infants delivered by cesarean section, three developed RDS (5.3 per cent), while of 197 living infants delivered vaginally, seven developed RDS (3.5 per cent). This difference is not statistically significant.

RDS represents the "sound barrier" in the care of the pregnant diabetic, and presently makes the greatest contribution to perinatal loss. The fact that seven of the ten cases of RDS occurred in complicated pregnancies has already been noted. These infants were reasonably mature by weight and dates and yet succumbed. Does this support the view that RDS is begotten in utero? If so, then it would appear that the greatest single contribution to be made to the reduction of perinatal loss lies in the prevention of toxemia and hydramnios.

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REFERENCES

1 Kyle, G. C.: Diabetes and pregnancy. Ann. Intern. Med. 59:Suppl. 3, p. 27, July, 1963.

2 White, P.: Pregnancy complicating diabetes. Amer. J. Med. 7:609, 1949.

3 Jones, W. F.: Management of the pregnant diabetic. Diabetes 7:6:439, 1958.

4 Pedersen, J.: Fetal mortality in diabetic pregnancies. Diabetes 3:199, 1954.

5 Hagbard, L.: Pregnancy and Diabetes Mellitus. Springfield, Ill., Charles C Thomas, 1961, p. 4.

6 White, P.: Pregnancy and diabetes. Medical aspects. Med. Clin. N. Amer. 49:4:1015, July, 1965.

7 Anderson, B.: Diabetes and pregnancy. Acta Med. Scand. 138:259, 1950.

8 Gellis, S. S., and Yi-Yung Hsia, D.: The infants of the diabetic mother. Amer. J. Dis. Child. 97:1, 1959.

9 Harley, J. M. G., Montgomery, D. A. D.: Management of pregnancy complicated by diabetes. Brit. Med. J. 1:14-18, 1965.

10 Streeter, G. L.: Weight; sitting height; head size; foot length, and menstrual age of human embryo. Contributions to Embryology 11:143, 1920.

BRIEF NOTES AND COMMENTS

Effect of Antagonistic Albumin on Insulin-stimulated Intracellular Metabolic Pathways

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SUMMARY

Previous data on incorporation of glucose C-14 into glycogen by the rat diaphragm had suggested that this synthetic pathway was unaffected by the albumin-associated insulin antagonist. To test the hypothesis that only the transport functions of insulin were blocked by this antagonist, studies on two insulin-stimulated intracellular pathways independent of transport were carried out. The increase in tissue levels of the I form of glycogen synthetase seen with insulin was not affected by antagonistic albumin (human fraction V). The D form of glycogen synthetase was also unaffected. However, insulin-stimulated adenine-8-C-14 incorporation into RNA was completely abolished by this albumin. A nonantagonistic albumin (bovine fraction V) by itself increased RNA synthesis indicating that the inhibition of insulin-stimulated RNA synthesis was not a general property of the albumin molecule. The physical-chemical and metabolic characteristics of the albumin-associated antagonist are summarized and are considered to be incompatible with the postulated physiological role for this insulin antagonist. DIABETES 15:835-38, November, 1966.

We recently published a paper[1] in this journal on the mechanism of insulin antagonism by albumin on rat diaphragm. These studies indicated that antagonistic albumin was bound to the diaphragm and blocked insulin-stimulated transport of glucose and AIB in a noncompetitive manner. Data on glycogen synthesis suggested that this pathway was relatively unaffected by the albumin antagonist. Thus: (1) Although human fraction V by itself depressed basal glucose uptake slightly, it increased glucose-C-14 incorporation into glycogen by 118 per cent; (2) in spite of a marked reduction of insulin-stimulated glucose uptake by albumin, 92 per cent of this decreased uptake was channelled into glycogen synthesis; and (3) although glycogen synthesis was stimulated

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by albumin alone, insulin was still able to increase it another 46 per cent in the presence of the antagonist.

Since insulin not only activates glycogen synthetase[2] but also increases RNA synthesis[3,4] independent of its effect on glucose transport, these two parameters were studied to determine the effect of the albumin antagonist on insulin-stimulated intracellular pathways. A measure of the tissue levels of glycogen synthetase would also furnish direct evidence that the glycogen synthetic pathway is unaffected by the antagonist.

MATERIALS AND METHODS

Although the method of incubation of the diaphragms has been detailed in a previous publication,[5] minor modifications were necessary in these studies. Glucose uptake was expressed as μg glucose/mg. diaphragm (wet wt.)/length of incubation.

RNA synthesis

In these experiments, the diaphragms were incubated for two hours with neither a preincubation period nor removal from the original flask. Each incubation flask contained 1 μc of adenine-8-C-14* (5.4-7.5 x 10^5 cpm). One of each pair of hemidiaphragms was incubated in Krebs-Ringer bicarbonate buffer (KRB) containing 2 mg. of glucose per ml. (B) or with added insulin,† 0.1 U./ml. (I). Albumin in a final concentration of 5 gm. per cent was added to some flasks (designated A and A + I, respectively). All four types of flasks were included in any one experiment to minimize day to day variation.

After the incubation period, the hemidiaphragms were quickly washed in cold KRB, blotted, weighed and homogenized in cold 10 per cent trichloroacetic acid (TCA). The nucleic acid fraction was isolated by a modification of the method of Schneider.[6] The centrifuged precipitate was washed three separate times in cold 10 per cent TCA (with at least five minutes of exposure of the tissue to the TCA each time) and extracted twice with 1.0 ml. of 5 per cent TCA at 95-100°. Two hundred lambda aliquots of the combined supernatants were dried on filter paper and counted in a liquid scintillation detector. The results were expressed as cpm/100 mg. diaphragm (wet wt.)/10^5 counts of standard. There was no incorporation of radioactivity in zero time control diaphragms.

*Purchased from New England Nuclear Corporation.

†Kindly supplied by Dr. O. K. Behrens of Eli Lilly and Company.

TABLE 1

Glycogen synthetase (I Form)

	Human fraction V							
	B	I	Δ	n	A	A+I	ΔH	n
Glucose uptake μg/mg./30 min.	1.7	3.2	1.5*	14	1.4	1.9	0.5*	14
±S.E.M.	±0.1	±0.2	±0.2	—	±0.1	±0.2	±0.1	—
Enzyme activity	†	†	—	—	‡	‡	—	—
cpm/mg./10^5 cts. std.	2,881	3,193	312	14	3,127	3,499	372	14
±S.E.M.	±206	±255	±118	—	±141	±207	±160	—

*$p<.001$ for Δ vs. ΔH using *t* test for difference between means.

†$p<.025$ for I vs. B using *t* test for difference between paired observations.

‡$p<.05$ for A + I vs. I using *t* test for difference between paired observations.

Glycogen synthetase

The incubations were carried out as above, except: (1) There were no radioactive substances in the media; (2) The insulin concentration was 500 μU/ml.; and (3) The incubation was terminated after thirty minutes. The hemidiaphragms were removed, quickly blotted, weighed and homogenized in 1.0 ml. of 0.25M sucrose-0.01M EDTA. Glycogen synthetase (both D and I forms) were determined by a slight modification of the method of Robbins et al.[7] Fifty lambda of the undiluted homogenate supernatant were added to a reaction mixture such that the final volume of 0.5 ml. of glycylglycine buffer (0.05M, *p*H 7.5) contained 0.01M $MgCl_2$, 1 per cent glycogen, 0.001M uridine diphosphate glucose (UDPG) and 0.2 μc. uridine diphosphate glucose C-14 (U.L.)* (2.4-3.7 × 10^5 cpm). Glucose-6-phosphate was present in a final concentration of 0.02M for the measurement of the D form of glycogen synthetase. The reaction tubes were incubated for twenty minutes at 30° C. without preincubation. A two hundred lambda aliquot of the final glycogen

*Purchased from New England Nuclear Corporation.

TABLE 2

RNA synthesis

					Human fraction V				Bovine fraction V			
	B	I	Δ	n	A	A+I	ΔH	n	A	A+I	ΔB	n
Glucose uptake (μg/mg./2 hrs.)	3.8	10.3	6.5*	12	3.1	6.9	3.8*	13	4.8	9.9	5.1	7
±S.E.M.	±0.2	±0.6	±0.6	—	±0.2	±0.3	±0.3	—	±0.3	±0.4	±0.3	—
Adenine C-14 incorporation (cpm 100 mg./10^5 cts. std.)	798†	931†	133*	12	756	738	—18*	13	1,145	1,095	—50	7
±S.E.M.	±46	±40	±35	—	±35	±25	±26	—	±49	±61	±33	—

*$p<.005$ for Δ vs. ΔH using *t* test for difference between means.

†$p<.005$ for I vs. B using *t* test for difference between paired observations.

solution was dried on filter paper and counted in a liquid scintillation detector. The results were expressed as cpm/mg. wet diaphragm/10^5 counts of standard.

Albumins

Antagonistic (human fraction V, Pentex) and nonantagonistic (bovine fraction V, Pentex) albumin[5] were dissolved directly into buffer without prior dialysis. Their concentrations were determined by the biuret method.

RESULTS

Glycogen synthetase

The first row of table 1 demonstrates that human fraction V significantly reduced insulin-stimulated glucose uptake over the thirty minute period of incubation. However, the second row shows that the insulin-stimulated activation of glycogen synthetase (I form) was unaffected by antagonistic albumin. Although this form of glycogen synthetase was higher in diaphragms incubated with albumin, these differences were not statistically significant because of rat to rat variation. The amount of the D form of glycogen synthetase in diaphragms incubated with human fraction V alone was no different from the amount in those tissues incubated without albumin (5274 vs. 5184 cpm/mg./10^5 cts std, n = 29).

RNA synthesis

The first row of table 2 demonstrates that human fraction V significantly reduced insulin-stimulated glucose uptake over the two-hour period of incubation. The second row shows that insulin-stimulated RNA synthesis was completely inhibited by this antagonistic albumin. Control experiments with bovine fraction V (the nonantagonistic albumin) were carried out to determine if this inhibition of RNA synthesis were a nonspecific effect of any albumin preparation. Since bovine fraction V alone stimulated RNA synthesis ($p < .001$),* the inhibition of the insulin effect by human fraction V was not a general property of the albumin molecule.

DISCUSSION

Glycogen synthetase has been shown to exist in two forms.[2] The I form is independent of glucose-6-phosphate and is stimulated by insulin. The D form is dependent on glucose-6-phosphate, is present in higher concentrations but does not respond to insulin. The two forms of the enzyme are interconvertible, the I→D reaction requiring Mg^{++} and ATP while inorganic phosphate is given up in the D→I reaction.[8] These results confirm the stimulation of the I form by insulin and the higher tissue levels of the D form. They further demonstrate that antagonistic albumin does not affect either form of glycogen synthetase. This furnishes direct evidence that the intracellular glycogen synthetic pathway is not depressed by the antagonist.

The increase in incorporation of adenine-8-C-14 into RNA in the presence of insulin was completely abolished by human fraction V. Since the transport of adenine into the muscle cell is independent of insulin,[9] at least one insulin-stimulated intracellular pathway is inhibited by the albumin antagonist.

Although the B chain of insulin has been suggested as the albumin-associated antagonist,[10] to date there are no *specific* physical-chemical or immunological criteria for identifying it. It can be removed from albumin by boiling for five minutes, by extracting with chloroform, ethanol, or glacial acetic acid in iso-octane,[11] and by passage through either cellulose[12] or Sephadex.[13] Recent observations[10] indicate that the antagonist is thermostable, that after dissociation from albumin by heat and extremes of *p*H, it behaves as a molecule of molecular weight under 4,000 on Sephadex G-25, and that it is cationic in nature since it is retained on Dowex 50 and eluted by a formate buffer of *p*H 3.1. In vitro, the albumin antagonist is firmly bound to the rat diaphragm,* inhibits glucose transport in a noncompetitive manner, blocks AIB transport and RNA synthesis, but does not depress the glycogen synthetic pathway.[1] The antagonist has also been shown to decrease insulin-stimulated lactic acid production[14,15] and lipid synthesis[15] in rat diaphragm but these effects can not be separated from its primary action on glucose transport. Oxygen consumption by the rat diaphragm is also diminished by the antagonist.[16] And finally, the albumin antagonist does not inhibit insulin-stimulated glucose uptake of adipose tissue.[17,18] Although no specific method of identification of the albumin-associated antagonist is yet available, many of its physical-chemical and metabolic properties have been elucidated.

The pattern of effects exhibited by antagonistic albumin, particularly on RNA synthesis, the noncompetitive inhibition of glucose transport and the depression of oxygen consumption, cast doubt on its postulated physiological role. In addition, the previously reported variability in the antagonistic activity of albumins prepared by slightly different technics and the frequent appearance of insulin-like stimulation by albumins suggest that preparative artifacts may either induce or mask antagonism. Recent studies in this laboratory attempting to demonstrate an effect of antagonistic albumin on carbohydrate metabolism in the whole organism under a variety of conditions have been completely negative.[19] Taken together, the present evidence suggests that the antagonist associated with albumin does not qualify as a physiologically active principle.

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REFERENCES

1 Davidson, M. B., and Goodner, C. J.: Studies on the mechanism of insulin antagonism by albumin in rat diaphragm. Diabetes *15*:373-79, June 1966.

2 Villar-Palasi, C., and Larner, J.: Insulin treatment and increased UDPG-glycogen transglucosylase activity in muscle. Arch. Biochem. Biophys. *94*:436-42, 1961.

*Using *t* test for differences between means.

*As previously discussed,[1] an alternative explanation for the reduction of insulin effect on diaphragms pre-exposed to antagonistic albumin is an irreversible alteration of the diaphragms' response to insulin without binding.

[3] Wool, I. G.: Insulin and incorporation of radioactivity into nucleic acid fractions of isolated diaphragm. Amer. J. Physiol. *199*:719-21, 1960.

[4] Wool, I. G.: Effect of insulin on nucleic acid synthesis in isolated rat diaphragm. Biochim. Biophys. Acta. *68*:28-33, 1963.

[5] Davidson, M. B., and Goodner, C. J.: Assay of insulin antagonism by serial incubation of paired rat hemidiaphragms. Diabetes *15*:380-87, June 1966.

[6] Schneider, W. C.: Methods in Enzymology, Vol. 3, New York, Academic Press, 1957, p. 680.

[7] Robbins, P. W., Traut, R. R., and Lipmann, F.: Glycogen synthesis from glucose, glucose-6-phosphate, and uridine diphosphate in muscle preparations. Proc. Nat. Acad. Sci. *45*: 6-12, 1959.

[8] Friedman, D. L., and Larner, J.: Interconversion of two forms of muscle UDPG-α-transglucosylase by a phosphorylation-dephosphorylation reaction sequence. Biochim. Biophys. Acta. *64*:185-86, 1962.

[9] Wool, I. G., and Munro, A. J.: An influence of insulin on the synthesis of a rapidly labeled RNA by isolated rat diaphragm. Proc. Nat. Acad. Sci. 50:918-23, 1963.

[10] Ensinck, J. W., Mahler, R. J., and Vallance-Owen, J.: Antagonism of insulin action on muscle by the albumin-bound B chain of insulin. Biochem. J. *94*:150-58, 1965.

[11] Vallance-Owen, J., and Lilley, M. D.: An insulin antagonist associated with plasma-albumin. Lancet *1*:804-06, 1961.

[12] Vallance-Owen, J., Dennes, E., and Campbell, P. N.: The nature of the insulin-antagonist associated with plasma albumin. Lancet *2*:696, 1958.

[13] Recant, L., and Alp, H.: Insulin inhibition and adipose tissue metabolism. Ann. N.Y. Acad. Sci. *131*:334-43, 1965.

[14] Buse, M. G., and Buse, J.: Insulin antagonism associated with albumin. Fifth Congress of the International Diabetes Federation. Abstract No. 336, p. 169, 1964.

[15] Unpublished observations.

[16] Davidson, M. B., and Goodner, C. J.: Effects of insulin antagonistic albumin on transport, glycogen synthetase, RNA synthesis and QO_2 in rat diaphragm. Clin. Res. *14*:176, 1966.

[17] Lowy, C., Blanshard, G., and Phear, D.: Antagonism of insulin by albumin. Lancet *1*:802-04, 1961.

[18] Alp, H., and Recant, L.: Effect of the insulin-inhibitory albumin fraction from normal and diabetic subjects on adipose tissue. Metabolism *13*:609-19, 1964.

[19] Davidson, M. B., and Goodner, C. J.: The failure of synalbumin to demonstrate insulin antagonism in vivo. Diabetes (Submitted).

Effect of Acute Muscular Exercise on Serum Immunoreactive Insulin Concentration

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SUMMARY

The change in circulating levels of immunoreactive insulin (IRI) was studied in a group of six normal, three obese, and eight obese diabetic subjects undergoing the acute exercise of stair climbing. Bloods were sampled immediately before and after the exercise period. Serum IRI concentration decreased in ten subjects and did not increase in the remainder of the subjects. The data suggest an exercise metabolite rather than increased insulin secretion accounts for the enhanced glucose assimilation produced by muscular activity. DIABETES *15*:838-41, November, 1966.

It has been demonstrated that the energy expenditure of muscular exercise is accomplished by utilization of glucose as a part of the source of fuel in the laboratory animal[1-4] and in man,[5,6] and that prolonged and severe exercise may produce hypoglycemia.[7] The precise role of body insulin in the metabolism of carbohydrate during exercise has, however, not been defined.

It has been reported that serum insulin-like activity (ILA) as assayed on the rat diaphragm is diminished by muscular activity.[8] In the present study we have utilized a more specific assay for immunoreactive insulin (IRI) to study the effect of acute muscular exercise on serum insulin concentrations in human subjects.

MATERIAL AND METHODS

Ambulatory normal, as well as hospitalized obese subjects, both diabetic and nondiabetic, were studied. All participants fasted overnight for ten to fourteen hours and rested at least fifteen minutes prior to the beginning of exercise. No hypoglycemic or other medication was being used by the group. Serum concentrations of glucose, insulin, lactate and pyruvate were determined on venous blood obtained by the method of Friedemann[9] immediately before and after the acute exercise study. Exercise consisted of climbing up and down six ¾-inch steps, at a rate of seventy to 100 steps per minute.

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