Effect of Insulin on the Formation of Glycogen by the Mouse Diaphragm in the Presence and Absence of Glucose

A Preliminary Report

A. J. Moody, M.A., and J. P. Felber, M.D., Lausanne, Switzerland

SUMMARY

The incubation of mouse hemidiaphragms in Krebs' buffer containing insulin, but no glucose, results in a stimulation of the formation of glycogen by the tissues; this stimulation is proportional to the concentration of insulin in the range 0 to 100 μU./ml.

The glycogen formed by mouse hemidiaphragm during incubation in buffer which contains insulin and (U. C-14) glucose, consists of a labeled and an unlabeled component. The total glycogen, the labeled glycogen and the unlabeled glycogen are all increased by an increase in the concentration of insulin in the incubation medium. Partial fractionation on a column of Sephadex G-200 of glycogen formed by hemidiaphragms incubated in the presence of (U. C-14) glucose with or without insulin, reveals that glucose is incorporated in the smaller glycogen molecules, whereas the larger glycogen molecules are, to a large extent, derived from an unlabeled precursor. DIABETES *15*:492-96, July, 1966.

Insulin increases the penetration of glucose from the medium into the perfused rat heart,[1] the diaphragm,[2,3] and the epididymal fat pad[4] in vitro. The presence of insulin in the incubation medium also increases the formation of glycogen, and the incorporation of glucose carbon into glycogen, by both the diaphragm[5] and the fat pad[6] in vitro. Incubation of the fat pad with insulin also increases the net gas exchange,[7] the liberation of C-14-O_2 from the carbon of (C-14) glucose (and in particular from C-1[6]) and the incorporation of glucose into fatty acids.[6] Intraperitoneal injection of insulin into the rat,[5] or mouse,[9] evokes increased formation of glycogen[8] and incorporation of (C-14) glucose into glycogen by both the diaphragm and the epididymal fat pad.[8]

A mechanism for the enzymatic synthesis of glycogen in vivo has been suggested by Leloir,[10] according to which the enzyme glycogen synthetase (UDPG-α-glucan transglucosylase) adds glucose, derived from glucose-1-phosphate, to a primer molecule of glycogen. The enzyme is readily extracted from muscle for the examination of its properties in vitro. Incubation of rat muscle with insulin, prior to the extraction of the glycogen synthetase, produces an enzyme of higher activity in vitro than the enzyme prepared from muscle which has not been incubated with insulin.[11] This apparent stimulation is occasioned by the conversion, during incubation of the muscle with insulin, of glycogen synthetase from a form requiring glucose-6-phosphate for activity to a form whose activity is independent of glucose-6-phosphate.[12] The conversion of one form of the enzyme to another is not dependent on the presence of glucose in the medium in which the muscle is incubated,[13] and the conversion involves a phosphorylation-dephosphorylation reaction sequence.[14]

The multiple effects of insulin on the carbohydrate metabolism of tissues, both in vivo and in vitro, cannot readily be explained if the sole action of insulin is to enhance the translocation of glucose from the interstitial fluid into the cells. In particular, the effect of insulin on the form of glycogen synthetase, which is independent of the presence of glucose, is inexplicable in terms of such a mode of action.

The investigations reported were undertaken to study the effects of the balance between the concentration of insulin and of glucose on the utilization of glucose by the mouse hemidiaphragm, and the rat epididymal fat pad in vitro.

It is found that on increasing the glucose concentration in the medium, the formation of glycogen and the

From the Clinique Medicale Universitaire, Lausanne, Switzerland.

Mr. Moody was winner of the 1965 Research Contest of the American Diabetes Association. This is his prize-winning paper. His present address is Novo Research Institute, Copenhagen, Denmark.

incorporation of glucose from the medium into glycogen are increased; at all levels of glucose tested insulin enhances these effects. Insulin also stimulates the formation of glycogen when incubations are carried out without glucose. Partial fractionation of glycogen suggests that two components of glycogen are affected by insulin. A fraction of glycogen, composed of the larger molecules, is not derived from glucose entering the tissues and is increased by the addition of insulin to the medium of incubation. The smaller molecules of glycogen are derived from glucose entering the tissues; this fraction is also increased by insulin.

MATERIALS AND METHODS

Hemidiaphragms were obtained from fasted male mice of between 10 and 20 gm.; for each experiment, the mice were chosen from a weight range of not more than 2 gm. The tissues were pre-incubated for ten minutes in Krebs' bicarbonate buffer, *p*H 7.4, containing anti-insulin serum equivalent to 50 μU.* of insulin per milliliter, to deplete endogenous insulin and glucose. Incubation media were prepared by adding insulin and glucose (or U. C-14 glucose) to Krebs' buffer containing 2 mg. of gelatin per milliliter. Duplicate flasks, each containing three hemidiaphragms, were prepared for each medium in some experiments and single flasks, each containing four tissues, were prepared for testing the response of the hemidiaphragms to several dilutions of insulin. The tissues were randomly allocated to the incubation media; the allocation was determined by drawing numbered cards from a shuffled pack. Incubations were carried out for one hour at 37° C.

After incubation the tissues were removed from the flasks, rinsed with buffer, blotted, weighed and dropped into separate centrifuge tubes containing 1 ml. of 30 per cent KOH. After heating for fifteen minutes at 100° C., the tubes were cooled and 1.2 ml. of 95 per cent alcohol added to each tube. The samples were placed at —20° C. for one hour and then centrifuged at +4° (35 min. at 1,400 x g) to precipitate the glycogen. The supernatants were rejected, the glycogen fractions dissolved in 2.5 ml. of water and analysed for glycogen (by the anthrone method[15]) and for radioglucose (by liquid scintillation counting using Bray's[16] scintillator).

Epididymal fat pads were obtained from fed rats of between 150 and 200 gm. The incubation media were prepared as for the diaphragms except that the gelatin was replaced by 20 mg. of albumin (freed from FFA[17]) per milliliter. The pads were sectioned and distributed in the incubation media according to Renold et al.[18] and the glycogen prepared from the tissues after an incubation of two hours at 37° C.

The results of the above determinations are presented as mμ moles of glucose (determined as glycogen), or mμ moles of (U. C-14) glucose, in the glycogen fractions per milligram wet tissue per hour of incubation. The glucose in the glycogen was calculated by reference to standards of glycogen whose glucose content had been determined by hydrolysis in 2 N H_2SO_4 followed by measurement of the glucose liberated. The data were then subjected to an analysis of variance according to Wardaw and Van Belle.[19]

Glycogen, prepared from groups of diaphragms incubated in buffer which contained (U. C-14) glucose with or without insulin, was fractioned by passing 2-ml. aliquots over a column (2 cm. x 48 cm.) of Sephadex G-200 in 1 per cent Na_2SO_4. The columns were eluted with 1 per cent Na_2SO_4, 2 ml. fractions collected and each fraction analysed for glycogen and radioglucose.

RESULTS

Table 1 (A) presents the results of an experiment in which diaphragms were incubated in media containing different concentrations of glucose, each concentration of glucose being combined with 0 and 125 μU. of insulin per milliliter. The glycogen in the diaphragms rises with increasing glucose concentration; at all levels of glucose used, including zero, insulin stimulates glycogen formation.

Table 1 (B) contains the results of a complementary experiment in which the diaphragms were incubated with three levels of insulin, each with and without added glucose. Increasing the concentration of insulin increases the formation of glycogen, in either the presence or the absence of glucose in the medium.

Table 2 (A) shows the response of hemidiaphragms to increasing concentrations of insulin when the incubations are carried out in the presence of (U. C-14) glucose. The glycogen, the incorporation of exogenous glucose into the glycogen and the glycogen which is not accounted for by the radioglucose incorporated increase with increasing concentration of insulin.

Table 2 (B) summarizes a similar experiment carried out with the epididymal fat pad; the glycogen, radioglucose incorporated into glycogen and the glycogen not accounted for by the incorporated radioglucose all increase with increasing insulin.

Crystalline beef insulin was the gift of Wellcome Research Ltd.

TABLE 1

The influence of the concentrations of insulin and of glucose on the formation of glycogen by the mouse diaphragm in vitro*

Total glycogen (as mμ moles of glucose/mg. wet tissue)

A.

Glucose in medium (mM)	Insulin in medium (μU./ml.) 0	125
0.0	4.87	14.91
5.4	7.04	17.85
11.4	10.55	19.47
17.1	14.53	23.74
D† = 2.57		

B.

Insulin in medium (μU./ml.)	Glucose in medium (mM.) 0	14.4
0.0	5.95	6.60
50.0	11.25	13.20
100.0	17.15	38.00
D† = 2.54		

*The glycogen content of unincubated tissues is 3.55 ± 0.26mμ moles of glucose/mg. wet tissue (mean ± S.D. for 40 tissues). The values are the means of six tissues.

†D is the minimum difference between means for the responses to be significantly different at p = 0.01.

TABLE 2

The influence of the concentration of insulin on the formation of glycogen and the incorporation of exogenous glucose into glycogen by the mouse diaphragm and the rat fat pad in vitro*

A.

Incubations with diaphragms

Insulin (μU./ml.)	Glycogen composition: Total glycogen	(U. C-14) glucose incorporated	Unlabeled glycogen
5	10.9	2.5	8.3
25	15.0	4.5	10.5
50	26.6	12.9	13.7
100	35.3	13.9	21.4
Glucose was 16.2 mM.			
D (p = 0.01)	4.0	1.9	7.2

The values are the means of four diaphragms.

B.

Incubations with epididymal fat pads

0	1.16	0.04	1.12
62	1.91	0.13	1.84
125	3.42	0.16	3.19
Glucose was 18 mM			
D (p = 0.05)			

The values are the means of three tissues

*All values are in mμ moles of glucose/mg. wet tissue.

TABLE 3

The effect of insulin on the formation and composition of glycogen by the mouse diaphragms in vitro*

Insulin (μU./ml.)	Glycogen composition: A. With 12.6 mM. (U. C-14) glucose: Total glycogen	(U. C-14) glucose incorporated	Unlabeled glycogen	B. Without glucose: Total glycogen
0	26.2	17.1	8.7	3.0
5	38.0	28.4	9.4	4.6
25	42.9	29.6	13.3	20.5
50	69.7	37.3	33.0	31.5
100	91.5	38.5	52.3	53.9
D	20.0	5.0	17.7	20.0

*All values are in mμ moles of glucose, (or of (U. C-14) glucose), per mg. wet tissue per hour of incubation. The data are the means of the results obtained by repeating the same experiment on two different days; five different concentrations of insulin were prepared in Krebs' buffer and in Krebs' buffer containing (U. C-14) glucose (12.6 mM, 40μ C./m. mole), and four tissues incubated in each incubation medium. The results of the two experiments were then combined, and subjected to an analysis of variation.

The difference between the results of the two different experiments is not significant at p = 0.05. The differences between the mean responses to the various concentrations of insulin are significant at p = 0.05 for the pairs 0 and 5 μU./ml., and 5 μU./ml., and 25 μU./ml. The remaining differences between mean responses are significant at p = 0.001.

The data shown in table 3 were obtained by combining the results of two experiments, carried out on separate days, in which diaphragms were incubated at five concentrations of insulin, each with and without (U. C-14) glucose. The glycogen is increased by insulin, in either the presence or absence of glucose, and this increase is proportional to the concentration of insulin in the range of concentration 0 to 100 μU. of insulin per milliliter. The radioglucose incorporated into glycogen is also increased by increasing the concentration of insulin, as is the difference between the total glycogen and the glycogen accounted for by the incorporation of radioglucose.

These results show clearly that insulin stimulates the formation of glycogen by the mouse diaphragm in vitro, this stimulation is statistically valid and occurs with or without glucose in the medium of incubation. In the presence of (U. C-14) glucose, not all the glycogen formed by the diaphragms is accounted for by the glucose incorporated, and the amount which is unlabeled increases with increasing insulin. Insulin would then appear to stimulate two modes of glycogen synthesis; the first involves the direct incorporation of exogenous glucose into glycogen, the second the incorporation of an endogenous compound into glycogen. Both routes of synthesis are active when both insulin and glucose are

TABLE 4

Analysis of the incorporation of radioglucose into the glycogen formed by diaphragms incubated with and without insulin*

Fraction No.	Glycogen formed by tissues incubated with glucose		Glycogen formed by tissues incubated with glucose and 125 μU. of insulin/ml.	
	Total glucose	(U. C-14) glucose	Total glucose	(U. C-14) glucose
1-7	0	0	0	0
8	20	12	0	0
9	88	76	130	28
10	130	102	220	105
11	25	12	94	86
12	13	10	84	61
13	10	10	50	29
14	10	3	18	16
15	10	4	6	7
16	5	4	10	4
Recovery by percentage	66	73	70	62

*The media for both incubations contained 12.6 mM. (U. C-14) glucose. 2-ml. fractions of the glycogen obtained from 10 hemidiaphragms were added to a 2 x 48 cm. column of Sephadex G-200 in 10 mg. Na_2SO_4/ml. The column was eluted with 10 mg./ml. Na_2SO_4 and 2-ml. fractions collected and analyzed for glycogen and radiocarbon contents.

The data are mμ moles of glucose (determined as glycogen) or mμ moles of (U. C-14) glucose/fraction.

available, and the latter functions in the absence of glucose.

The results of the partial fractionation of glycogen on columns of Sephadex G-200 are contained in table 4. Diaphragms incubated in buffer containing glucose alone produce a glycogen which gives nearly superimposable peaks of radioactivity and of glycogen. On the other hand, glycogen isolated from diaphragm incubated with glucose and insulin is separable into two zones, the larger molecules are associated with but little radioactivity and the smaller molecules which are associated with the bulk of the radioactivity. Insulin then facilitates the formation of the high molecular weight glycogen from nonradioactive precursors.

DISCUSSION AND CONCLUSIONS

The evidence presented here shows that insulin alone stimulates the formation of glycogen (defined as the polysaccharide which is precipitated by 52 per cent alcohol from a KOH extract of the tissues) by the mouse diaphragm in vitro. The stimulation is proportional to the concentration of insulin in the medium in the range 0 to 100 μU. of insulin per milliliter. This stimulation could occur in two ways; insulin may stimulate glycogen synthetase as described by Villar-Palasi and Larner,[11] thereby stimulating the formation of glycogen from endogenous glucose-1-phosphate, or insulin may stimulate the formation of glycogen from a precursor which is, as yet, unknown. The results obtained with the Sephadex fractionation suggest that insulin favors not only the incorporation of glucose into glycogen, but also the appearance of high molecular weight glycogen which is not derived from glucose entering the tissue. This high molecular weight fraction may correspond to the unlabeled glycogen which is formed by the diaphragms when they are incubated with insulin and radioglucose. The increase in glycogen synthesis obtained by increasing the concentration of glucose in the medium is not dependent on the presence of insulin in the tissues; glucose would then seem to be directly incorporated into the hemidiaphragm in vitro in proportion to the glucose entering the tissue. Insulin, as has been shown here and elsewhere,[5] stimulates this incorporation as well as the incorporation of an endogenous precursor into glycogen. Villee and Hastings[20] observed differences between total glycogen and that derived from labeled glucose; our results (table 2) confirm these earlier findings.

On the evidence presented here, it is not yet possible to decide if insulin stimulates glycogen synthesis by two pathways, one from glucose entering the tissue and the other from an endogenous precursor which is not directly derived from glucose, or whether the experimental findings can be explained by assuming that a pool of glycogen precursor (which equilibrates with a derivative of glucose) is converted to glycogen in increasing quantities by the action of insulin.

Certain well-established effects of insulin, for example the stimulation of amino-acid incorporation into protein,[21] the conversion of the form of glycogen synthetase[13] and, as is reported above, the stimulation of glycogen synthesis, are not dependent on the presence of glucose in the medium in which the tissues are incubated. The question now arises whether the enhancement of hexose translocation by insulin represents a primary effect of insulin or whether this effect is secondary to the effects which can be demonstrated in the absence of glucose.

A further possibility is that the effects of insulin which have been characterized are all secondary to a single interaction of insulin with either the structure or the metabolism of the cell.

Further studies are in progress to characterize the glycogen formed in the presence and absence of glucose.

ACKNOWLEDGMENT

This research was supported by Grant No. 3279 from the Swiss National Funds for Scientific Research.

REFERENCES

[1] Bleehen, N. M., and Fisher, R. B.: The action of insulin in the isolated rat heart. J. Physiol. *123*:260, 1954.

[2] Gemmill, C. L.: The effects of glucose and of insulin on the metabolism of the isolated diaphragm of the rat. Bull. Johns Hopkins Hosp. *68*:329, 1941.

[3] Gemmill, C. L., and Hamman, L.: The effect of insulin on glycogen deposition and on glucose utilization by isolated muscles. Bull. Johns Hopkins Hosp. *68*:50, 1941.

[4] Krahl, M. E.: The effect of insulin and pituitary hormones on glucose uptake in muscle. Ann. N.Y. Acad. Sci. *54*:649, 1951.

[5] Moody, A. J., and Felber, J.-P.: A diaphragm bioassay for the measurement of total insulin-like activity and of antigenic insulin in serum. Experientia (Basel) *20*:105, 1964.

[6] Cahill, G. F., Jr., Leboeuf, B., and Renold, A. E.: Studies on rat adipose tissue in vitro. III. Synthesis of glycogen and glyceride-glycerol. J. Biol. Chem. *234*:2540, 1959.

[7] Ball, E. G., Martin, D. B., and Cooper, O.: Studies on the metabolism of adipose tissue. I. The effect of insulin on glucose utilization as measured by the manometric determination of carbon dioxide output. J. Biol. Chem. *234*:774, 1959.

[8] Rafaelsen, O. J., Lauris, V., and Renold, A. E.: Localized intraperitoneal action of insulin on rat diaphragm and epididymal tissue in vivo. Diabetes *14*:19, 1965.

[9] Moody, A. J., and Felber, J.-P.: A bioassay for insulin using the in situ mouse diaphragm. Experientia (Basel) *20*: 646, 1964.

[10] Leloir, L. F., and Cardini, C. E.: Biosynthesis of glycogen from uridine diphosphate glucose. J. Amer. Chem. Soc. 79: 6340, 1957.

[11] Villar-Palasí C., and Larner, J.: Insulin-mediated effect on the activity of UDPG-glycogen transglucosylase of muscle. Biochim. Biophys. Acta *39*:171, 1960.

[12] Rosell-Perez, M., and Larner, J.: Evidence for two forms of UDPG-glycogen transglucosylase activity in muscle. Federation Proc. *20*:193, 1961.

[13] Villar-Palasí, C., and Larner, J.: Insulin treatment and increased UDPG-glycogen transglucosylase activity in muscle. Arch. Biochem. Biophys. *94*:436, 1961.

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

[15] Wardlaw, A. C., and Moloney, P. J.: The assay of insulin with anti-insulin and mouse diaphragm. Canad. J. Biochem. Physiol. *39*:695, 1961.

[16] Bray, G. A.: A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. *1*:279, 1960.

[17] Goodman, D. S.: Preparation of human serum albumin free of long-chain fatty acids. Science *125*:1296, 1957.

[18] Renold, A. E., Martin, D. B., Dagenais, Y. M., Steinke, J., Nickerson, R. J., and Sheps, M. C.: Measurement of small quantities of insulin-like activity using rat adipose tissue. I. A proposed procedure. J. Clin. Invest. *39*:1487, 1960.

[19] Wardlaw, A. C., and Van Belle, G.: Statistical aspects of the mouse diaphragm test for insulin. Diabetes *13*:622, 1964.

[20] Villee, C. A., and Hastings, A. B.: Metabolism of C-14-labeled glucose by rat diaphragm in vitro. J. Biol. Chem. *179*:673, 1949.

[21] Sinex, F. M., MacMullen, J., and Hastings, A. B.: The effect of insulin on the incorporation of C-14 into the protein of rat diaphragm. J. Biol. Chem. *198*:615, 1952.

Hypermetabolism and Undernutrition

(*Continued from page* 470)

proved to be —0.74, indicating direct relationship between the degree of growth failure and the degree of hypermetabolism.

Five infants were restudied after surgical repair of the cardiac defect. Oxygen consumption remained the same or increased. This finding would at first glance seem to speak against the hypothesis offered by the authors. However, the defect was completely corrected in only two of the five operated patients, and one of these was gaining weight at the time the second measurement was made. In this connection it is known that malnourished infants exhibit a rise in oxygen consumption during the early period of recovery (R. D. Montgomery, J. Clin. Invest. *41*:1653, 1962).

The study of Lees and co-workers points up another possible reason for growth failure in infants with severe cardiac defects. Hypermetabolism of this degree, in which resting oxygen consumption ranged as high as 160 per cent of normal, could well place an undue metabolic burden on the sick individual. A number of cardiologists, recognizing this phenomenon in their adult patients, have attempted to reduce thyroid function. Such a maneuver could not be seriously entertained for infants, however, in view of the likely effects of thyroid hypofunction on central nervous system development.

From *Nutrition Reviews,* Vol. 24, No. 3, March 1966, pp. 68-69