965, v11, 144.
6. Oyama, J., Platt, W. T., Endocrinology, 1965, v76, 203.
7. Leon, H. A., Feller, D. D., Neville, E. D., Daligcon, B., Life Science, 1965, v4, 737.
8. Feller, D. D., Neville, E. D., Oyama, J., Averkin, E. G., Proc. Soc. Exp. Biol. and Med., 1965, v119, 522.
9. Abraham, S., Kopelovich, L., Chaikoff, I. L., Biochim. Biophys. Acta, 1964, v93, 185.
10. Nejad, N. S., Chaikoff, I. L., Endocrinology, 1964, v75, 923.
11. ———, Am. J. Physiol., 1963, v204, 137.

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Evidence of an Insulin Inhibitor in Human and Rat Serum.* (30743)

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A number of *in vitro* methods using isolated rat diaphragm or epididymal fat pad have been described and extensively used in determination of serum insulin(1,2,3,4). These methods are not specific for insulin and values obtained are usually reported in units of insulin-like activity. Recent reports have suggested(5,6) that insulin circulates in blood as a free form similar to crystalline insulin with a molecular weight of approximately 6000 and a bound form with a molecular weight of approximately 60,000 to 100,000. Isolated diaphragm assays only estimate free serum insulin while adipose tissue assays determine both free and bound insulin.

This difference in the activity of diaphragm and epididymal adipose tissue probably accounts for some of the discrepancies reported in assays of insulin-like material by diaphragm and adipose tissue. A further difficulty in the assay of insulin has been encountered with diluted sera which show a far higher insulin-like activity than undiluted sera. This has been observed by Groen *et al* (7), Randle(8), and Willebrands *et al*(9), using the rat diaphragm assay. It was also observed and discussed by Sheps *et al*(10) and Ball and Merrill(4) for insulin assays performed on the epididymal fat pad of rats. These reports suggested that there might be an inhibitor of insulin-like activity present in serum.

The following experiments provide additional evidence that human and rat sera contain an inhibitor of insulin activity and that this inhibitor may be removed by dialysis.

Experimental. Human sera were obtained from 9 normal subjects. Rat sera were obtained from adult rats on a purified control diet by pooling blood, taken under ether anesthesia from the inferior vena cava and centrifuged. The sera were refrigerated and were used as soon as possible. To remove inhibitor, the sera were dialyzed in Visking casing against 100 times their volume of Krebs-Ringer bicarbonate buffer, pH 7.4 at 3°C, overnight.

Insulin activity was determined on rat epididymal fat pads using the manometric Warburg technique of Ball *et al*(3). To the Warburg vessels were added two ml of serum containing 3 mg per ml of glucose to which uniformly labeled glucose was added to give a final concentration of 0.08 μcuries per ml. To some sera, 1 or 0.5 mUnit of crystalline insulin per ml was added. The blank flasks

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TABLE I. Human Serum.

Flask contents	N	Net gas exchange* μl CO_2/3 hr/100 mg epididymal fat pad	Fat synthesis* counts/min/μEq ester
Blank	6	5.2 ± 2.5	82 ± 4
1 mUnit/ml crystalline insulin	9	78.3 ± 3.6	242 ± 34
Serum	9	29.1 ± 6.6	150 ± 27
Serum + 1 mUnit/ml crystalline insulin	9	51.0 ± 6.0	211 ± 18
Serum dialyzed	9	57.1 ± 7.7	329 ± 14
Serum dialyzed + 1 mUnit/ml crystalline insulin	9	87.0 ± 6.4	372 ± 18
Effect of dialysis: Serum / Serum dialyzed		P <.02	P <.001
Serum + insulin / Serum dialyzed + insulin		P <.001	P <.001

* Results represent means ± standard error of mean.

contained Krebs-Ringer bicarbonate buffer with 3 mg of labeled glucose per ml as above and either no insulin or 0.5 or 1 mUnit of crystalline insulin and 0.1 mg gelatin per ml.

Epididymal fat pads of rats weighing approximately 220 g (205-241 g) were used. The animals were decapitated and the fat pads removed with minimum handling. Pieces weighing approximately 130 mg were weighed on a torsion balance and placed in Krebs-Ringer bicarbonate buffer containing 3 mg of glucose per ml at room temperature. The fat pads were transferred to the Warburg vessels at approximately the same time. The flasks were gassed with 95% O_2, 5% CO_2 for ten minutes at 37°C. After another 10-minute equilibration period, the stopcocks were closed and gas exchange measured every 30 minutes for 3 hours. At the end of the incubation period the fat pads were removed, thoroughly washed with 5 changes of distilled water and extracted overnight with a chloroform methanol mixture (2:1). On aliquots of these extracts, lipid ester determinations were made according to Antonis(11) and the radioactivity measured in a liquid scintillation counter. The specific activity of the fat was expressed as counts per minute per μ equivalent of ester.

In a second series of experiments, 60 ml of human and rat sera were each dialyzed against 60 ml of Krebs-Ringer bicarbonate buffer overnight. Pieces of rat epididymal fat pad were incubated as described above either in buffer or the dialysates containing glucose and varying levels of crystalline insulin and net gas exchange was measured to determine whether the dialysates contained an inhibitor of insulin activity.

Results and discussion. Net gas exchange and fat synthesis by epididymal adipose tissue treated with human and rat sera are given in Tables I and II. These data show that dialysis of sera with or without added crystalline insulin significantly increased net gas exchange and the incorporation of labeled glucose into fat by epididymal tissue.

The data shown in Table III indicate that dialysates from both rat and human sera inhibit the response of rat epididymal adipose tissue to varying levels of crystalline insulin.

The data obtained indicate that rat and human sera contain a dialyzable inhibitor of insulin activity. The increased activity of dialyzed sera was not caused by a concentration effect. Care was taken to maintain constant sera volumes before and after dialysis.

As yet, the inhibitor has not been characterized. The fact that it is dialyzable suggests that it has a relatively small molecular weight and is not a protein or the lipoprotein inhibitor of insulin activity which Bornstein (12) has reported. Fat solvents do not remove it from whole serum. It is not stable.

TABLE II. Rat Serum.

Flask contents	N	Net gas exchange* μl CO_2/3 hr/100 mg epididymal fat pad	Fat synthesis* counts/min/μEq ester
Blank	8	7.6 ± 1.0	96 ± 4
500 μunits/ml crystalline insulin	8	54.8 ± 1.7	193 ± 12
Serum	8	20.0 ± 4.3	111 ± 4
Serum + 500 μunits/ml crystalline insulin	8	33.3 ± 3.9	133 ± 4
Serum dialyzed	8	39.9 ± 5.5	154 ± 9
Serum dialyzed + 500 μunits/ml crystalline insulin	8	66.7 ± 7.7	210 ± 10
Effect of dialysis: Serum / Serum dialyzed		P <.01	P <.001
Serum + insulin / Serum dialyzed + insulin		P <.01	P <.001

* Results represent means ± standard error of mean.

In 2 experiments the boiling of dialysate from human sera for ten minutes resulted in a 63 and 54% loss of inhibitory action and 90% of the inhibitory action of dialysate was lost following freeze drying. We have not been able to prepare a stable sample of the inhibitory material. It is obvious that more work is needed to elucidate the nature of this insulin inhibitor and its physiological role.

Summary. Greater insulin-like activity was observed in dialyzed rat and human sera than undialyzed sera, using an isolated rat epididymal adipose tissue insulin assay technique. Dialysates from both rat and human sera decreased the activity of rat epididymal adipose tissue in the presence of crystalline insulin.

TABLE III. Effect of Dialysates from Rat and Human Sera on the Activity of Rat Epididymal Adipose Tissue in Presence of Insulin.

μU insulin/ml	μl CO_2/100 mg fat pad/3 hr K-R buffer	Dialysates
Rat serum		
0	7	1
100	48	32
200	66	50
500	77	57
Human serum		
50	20	10
100	34	18
600	55	50

Each value represents the mean of 4 experiments.

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1. Vallance-Owen, J., Hurlock, B., Lancet, 1954, v1, 68.
2. Renold, A. E., Martin, D. B., Dagenais, Y. M., Steinke, J., Nickerson, R., Sheps, M. C., J. Clin. Invest., 1960, v39, 1487.
3. Ball, E. G., Martin, D. B., Cooper, O., J. Biol. Chem., 1959, v234, 774.
4. Ball, E. G., Merrill, M. A., Endocrinology, 1961, v69, 596.
5. Antoniades, H. N., Huber, A. M., Boshell, B. R., Saravis, C. A., Gershoff, S. N., ibid., 1965, v76, 709.
6. Antoniades, H. N., Bougas, J. A., Camerini, R., Pyle, H. M., Diabetes, 1964, v13, 230.
7. Groen, J., Kamminga, C. E., Willebrands, A. F., Blickman, J. R., J. Clin. Invest., 1952, v31, 97.
8. Randle, P. J., Ciba Foundation Colloquia on Endocrinology, 1957, v11, 115.
9. Willebrands, A. F., Geld, H. V. D., Groen, J., Diabetes, 1958, v7, 119.
10. Sheps, M. C., Nickerson, R., Dagenais, Y. M., Steinke, J., Martin, D. B., Renold, A. E., J. Clin. Invest., 1960, v39, 1499.
11. Antonis, A., J. Lipid Research, 1960, v1, 485.
12. Bornstein, J., J. Biol. Chem., 1953, v205, 513.

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