able and showed clearly the Z-lines and A- and I-bands. The distance between Z-lines was about 2.5 μ, whereas the length of the A-band was approximately 1.5 μ. After contraction in potassium iodide solutions the bands were more vague; both the A-band and the Z-Z distance contracted to approximately half their original length. This result contrasts with the microscopic results (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954) obtained with light optics for contraction under physiologic conditions, for which the A-band has been reported to remain constant in length, whereas the Z-Z distance decreases. However, the results of Knappeis and Carlsen (1956), obtained by electron microscopy of muscle contracted under physiologic conditions and staining techniques similar to ours, indicate contraction of the A-bands also. Of course, this is not to be construed as proof that the mechanism of contraction under our conditions is identical to that under physiologic conditions. However, in view of the demonstrated ability of muscle to contract as a result of the transition from an oriented, crystalline state to an amorphous phase, this mechanism deserves serious consideration for contraction under physiologic conditions (see also the following paper [Hoeve *et al.*, 1963]).

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Evidence for a Phase Transition in Muscle Contraction*

C. A. J. Hoeve, Y. A. Willis, and D. J. Martin

From the Mellon Institute, Pittsburgh, Pennsylvania

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The length of glycerinated muscle fibers in solutions of ATP in aqueous glycerol and ethylene glycol is measured as a function of water content and temperature. Abrupt contraction is observed with small changes in one of the variables while the other remains fixed; therefore, a phase change underlies contraction. Since such phase changes are not inherent in the mechanisms proposed by Riseman and Kirkwood, Morales and Botts, Kuhn *et al.*, and Huxley, these mechanisms, unless modified, are unsatisfactory. Among the models proposed by Hill, Pauling and Corey, and Flory, all assuming phase transitions, the last appears the most likely; in it a phase change from oriented, crystalline to random, amorphous protein is the basis for contraction. In accordance with Bowen's conclusions, our results show no correlation between ATP splitting and contraction.

It is well known that glycerinated muscle fibers can be made to contract in solutions of adenosine triphosphate (ATP). Furthermore, this substrate is enzymatically hydrolyzed into adenosine diphosphate or orthophosphate. The opinion is widespread that the free energy change in this reaction must drive muscle contraction *in vivo* as well as *in vitro*, although no clear *molecular* mechanism has been advanced for this mechanochemical coupling and experimental evidence in its favor is lacking. Several suggestions have been made for the molecular mechanism underlying contraction. To facilitate discussion the most important models are here summarized.

Astbury (1947) supposed, in analogy to supercontraction in wool, that muscle contraction is accompanied by a change from a crystalline α-protein, oriented parallel to the fiber axis, to the cross-β state, partly oriented perpendicular to this direction. On the other hand, Pauling and Corey (1951) proposed a phase transition between two crystalline states: the extended pleated sheet form and the shorter α-helical form.

Riseman and Kirkwood (1948) supposed that the high charge of adsorbed ATP keeps the amorphous muscle proteins in the extended state. As a consequence of ATP splitting the charge would be reduced, resulting in contraction of the proteins; however, contraction should not in this case be accompanied by a phase change. These ideas were modified and elaborated by Morales and Botts (1953) and later by Morales *et al.* (1955). Kuhn *et al.* (1960) showed the feasibility of these models by inducing contractions and elongations in polyacrylic acid samples by changing the *p*H. In this case the electrical interactions are short-range and may be treated as Donnan effects.

Hill (1953) described several possible contractile systems, including models in which separation into two liquid phases occurs. By imposing geometrical restrictions on the polymer phases, he constructed theoretical

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models in which this phase separation is coupled with length changes.

In another model, hinted at by Pryor (1950) and put on a firm basis by Flory (1956a,b, 1957), a phase transition from an oriented, crystalline to a random, amorphous state underlies contraction. When immersed in potassium iodide solutions, glycerinated muscle indeed contracts according to this mechanism (Hoeve and Willis, 1963). Some experimental indications exist at present (Mandelkern *et al.*, 1959) that muscle may contract according to the same mechanism under physiologic conditions.

On the basis of optical and electron microscopic results Huxley and Hanson (1954) and Huxley and Niedergerke (1954) proposed that on contraction the thin actin filaments slide with respect to the thicker myosin filaments. Huxley (1957) made an effort to bring under one scheme this evidence and Hill's experiments (1938) on the rate of contraction. He assumed that side-pieces situated on actin form temporary bonds with those on myosin and explained sliding by making the *ad hoc* assumption that rates of bond formation and breakage depend on whether the side-pieces have passed each other. Since ATP splitting would be required for bond breaking, sliding and ATP *splitting* would be coupled; in this respect his mechanism distinguishes itself from the others mentioned.

An important difference between the mechanisms proposed is that phase changes are inherent in some of them but not in others; this difference allows us to make a choice by carrying out relatively simple experiments. If a one-component sample in equilibrium undergoes a phase transformation, *extensive* variables (volume, energy, length, *etc.*) are discontinuous with change in *intensive* variables (pressure, temperature, force, *etc*). It can be shown similarly that if the sample undergoing a phase transition is immersed in a large bath and swelling equilibrium is established, discontinuities in extensive variables occur, not only for alterations in temperature at fixed fluid composition, but also at fixed temperature for alterations in fluid composition.

Although in Astbury's model (1947) contraction results in the cross-β state, it is difficult to imagine how the α-state could be transformed directly into the β-state along an equilibrium path. It is more likely that, if formed at all, the cross-β state results in a secondary process, whereas one of the other mechanisms mentioned underlies contraction directly. Among the legitimate phase transitions proposed, Hill's models (1953) involving two liquid phases are to be distinguished from those of Pauling and Corey (1951) and Flory (1956a,b, 1957), in that in the first discontinuities in length are not to be expected at zero force, in contrast with the last two. Without further assumptions no phase changes are to be expected for the models proposed by Riseman and Kirkwood (1948), Morales and Botts (1953), Kuhn *et al.* (1960), and Huxley (1957). It is therefore of great importance to establish whether abrupt length changes occur under conditions favorable to attainment of equilibrium. This is the major object of this paper; in addition the relation between ATP splitting and contraction is being explored.

Experimental

All muscle samples used were obtained by glycerination of rabbit *psoas* fibers at their resting lengths according to methods described by Szent-Györgyi (1951). The samples were stored in 50% glycerol-water mixtures for periods ranging from 1 week to 6 months before being used; no decrease in activity with time was noticeable. Fiber bundles of 5 to 8 cm in length and with cross sections of approximately 0.02 cm^2 were prepared. The rate of dephosphorylation was measured for these samples or for suspensions obtained from glycerinated bundles. After glycerinated muscle samples were washed in water for half an hour they were mashed in a Waring Blendor for a total of 10 minutes, the suspension being cooled each time after 2 minutes of blending. This suspension could be pipetted and contained approximately 4×10^{-3} g of protein per ml; the particles were about 2 μ in diameter. Protein concentrations were obtained by drying a specified volume to constant weight at 100° and weighing. Deionized water was used throughout. Solutions of ATP and other salts were made up in aqueous glycerol or ethylene glycol solutions of various water contents. Two sets of solutions, 1 and 2, were used, with the following salt compositions: solution 1: ATP, 3.06×10^{-3} M; $MgCl_2$, 1.5×10^{-3} M; NaCl, 0.13 M; Na_3PO_4, 5×10^{-3} M; solution 2: ATP, 3.22×10^{-3} M; $MgCl_2$, 1.5×10^{-3} M; NaCl, 0.13 M; tris(hydroxymethyl)-aminomethane, 0.1 M. As a result of the relatively high buffer concentration in solution 2, the *p*H is essentially constant during splitting of ATP. The *p*H was adjusted to 7 in all cases. In some experiments, indicated under Results and Discussion, other concentrations of ATP were used, but concentrations of the rest of the salts remained as indicated for solutions 1 and 2. ATP used in solutions 1 and 2 was obtained from the Mann Research Laboratories and the Nutritional Biochemicals Corporation, respectively.

All experiments were performed in thermostats held constant to within 0.1°. Lengths were measured with a cathetometer. The bundle was suspended from a fixed upper clamp, and a small piece of lead shaving with negligible weight attached to the lower end of the bundle served as marker. Rates of shrinkage were unaffected by replacement of the solutions with fresh ATP solutions of the same initial concentration. In order to approach equilibrium conditions the greatest possible time for equilibration was allowed without the experiments' being so prolonged that the fibers lost their ability to shrink. The shrinking ability was checked after each experiment by placing the fiber in a freshly prepared ATP solution in water. If shrinkage was less than 40% of the original length, the experiment was discarded.

Phosphate determinations were carried out according to the Fiske-Subbarow method (1925). A fresh muscle sample was used for each splitting experiment.

Results and Discussion

Results of length measurements on bundles in type 1 ATP solutions of different glycerol content are given in Figure 1. As is observed, no length changes occurred for concentrations of glycerol higher than 50%, although the bundle was kept in these solutions for more than 6 hours in some cases. The major portion of shrinkage occurred within a rather small range between 40 and 20% glycerol, whereas only a small additional shrinkage was indicated at lower concentrations. A similar experiment with higher ATP concentration (4.5×10^{-3} M) gave virtually identical results. In order to examine whether equilibrium conditions prevailed, the period of immersion in each solvent mixture was increased from 80 minutes in one experiment to more than 6 hours in another; in the latter case a separate sample was chosen for each concentration, thus preventing inactivation. As seen in Figure 1, the results obtained were practically identical. Experimental results for mixtures of ethylene glycol

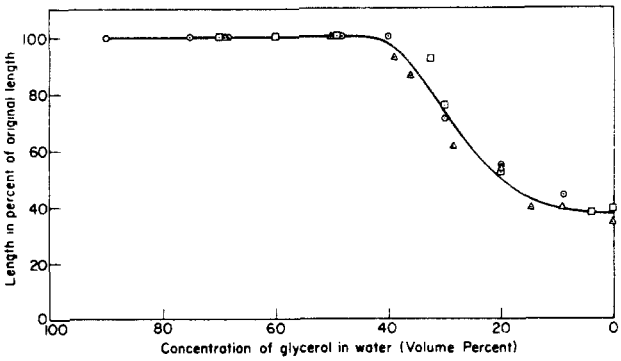

FIG. 1.—Length plotted *versus* glycerol concentration at constant temperature. □, The sample was immersed at 25° for 80 minutes in Solution 1 (3.06×10^{-3} M ATP) of different glycerol contents; the same sample was used throughout. ○, The sample was immersed at 25.5° for 90 minutes in Solution 1 (4.5×10^{-3} M ATP) of different glycerol contents; the same sample was used throughout. △, A fresh sample was immersed at 21.5° for 390 minutes in Solution 1 (4.5×10^{-3} M ATP) for each glycerol content.

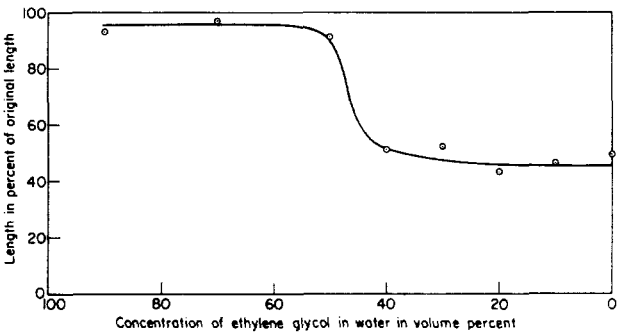

FIG. 2.—Length plotted *versus* ethylene glycol concentration at constant temperature (24°). A fresh sample was immersed for 3 hours in Solution 1 for each ethylene glycol content.

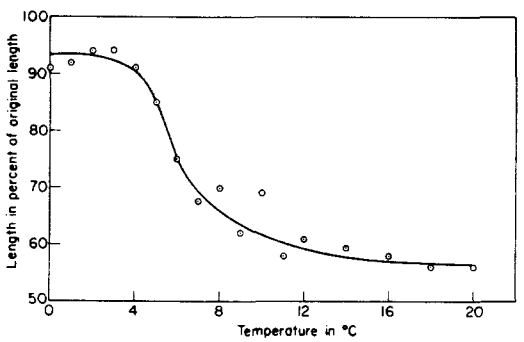

FIG. 3.—Length plotted *versus* temperature at constant concentration of ethylene glycol. Each point represents the average over the results for 4 fresh samples immersed for 22 hours in Solution 1 containing 45 volume % of ethylene glycol.

and water are shown in Figure 2. Appreciable length changes in these mixtures are confined to an even narrower range of concentrations.

In another set of experiments (Fig. 3) the temperature was changed in intervals of 1 or 2 degrees from 0 to 20° while the ethylene glycol concentration was kept fixed. Four fresh bundles were immersed for 22 hours at each temperature. Each point represents the average of the four samples. Again a sharp transition is indicated, although a certain amount of scattering of the data was unavoidable.

Several attempts were made to observe elongations of contracted fibers at higher glycerol concentrations or lower temperatures. However, within experimental error such attempts failed. This does not necessarily mean that no equilibrium values were observed for contraction. For example, *slightly* cross-linked collagen fibers are known to shrink abruptly with changes in temperature, and equilibrium measurements have been performed (Oth *et al.*, 1957), although once fully contracted these samples elongate only slightly at lower temperatures (see also results of the preceding paper [Hoeve and Willis, 1963] for muscle fibers).

Figure 4 shows length changes and amount of phosphate split per hour as a function of glycerol concentration. Shrinkage in solution 2 occurs at higher glycerol concentration than in solution 1 (Fig. 1). Since the buffer concentration in solution 2 is relatively high, this observation is in keeping with results of Bowen (1957), who noticed that higher salt concentrations promote shrinkage. The amount of phosphate split per hour for mashed samples is approximately ten times higher than that for fiber bundles. The lower rate of splitting by fiber bundles is in qualitative accord with results of Blum *et al.* (1957) and reflects low diffusion rates. Unfortunately, splitting rates for fiber bundles display rather large scattering (Fig. 4). Nevertheless, apart from a factor of ten, the *functional* dependence of ATP splitting on glycerol concentration is not appreciably affected by the degree of dispersion of the sample. The scattering in the results of fiber bundles is probably a result of different sizes and therefore of different diffusion rates. Results for ethylene glycol–water mixtures are given in Figure 5. Values of length and rate of splitting in an ethylene glycol–water mixture of fixed composition are plotted as functions of temperature in Figure 6.

The foregoing results indicate that large, abrupt changes in length occur with relatively small changes in either solvent composition or temperature as one of the variables, for fixed values of the other. *It follows from this evidence alone that a phase transition underlies contraction under the conditions of our experiments.* The somewhat diffuse character observed for the length changes—in contrast to discontinuities to be expected for first-order transitions in pure substances—may in part be due to failure to achieve equilibrium. In accordance with results of Blum *et al.* (1957), the length changes may have been affected by low rates of diffusion of ATP. These effects, if present, tend to broaden the transition; under true equilibrium conditions the transition may be even sharper. Unfortunately the experiments could not be more prolonged, since the contractility of samples decreases in time. Apart from possible diffusion effects, it is to be noted that a slow approach to equilibrium is the rule for polymers, rather than the exception.

We observe in Figures 4–6 that the rate of dephosphorylation as function of temperature or solvent composition is smooth; *the abrupt length changes are not accompanied by any increase above this smooth curve.* At high concentrations of glycerol or ethylene glycol the bundles do not contract appreciably, but dephosphorylation occurs, as seen in Figures 4 and 5. Even more remarkably, with high water content no further contraction occurs, although the rate of dephosphorylation increases steeply. Similar results are apparent (Fig. 6) with the temperature as variable. In order to study the relative rates of length changes and dephosphorylation, we carried out some experiments in a 20% ethylene glycol solution, in which the length changes slowly enough to be measurable as a function of time (Fig. 7). In accordance with the results of

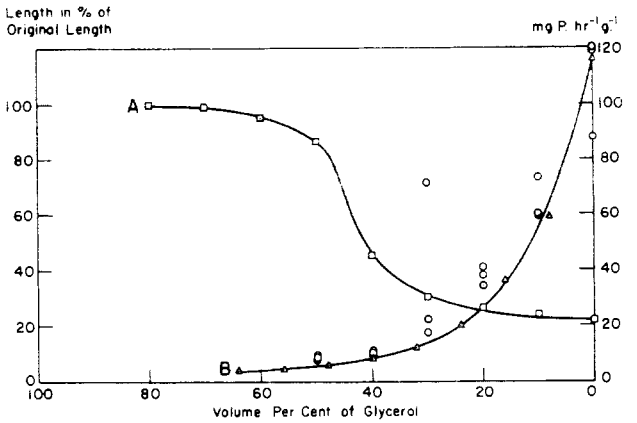

FIG. 4.—Length (curve A) and rate of ATP splitting (curve B) plotted *versus* glycerol concentration at constant temperature (24.8°). □, At each concentration the sample was immersed in Solution 2 for one hour before the length was measured (left ordinate); the same sample was used throughout. ○, Rate of ATP splitting multiplied by ten (right ordinate) for fresh fiber bundles in Solution 2. △, Rate of ATP splitting (right ordinate) for fresh, mashed samples in Solution 2.

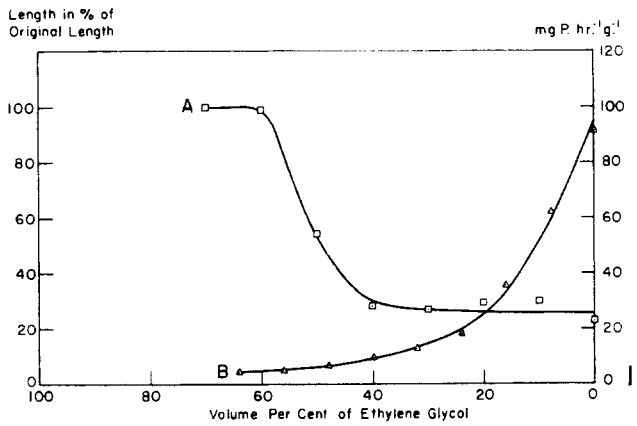

FIG. 5.—Length (curve A) and rate of ATP splitting (curve B) plotted *versus* ethylene glycol concentration at constant temperature (24.8°). □, At each concentration the sample was immersed in Solution 2 for 15 minutes before the length was measured (left ordinate); each point represents a fresh sample. △, Rate of ATP splitting (right ordinate) for fresh, mashed samples in Solution 2.

Bowen (1954, 1957) for water, the rate of dephosphorylation is constant even after the bundle is fully contracted. Thus, our results support Bowen's conclusions (see also Bowen and Martin, 1958, and Bowen, 1951) that dephosphorylation and contraction are essentially independent events.

Unless major modifications are made, the mechanisms proposed by Riseman and Kirkwood (1948), Morales and Botts (1953), Kuhn *et al.* (1960), and Huxley (1957) cannot explain the phase transition indicated in our experiments. Moreover, contrary to the assumptions in Huxley's model, the amount of ATP split is not related to changes in length. The abrupt length changes *at zero force* are not in agreement with expectation for Hill's models (1953). Wide-angle x-ray studies by Astbury (1947) and Pollack (1960, unpublished) before and after contraction do not indicate a change to the pleated sheet structure proposed by Pauling and Corey (1951). On the basis of this evidence alone, however, their model cannot be ruled out. As a result of the diffuseness in the x-ray patterns it is even more difficult to judge whether changes in degree of crystallinity occur, as required by Flory's model (1956a,b, 1957). Although Mandelkern *et al.* (1959) interpreted their results as showing a definite change to amorphous protein, Pollack's results (1960) do not warrant such conclusions.

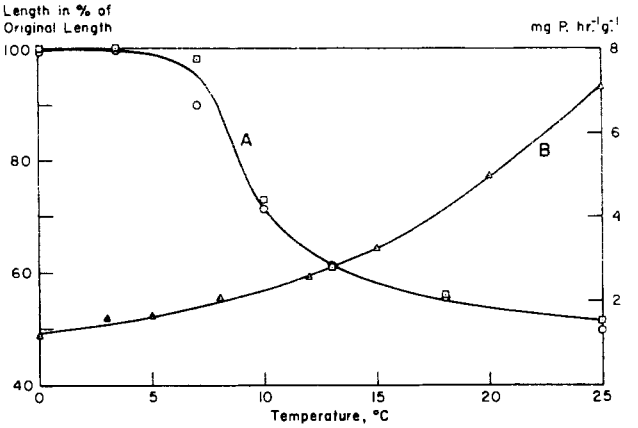

FIG. 6.—Length (curve A) and rate of ATP splitting (curve B) plotted *versus* temperature at constant concentration (32 volume %) of ethylene glycol. □ and ○, Lengths of two samples (left ordinate) after immersion for one hour in Solution 2. The same two samples were used throughout. △, Rate of ATP splitting (right ordinate) for fresh, mashed samples in Solution 2.

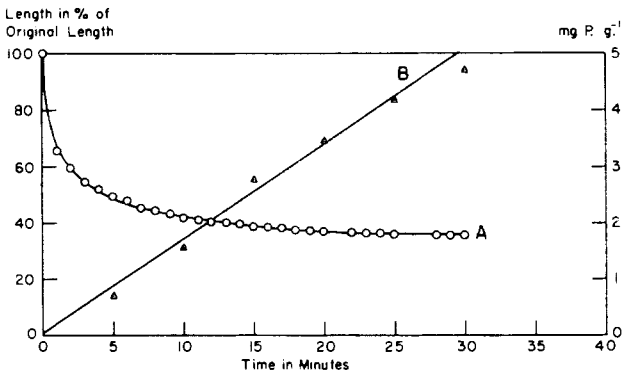

FIG. 7.—Length (curve A) and amount of ATP split (curve B) *versus* time at constant temperature (24.8°) and fixed concentration (20% by volume) of ethylene glycol. ○, Length of the fiber bundle (left ordinate) in Solution 2. △, Amount of ATP split (right ordinate) by the fiber bundle in Solution 2.

On the other hand, ample evidence exists that different polymers, including glycerinated muscle in potassium iodide solutions (Hoeve and Willis, 1963), contract as a result of the transition from an ordered, oriented to a random, amorphous state, as proposed by Flory (1956a,b, 1957). The inability of our muscle samples to elongate spontaneously after contraction, seemingly in disagreement with expectation, is frequently observed for this mechanism. If the density of crosslinks in the oriented, crystalline state is low, the transition to an amorphous phase is accompanied by a large decrease in length; recrystallization from this phase under zero tension is, however, mainly unoriented and is therefore not reflected in a correspondingly large increase in length. In contrast, the failure to reelongate is difficult to explain with Pauling and Corey's model (1951), in which both crystalline phases are oriented. Although more experimental evidence is required, we conclude that the results described in this paper are in complete harmony only with the mechanism proposed by Flory (1956a,b, 1957).

We can only speculate on the role played by ATP,

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

WALTER N. SHAW AND ELDON W. SHUEY

From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

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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.