or its splitting products, in the contraction mechanism. When one of these phosphates is adsorbed, its high electrical charge may disrupt the crystalline helices and thereby induce contraction. Such charge effects have been observed in solutions of several polypeptides, including polyglutamic acid (Doty *et al.*, 1957) and polylysine (Doty *et al.*, 1958).

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The Presence of Two Forms of Insulin in Normal Human Serum

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The mean insulin content of undiluted serum from normal subjects was found to be about 50 microunits per ml as measured by the rat diaphragm assay. When these sera were preincubated with an adipose tissue extract, the mean insulin content rose to over 600 microunits per ml. Treatment of a serum fraction obtained by Dowex 50W-X8 chromatography with the adipose tissue extract led to a similar increase in insulin activity. Insulin antiserum neutralized this increase in insulin activity in both instances. Furthermore, the insulin activity released by the action of the adipose tissue extract enhanced the glycogen synthesis as well as the glucose uptake of the rat diaphragm. Both of these observations provide evidence that the adipose tissue extract released insulin and not some other substance with insulin-like activity. These data confirm and extend the original experiments of Antoniades and his associates.

Evidence has been presented recently that insulin occurs in the blood in several forms. Ramseier *et al.* (1961) and Samaan *et al.* (1962) have suggested the existence of various forms of insulin in human and dog serum, based on an insulin assay which employs the epididymal fat pad of the normal rat. Antoniades and his associates (Antoniades, 1961; Antoniades and Gundersen, 1961; Antoniades *et al.*, 1961a) have found evidence for a "bound" or complex form of insulin and a "free" form of insulin in the serum of normal human subjects as measured by the rat diaphragm assay for insulin. "Free" insulin is presumed to be similar to or identical with crystalline pancreatic insulin, since both have the same effect upon the glucose uptake of the rat diaphragm. The terms "bound" insulin or insulin complex are used to describe a material present in diluted serum that does not produce a response in the diaphragm assay but that may be converted to a biologically active form, or "free" insulin, by treatment with an aqueous extract of adipose tissue. Antoniades *et al.* (1958) have found that "bound" insulin in serum is adsorbed to Dowex 50W-X8 resin under conditions where "free" insulin is not adsorbed. The "bound" insulin recovered from this resin may be converted into biologically active insulin by treatment with strong base or with the adipose tissue extract.

The purposes of this paper are (*a*) to report that treatment of either undiluted serum or a purified serum fraction with an adipose tissue extract results in a highly significant increase in insulin activity as measured by the rat diaphragm assay, and (*b*) to present evidence that this increased activity is due to insulin and not to some other substance. To avoid the introduction of new terms, the terminology of Antoniades will be used. Thus, free insulin produces a response in the diaphragm, while bound or complex insulin is inactive until treated with the adipose tissue extract.

Materials and Methods

Serum Samples.—Peripheral venous blood was collected from normal human subjects after 12 to 14 hours of fasting. The blood was allowed to clot at room temperature; the serum was separated by centrifugation and stored at 5° until assayed. The insulin content of each sample was determined within a few days of collection. Our preliminary experiments suggested that storage of serum by freezing abolished the effect of the adipose tissue extract.

Adipose Tissue Extract.—An extract of epididymal fat pads from normal rats was prepared by a method similar to that described by Antoniades and Gundersen (1962). The epididymal fat pads from rats weighing about 125 g were removed immediately after decapitation. They were homogenized with a Teflon pestle tissue homogenizer, 1 ml cold physiological saline being used per fat pad. The homogenate was filtered through nylon cloth and the filtrate was centrifuged at 800 × *g* at 5° for 15 minutes. The supernatant solution was chilled to freezing, and cold 95% ethanol was added to give a final concentration of 60% ethanol. The precipitate was removed by centrifugation at −5° at 2200 × *g* for 15 minutes and discarded. The supernatant fraction was diluted with water to approximately 5% alcohol and was lyophilized. This material was reconstituted with distilled water to the original volume of the homogenate. To avoid repeated freezing and thawing, 1.1 ml aliquots of the adipose tissue extract were placed in separate vials, frozen, and stored at −16°. Adipose tissue extract prepared and stored in this manner was active for several months without significant loss of activity.

Extraction of Bound Insulin from Serum.—Serum was passed through a column of Dowex 50W-X8 resin according to the method of Antoniades and Gundersen (1962). The biologically inactive material was eluted from the resin with 0.02 N ammonium hydroxide, neutralized with 0.1 N hydrochloric acid, lyophilized, and stored at 4° as the dry lyophilizate. For assay, the preparation was dissolved in a volume of Gey and Gey buffer (1936) equal to the volume of serum extracted and was dialyzed in Visking tubing at 4° for 16 hours against 10 volumes of Gey and Gey buffer.

Determination of Insulin Activity.—The insulin activity was determined by the bioassay procedure of Vallance-Owen and Hurlock (1955) employing the isolated diaphragm of the normal rat. Rats weighing 110 to 125 g were starved for 24 hours and were then used for the assay. Gey and Gey buffer served as the basic medium. One mg per ml of gelatin was suspended in the buffer and stirred at room temperature for 15 minutes. After removal of the excess gelatin by filtration, 3 mg glucose per ml was added. The buffer with gelatin and glucose constituted the medium used in the assay.

The total insulin content was determined in the serum by the incubation of 5.5 ml of undiluted serum with 1 ml rat adipose tissue extract for 30 minutes at 37°. The solution was then divided into three 2-ml aliquots and each was placed in a 30-ml beaker. The rat hemidiaphragms, which were prewashed for 15 minutes in Gey and Gey buffer containing no glucose or gelatin, were added to the beakers. The incubation was carried out in a Dubnoff metabolic shaker for 90 minutes with a gas phase of 95% oxygen and 5% carbon dioxide. The glucose uptake during the 90-minute incubation was determined by measuring the glucose concentration of the medium with a Technicon Autoanalyzer by a modification of the method described by Hagedorn and Jensen (1923). In all experiments, one of the paired hemidiaphragms from each rat was incubated in the buffer containing glucose and gelatin while the other hemidiaphragm was incubated with the experimental sample. Hence, the basal uptake was determined separately for the diaphragm of each rat.

The amount of insulin in the serum samples was determined by comparing the increase in glucose uptake of the rat diaphragm with that produced by known concentrations of insulin. A porcine insulin standard (Lilly zinc insulin crystals, Lot 499667, 25.1 International Units per mg) of 1000 microunits per ml was run with each assay, and two standards of 100 and 1000 microunits per ml were run in the same assay once each week. As was found by Vallance-Owen and Hurlock (1955), the slope of the standard line between 100 and 1000 microunits per ml does not change when the cube root of the insulin concentration is plotted against the glucose uptake, expressed as mg of glucose taken up per 100 ml per 10 mg dry weight of the diaphragm. Under our experimental conditions, the greatest accuracy was obtained between 100 and 1000 microunits insulin per ml.

Free insulin was determined in the same manner as total insulin except that 1.0 ml of saline was added to the serum in place of the adipose tissue extract for the 30-minute incubation period. The only dilution of the samples was that incurred by the addition of the adipose tissue extract or saline. The amount of bound insulin in the sample was determined by subtraction of the value for free insulin from the total value.

Total and free insulin-like activities in the material prepared from serum with the Dowex 50W-X8 resin were determined by the same procedures used for whole serum.

The effect of insulin antiserum on the additional activity obtained after the treatment with adipose tissue extract was determined. Insulin antibodies to crystalline bovine insulin were prepared in guinea pigs as described by Wright (1959). A 0.1-ml aliquot of antiserum diluted 1:100 was added to the serum or resin eluate *after the 30-minute incubation period with rat adipose tissue extract.* The assay was completed in the usual manner. The amount of antiserum added to the assay medium had no effect on the basal glucose uptake of the rat diaphragm, but it neutralized the effect of 1800 to 2000 microunits of bovine insulin per ml.

Results

The effect of rat adipose tissue extract upon the insulin content of normal serum as determined by the rat diaphragm assay is shown in Table I. After treatment of the *undiluted* serum with rat adipose tissue extract, the insulin activity was found to increase about 12-fold as compared to the sample incubated with saline. The magnitude of the increase is indicated in the third column of Table I labeled bound insulin.

The insulin complex was obtained by the resin fractionation technique from six of the serum samples shown in Table I. The data in Table II show the

Table I

The Effect of Rat Adipose Tissue Extracts (ATE) Upon the Insulin-Like Activity in Sera of Normal Fasted Subjects

	Insulin Concentration (microunits per ml serum)		
Subject	Free (Serum)	Total (Serum + ATE)	Bound
RH	20	660	640
WB	20	740	720
WS	40	650	610
NS	50	290	240
DG	70	650	580
SB	120	1130	1110
RE	10	400	390
HG	0	440	440
MS	90	860	770
HJ	70	340	270
Mean	49	616	567

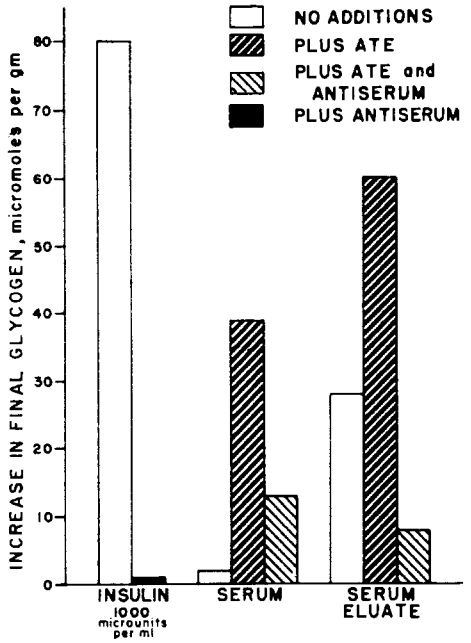

FIG. 1.—Effects of rat adipose tissue extract (ATE) and insulin antiserum on stimulation of glycogen synthesis in rat diaphragm by serum or serum eluates of normal subjects. The values are calculated as the difference between the glycogen content of paired hemidiaphragms, one equilibrated in buffer and the other in the experimental sample. The data represent the mean glycogen changes in the hemidiaphragms used in the assays for subjects MS and HJ shown in Table III.

TABLE II

THE EFFECT OF RAT ADIPOSE TISSUE EXTRACT (ATE) UPON THE INSULIN-LIKE ACTIVITY OF THE BOUND OR COMPLEX INSULIN DERIVED FROM THE SERUM OF NORMAL FASTED SUBJECTS

	Insulin Concentration (microunits per ml)		
Subject	Free (Insulin Complex)	Total (Insulin Complex plus ATE)	Bound
WB	0	960	960
WS	60	710	650
NS	60	760	700
DG	70	280	210
MS	20	350	330
HJ	70	740	670
Mean	47	633	586

results obtained when the resin eluates were assayed. The insulin complex of the serum had little activity until rat adipose tissue extract was added. The small amount of free insulin observed could have resulted from alkaline breakdown of the complex during elution from the resin (Antoniades and Gundersen, 1962). Again an increase in insulin activity of approximately 12-fold was observed after treatment with rat adipose tissue extract. Some differences were found between the amount of bound insulin in individual whole sera and the quantity of the insulin complex isolated from the same sera. These differences may be due to the fact that optimal conditions for the use of adipose tissue extract have not been established. However, the same qualitative pattern was observed, and the mean bound insulin values of sera and of the resin eluates did not differ significantly.

It should be emphasized that rat adipose tissue extract alone in the concentration used in these experiments had no effect on the glucose uptake of the rat diaphragm. Also, when adipose tissue extract was added to known amounts of crystalline porcine insulin, there was no potentiation of the effect of this insulin.

The effect of insulin antiserum upon the insulin activity released by treatment of the serum or the serum resin eluates with adipose tissue extract is shown in Table III. It is evident that the increased insulin activity is abolished by insulin antiserum. This observation strongly suggests that treatment with adipose tissue extract releases a substance similar to or identical with crystalline insulin. The insulin antiserum had no effect upon the basal glucose uptake of the rat diaphragm.

TABLE III

THE EFFECT OF INSULIN ANTISERUM UPON THE INSULIN-LIKE ACTIVITY OF SERUM AND INSULIN COMPLEX FROM SERUM OF NORMAL FASTED SUBJECTS AFTER TREATMENT WITH RAT ADIPOSE TISSUE EXTRACT (ATE)

		Insulin Concentration (microunits per ml)		
Sample	Subject	No ATE	With ATE	With ATE and Anti-serum
Serum	RE	10	400	0
	HG	0	440	0
	MS	90	860	0
	HJ	70	340	51
Insulin Complex	MS	20	350	0
	HJ	70	740	15

Additional evidence that insulin activity released by rat adipose tissue extract is due to insulin is derived from the specificity of the diaphragm response. Salicylate (Manchester *et al.*, 1958), cyanide, 2,4-dinitrophenol, arsenite (Randle and Smith, 1957), synthalin, and phenethylbiguanide (Williams *et al.*, 1957; Wright, 1959) as well as insulin increase the glucose uptake of the rat diaphragm. However, the noninsulin compounds are not ordinarily present in a concentration sufficient to influence the diaphragm. Furthermore, insulin alone among these compounds promotes glycogen synthesis in the diaphragm. Hence, in some of the experiments reported in Table III the glycogen content was determined in the pooled triplicate hemidiaphragms used in the assay. The results are given in Figure 1. These data show that the insulin-like activity liberated from the serum complex increases the glycogen content of the hemidiaphragm. The addition of insulin antiserum greatly reduces the glycogen found. These data were obtained incidental to the main purpose of the experiment and consequently are semiquantitative in nature. Nevertheless, the effects are in accord with the idea that insulin itself is being measured.

DISCUSSION

It is clear from the data presented that incubation of adipose tissue extracts with serum and resin eluates from fasted normal subjects results in a significant increase in the insulin activity of the serum as measured by the rat diaphragm assay. Furthermore, the neutralization of this insulin activity by insulin antiserum provides strong evidence that the increased activity is

due to insulin itself and not to some other substance that enhances the glucose uptake of the diaphragm. The preliminary studies of glycogen synthesis by the diaphragm under the conditions described further substantiate this conclusion, since insulin is the only substance known to increase glycogen synthesis in the isolated rat diaphragm.

These observations confirm and extend some of the experiments of Antoniades and co-workers. The present results in no way contradict the concept of Antoniades that serum insulin exists in a free form and in a complex or bound form. However, the present data may also be considered in the light of other current concepts on the state of insulin in blood.

Vallance-Owen *et al.* (1958) have reported that normal human plasma contains an insulin inhibitor as measured by the rat diaphragm assay. Such an inhibitor could affect the interpretation of the present data in several ways. Adipose tissue extract could exert an effect by destroying the inhibitor or neutralizing the effect of the inhibitor on free insulin. This assumption may explain the data obtained in the serum but not those obtained with the resin eluate for the following reasons. First, it must be assumed that the inhibitor would be adsorbed to and eluted from the resin under the conditions used to prepare bound insulin. Second, and more important, free insulin or crystalline insulin is not adsorbed to the resin under the conditions used to obtain bound insulin. Thus, if adipose tissue extract destroyed or neutralized the inhibitor, free insulin would not be present in the eluate to exert its effect on the diaphragm.

There are two other ways in which the inhibitor concept could affect the interpretation of the data. The inhibitor could be a basic substance to which insulin is bound. In this instance, the concepts of insulin inhibitor and bound insulin become the same. It is also possible that insulin may form a biologically active complex in serum whose effect is masked by the inhibitor. The physical properties of this complex may be such that it would be adsorbed to the cationic resin. Under this circumstance, the active complex and the inhibitor would be adsorbed to and eluted from the resin at the same time.

Correlation of the data reported here with those of Samaan *et al.* (1962) is difficult because different assay methods for insulin activity have been employed. Samaan *et al.* have described "typical" and "atypical" forms of insulin in plasma of normal dogs. Typical insulin is neutralized by guinea pig antiserum to ox insulin, whereas atypical insulin is unaffected by the antiserum. It is possible that atypical insulin represents an insulin complex that is active in adipose tissue but is not neutralized by insulin antibodies.

Aside from the implication of the work of Samaan *et al.* (1962), bound and free insulin in serum have been characterized almost completely through their activity in the rat diaphragm assay. Whether this activity represents a phenomenon peculiar to the diaphragm is unknown.

The nature and the biological significance of the insulin complex remain to be elucidated. The more recent work of Antioniades and his associates (1961b) is intriguing because it suggests a defect in some diabetic patients in the conversion of bound to free insulin.

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