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The Carbohydrate Metabolism of the Isolated Diaphragm of the Rat. Glucose Uptake and Body Size

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Since Gemmill (1940, 1941) and Gemmill & Hamman (1941) have shown that the isolated diaphragm of the rat is a suitable tool for the experimental study of the glucose uptake and glycogen synthesis by muscle tissue, and demonstrated that these processes are stimulated by *in vitro* addition of insulin many investigators have used the method to study the hormonal control of carbohydrate metabolism. More recently, attempts have been made to estimate small amounts of insulin by that method (Groen, Kamminga, Willebrands & Blickman, 1951–2). The experimental conditions under which reproducible results may be expected have been the subject of several studies (Stadie & Zapp, 1947; Krahl & Cori, 1947; Krahl & Park, 1948), but for some time the stimulation of the glucose uptake by insulin could not be repeated in this laboratory. Similar difficulties were encountered by Perlmutter, Weisenfeld & Mufson (1952). Therefore it was felt necessary to re-examine the effect of some factors on the carbohydrate metabolism of the isolated diaphragm of the rat and on the stimulating effect of insulin on its glucose consumption.

The present paper deals with the relation existing between the size of the rats and the uptake of glucose measured. It was found that the uptake of glucose per g. of tissue was high if small rats were used, and low if large rats were used. Possible explanations are presented. Differences between insulin effects obtained with animals of different sizes could not be demonstrated. A preliminary note has appeared elsewhere (Liébecq, 1953). Later work will show the effect of other factors on the extent of the insulin effect.

METHODS

All rats were obtained from Brofo, Zevenaar, Holland, and were of the Epymus Albino Wistar strain. They were fed a mixture of oat flakes (50%), skimmed dried milk (35%), wheat germ (6%), dried yeast (6%), CaCO_3 (1.5%), NaCl (1%) and cod-liver oil (0.5%) *ad lib.*, supplemented with fresh vegetables. This diet contains 25–27% protein, 5–6% lipid, 60–65% carbohydrate and 5–6% ash. Rats were fasted 20–24 hr. before use.

The animals were killed by decapitation and the two hemidiaphragms were carefully excised and immediately transferred into two Warburg flasks kept in ice both containing 1 ml. of glucose (1.4 mg./ml.) in Krebs & Henseleit's (1932) bicarbonate medium (but with 6 mg. calcium/100 ml. instead of 10). Excess of Novo insulin (5 $\mu\text{g.}$, i.e. approx. 0.1 unit/ml.) free of hyperglycaemic factor was present in one flask only. The flasks were filled with 95% O_2 + 5% CO_2 and incubated for 30 min. at 37° while shaken at 140–145 strokes of approx. 5 cm./min., then cooled. For glucose determination, 0.3 ml. samples (in duplicate) were diluted with 5 ml. water and deproteinized by addition of 0.45 ml. 0.3N- Ba(OH)_2 and 0.40 ml. 0.15M- ZnSO_4 (9 ml. of the Ba(OH)_2 solution added dropwise should neutralize 10 ml. of the ZnSO_4 solution in the presence of 100 ml. water and 10 drops of 0.1% (w/v) phenolphthalein in ethanol). Glucose was determined on 1 ml. of the filtrate, in duplicate, by the method of Nelson (1944) as modified by Somogyi (1952). The hemidiaphragms were blotted on filter paper at the end of the experiment and weighed. The uptake of glucose was calculated in mg./g. of moist tissue/hr.

All solutions were prepared with reagents of analytical grade purity and with glass-distilled water. To remove residual insulin, stainless steel instruments were soaked in a concentrated solution of a commercial preparation of alkaline detergent, pipettes in 2N-NaOH, then washed carefully and dried in an oven at 120–130°. Warburg flasks were boiled in the solution of alkaline detergent for at least 10 min. before washing.

Table 1. *Uptake of glucose by two hemidiaphragms of rats weighing 50–160 g.*

Figures represent the mean $\pm$ S.E.M., the number of experiments being given in parentheses.

Weights of rats (g.)	Without insulin		With insulin	
	Weight of tissue (mg./flask)	Glucose uptake (mg./g./hr.)	Weight of tissue (mg./flask)	Glucose uptake (mg./g./hr.)
50–60	67.4 $\pm$ 1.3 (12)	7.42 $\pm$ 0.64 (12)	68.3 $\pm$ 1.2 (12)	10.37 $\pm$ 0.57 (12)
75–85	91.1 $\pm$ 2.7 (12)	5.55 $\pm$ 0.35 (12)	96.2 $\pm$ 2.8 (12)	8.08 $\pm$ 0.30 (12)
100–110	123.5 $\pm$ 3.4 (12)	4.01 $\pm$ 0.20 (12)	127.0 $\pm$ 3.7 (11)	6.12 $\pm$ 0.18 (11)
125–135	157.9 $\pm$ 4.8 (6)	3.58 $\pm$ 0.18 (6)	159.0 $\pm$ 5.2 (6)	5.08 $\pm$ 0.27 (6)
150–160	189.3 $\pm$ 5.1 (6)	3.33 $\pm$ 0.11 (6)	189.8 $\pm$ 4.9 (6)	4.87 $\pm$ 0.17 (6)

Fig. 1. Uptake of glucose by two hemidiaphragms of five groups of rats weighing 50–60, 75–85, 100–110, 125–135 and 150–160 g., respectively. The black quadrangles represent the mean weights of tissue and glucose uptakes $\pm$ S.E.M. The upper and lower curves are rectangular hyperbolas calculated for a 'weight $\times$ glucose uptake' product of 750 and 500, respectively.

When applied, the test for significance of differences between means was Student's *t* test (see Snedecor, 1946).

RESULTS

In the first set of experiments, five groups of rats were used, weighing 50–60, 75–85, 100–110, 125–135 and 150–160 g., respectively. The hemidiaphragms of two rats were used in each experiment. One Warburg flask containing one hemidiaphragm from each rat was incubated without insulin, the other flask containing the remaining hemidiaphragms was incubated with insulin: in this way, left and right hemidiaphragms were equally distributed between the control and insulin-containing flasks. The thickness of the pieces of tissue was not determined in all cases but the thinnest (among rats weighing 50–60 g.) were approx. 0.25 mm., the thickest (among heavy rats) approx. 0.55 mm. It is

clear from the results given in Table 1 that thin hemidiaphragms of small rats take up much more glucose than the thick hemidiaphragms of larger rats. For the first three groups of rats, the actual amount of glucose taken up (per manometer flask, not per g./hr.) was practically independent of the amount of tissue per flask: 65–125 mg. of tissue took up 0.24–0.26 mg. of glucose in 30 min. without insulin, and 0.35–0.39 in the presence of insulin. Fig. 1 shows that, with amounts of tissue up to 120–130 mg. per flask, the experimental values approximately follow the course of rectangular hyperbolas: the product 'glucose uptake (mg./g./hr.) $\times$ weight of tissue (mg./flask)' is essentially constant. Indeed, this is not too surprising, since the diffusion of glucose, oxygen and possibly other metabolites depends on the thickness of the preparation. However, the effect of tissue thickness on the course of the reaction is so important that two groups of results are not comparable unless the mean weights of tissue of the two groups are really identical. Thus, it is not permissible to assess the effect of insulin by comparing directly, for instance, the two values of line 2, Table 1 (5.55 and 8.08 mg./g./hr.), since the mean weights of tissues are in these cases 91.1 and 96.2 mg./flask, respectively. The relation existing between weight of tissue and glucose uptake makes it possible to predict what the mean uptake would be if the mean weight of tissue were 93.7 mg./flask in both cases:

$$91.1 \times 5.55 = 93.7 \times x; \quad x = 5.40.$$

$$96.2 \times 8.08 = 93.7 \times y; \quad y = 8.30.$$

The stimulating effect of insulin on the uptake of glucose by hemidiaphragms of rats weighing 75–85 g. is thus: $8.30 - 5.40 = 2.90$ mg. extra glucose taken up/g. of tissue/hr., i.e. a 54% increase. Statistical analysis however requires that all corrections be applied to individual values before calculation of the means. If this is done (see Table 2), all mean values are slightly modified and the standard errors are generally diminished. From such comparable figures, the effect of insulin on the uptake of glucose may be calculated. If expressed as extra glucose uptake, the maximum effect of insulin was obtained with the thinnest hemi-

Table 2. *Effect of insulin on the uptake of glucose by hemidiaphragms of rats weighing 50–160 g.*

Weights of rats (g.)	Weight of tissue* (mg./flask)	Glucose uptake†		Effect of insulin‡	
		Without insulin (mg./g./hr.)	With insulin (mg./g./hr.)	Extra uptake of glucose (mg./g./hr.)	Stimulation (%)
50–60	67.9	7.23±0.61 (11)	10.49±0.56 (11)	3.26±0.39 (11)	50.6± 8.4 (11)
75–85	93.7	5.36±0.30 (11)	8.32±0.28 (11)	2.96±0.16 (11)	57.2± 4.2 (11)
100–110	125.2	3.96±0.22 (11)	6.22±0.11 (11)	2.26±0.20 (11)	61.1± 7.6 (11)
125–135	158.5	3.56±0.16 (6)	5.08±0.23 (6)	1.52±0.33 (6)	44.9±11.0 (6)
150–160	189.5	3.32±0.13 (6)	4.89±0.26 (6)	1.57±0.29 (6)	48.3± 9.4 (6)

* Mean of the weights given in Table 1 for the experiments with and without insulin.
† Compare with the data of Table 1.
‡ In all cases, the effect of insulin is statistically significant ($P < 0.01$); differences between insulin effects expressed as extra uptake of glucose are also significant but differences between insulin effects expressed in percentage stimulation are not.

Table 3. *Uptake of glucose by 1–4 hemidiaphragms of rats weighing 75–85 g.*

All individual values were recalculated as if the weights of tissue per flask were really multiples of 46.9 mg., mean weight of a single hemidiaphragm in this set of experiments. In all cases, the effect of insulin is significant ($P < 0.01$). Glucose uptake in the presence of insulin and insulin effects (expressed as extra uptake of glucose or in percentage stimulation) are significantly lower ($P < 0.01$) if four hemidiaphragms are incubated together instead of two.

No. of hemidiaphragms per flask	Glucose uptake		Effect of insulin	
	Without insulin (mg./g./hr.)	With insulin (mg./g./hr.)	Extra uptake of glucose (mg./g./hr.)	Stimulation (%)
1	5.71±0.57 (11)	7.84±0.34 (12)	1.98±0.51 (11)	47.1±16.0 (11)
2	5.35±0.28 (12)	8.26±0.26 (12)	2.96±0.16 (11)	57.1± 4.2 (11)
3	4.88±0.13 (8)	7.75±0.34 (8)	2.48±0.35 (6)	51.9± 8.3 (6)
4	4.94±0.07 (8)	6.83±0.19 (8)	1.90±0.16 (6)	38.4± 3.1 (6)

Fig. 2. Uptake of glucose by four hemidiaphragms of rats weighing 75–85 g.

diaphragms; but if considered as the stimulation of a basic glucose uptake, and therefore expressed in percentage stimulation, the maximum effect of insulin was obtained with rats weighing 100–110 g.: differences between insulin effects expressed in this way were however not statistically significant. Reasons for preferring the second mode of expression will be given in the Discussion. The results

of Table 2 also show that the individual values of glucose uptake are very scattered when hemidiaphragms of small rats are used.

In a second set of experiments, material from rats all weighing 75–85 g. was used; one, two, three or four hemidiaphragms, respectively, were placed in a flask and incubated, care being taken to ensure equal distribution of left and right hemidiaphragms between control and insulin-containing flasks. In this set of experiments, the mean weight of a single hemidiaphragm was 46.9 mg. Using the correction outlined above, all individual values were recalculated as if the weights of tissue per flask were multiples of 46.9 mg. The results show (Table 3) that the uptake of glucose is practically the same in each case except when four hemidiaphragms are incubated together in the presence of insulin. This lower figure, together with the corresponding lower effect of the added insulin, might have been due to a limiting effect of the glucose concentration which is known to affect both the uptake of glucose and the synthesis of glycogen (Gemmell & Hamman, 1941; Hechter, Levine & Soskin, 1941; Stadie & Zapp, 1947; Riesser, 1947; Mentha & Voegtli, 1947) since the final glucose concentration had fallen, in this case, to a mean value of 0.76 mg./ml. against the initial value of 1.4 mg./ml. An experiment was carried out to follow the course of the reaction during shorter periods of incubation (10, 20 and

30 min.) and four hemidiaphragms were incubated to produce a sufficient uptake of glucose; it was found however, that (see Fig. 2) the extra uptake of glucose produced by addition of insulin was not diminished in rate after 20 min. as it would have been if low glucose concentration had limited the glucose consumption. It is possible that shaking is not efficient enough when four pieces of diaphragm are incubated together in the same flask; Brown, Park, Daughaday & Cornblath (1952) have shown that the rate of shaking is critical.

DISCUSSION

It is well known that the relative metabolic rate of large animals is lower than that of smaller animals and that the energy exchange is more or less proportional to the surface area rather than to the weight of the animal. From a comprehensive study of the respiration of isolated tissue preparations, Krebs (1950) concluded that for most tissues, the Q_{O_2} changed much less with the body weight than the rate of basal heat production of the intact animal; Krebs believes that the differences in the total rate of heat production in animals of different size are mainly due to variations in the Q_{O_2} of the musculature: this conclusion was reached by exclusion and not by direct evidence. Von Bertalanffy & Pirozynski (1951) and von Bertalanffy & Estwick (1953), who used slices of tissues or isolated diaphragms of the rat, recently showed that the Q_{O_2} of isolated tissues is higher when young animals are used. Statistical evaluation of their data for diaphragm demonstrates a negative correlation between the rate of respiration and the body size: although these results, as well as those of Kayser, Le Breton & Schaeffer (1925), seem to corroborate Krebs's conclusion, it is felt that the evidence so far presented is not satisfactory since the variable thickness of the preparations was not considered: diaphragms of rats weighing more than 100 g. are definitely too thick for reliable manometric measurements. It is interesting to note in this respect that the slope of the regression line is negligible (-0.065 , with a correlation coefficient of -0.66 only) when fibres of skeletal muscle are used instead of diaphragms where the slope of the regression lines ranged from -0.15 to -0.26 according to the medium used. Therefore, it is felt reasonable to assume that the metabolism of the isolated diaphragm is practically independent of the size of the rats used. In contrast with this conclusion, the uptake of glucose is highly dependent upon the size of the rats used: calculated in the same way but using glucose uptakes instead of oxygen uptake, the slope of the regression line shows a very marked negative correlation between glucose uptake and body size, whether insulin be present or not (-0.72

and -0.77 , respectively, with correlation coefficients of -0.98 and -0.99), and it is extremely unlikely that this reflects a reduction of the carbohydrate metabolism in the isolated diaphragm of large animals.

The hemidiaphragms of large rats could differ from those of small rats in three ways: (1) their true activity may be different, (2) their surface is larger, but not very much so apparently, and (3) they are thicker. The first possibility is not supported by the arguments developed in the first part of this discussion. It is clear from the experimental results reported in Table 3 that the relative uptake of glucose (glucose taken up/g. of tissue) is essentially unaffected by the number of hemidiaphragms used when they are of about the same size, i.e. the absolute uptake of glucose is proportional to the number—or to the surface—of the hemidiaphragms. The low glucose uptakes by hemidiaphragms of larger rats may probably be best explained by differences in rates of diffusion in preparations of varying thickness; the possibility that an inhibitor present in the hemidiaphragms diffuses out more easily from thin preparations may also have to be considered. The results presented support the view that the absolute uptake of glucose is apparently proportional to the surface of the diaphragms—independent of the thickness of the preparation—rather than to the weight of tissue. This would mean that only the superficial layers consume glucose, possibly because the superficial layers only are in contact with enough glucose. The fact that the centre of the preparation may be lacking oxygen cannot explain these results since the uptake of glucose is not diminished in an atmosphere of nitrogen, although the stimulating effect of insulin nearly disappears (Walaas & Walaas, 1952).

The stimulating effect of insulin could be demonstrated and was highly significant in all cases ($P < 0.01$) but its magnitude varied slightly with the type of preparation used (see Table 2); the differences observed were however not statistically significant. When considering the results obtained without added insulin, there is probably no need to take into account the amount of insulin possibly present in the hemidiaphragms. If we assume that insulin is evenly distributed between plasma and extracellular fluid, and, since the concentration of insulin in normal (human) plasma was found to be of the order of 1×10^{-4} units/ml. (i.e. approx. 5×10^{-3} $\mu\text{g./ml.}$) by Groen *et al.* (1951–2) using the diaphragm technique and by Bornstein (1950b) who measured the hypoglycemia produced by injection of insulin to alloxan-diabetic, hypophysectomized, adrenalectomized rats (Bornstein, 1950a), the amount of insulin present in 50–200 mg. of diaphragm would be of the order of $1\text{--}5 \times 10^{-6}$ units: this amount, diluted by the addition of 1 ml. of glucose-contain-

ing medium would be unable to produce any measurable stimulating effect (Krahl & Cori, 1947; Groen *et al.* 1951-2).

There is no possible comparison between the extra oxygen uptake produced by the addition of a substrate to a slice of surviving tissue and the extra glucose uptake produced by the addition of insulin to an isolated tissue incubated in a glucose-containing medium. In the first case, the control without substrate is due to the oxidation of various endogenous metabolites and the stimulating effect of the added substrate on the respiration rate is generally the result of a different and additional reaction. In the case of insulin, there are no reasons to believe that the extra glucose uptake is produced by a different enzymic system. In fact there are good reasons for believing that the glucose disappears as the result of a chain of reactions of which the first is catalysed by hexokinase. Unpublished measurements from this laboratory show that the hexokinase activity of water extracts of rat skeletal muscle reinforced with Mg^{2+} and adenosine triphosphate corresponds to a glucose uptake of approx. 15 mg./g./hr., i.e. 2-3 times as much as the values found for the isolated diaphragm. From the figures of Long (1952) and the calculations he made of the results of Stadie & Haugaard (1949), it may be inferred that the activity of whole homogenates of rat skeletal muscle would be of the order of 30 mg./g./hr. Although the activity of water extracts of muscle measured by other authors are not as high as ours (Stadie & Haugaard (1949) found approx. 11 mg./g./hr. and Gerritsen (1951) only 8-9 mg./g./hr.) there is no doubt that the uptake of glucose by the isolated diaphragm in the absence of added insulin is not limited by the rate of the hexokinase activity; the highest values found in the presence of insulin are still below the level of hexokinase activity found by us in muscle extracts and by Long (1952) in muscle homogenates. Therefore it is felt preferable to express the effect of insulin as the stimulation of the control glucose uptake and not as an extra glucose uptake. Rats weighing about 100 g. will provide hemidiaphragm preparations most sensitive to insulin and more accurate determinations will be made with such rats. Data on the uptake of glucose by homogenates of rat diaphragms have not been published; an approximate value of 10-12 mg./g./hr. is given by Krahl (personal communication).

SUMMARY

1. Diaphragms from rats of varying weights were used for measurements of the rates of glucose uptake and their stimulation by insulin.

2. The reaction has been studied as a function of the amount of tissue used per manometer flask. The uptake of glucose was found to be proportional to

the number of hemidiaphragms used when they were of about the same size, but thin hemidiaphragms of small rats took up as much glucose as thick hemidiaphragms of heavy rats; the product 'glucose uptake/g. of tissue $\times$ weight of tissue/flask' was found to be essentially constant. This is taken as evidence that the superficial layers of the preparations take up much more glucose than the centre of the preparations, although this cannot be the only explanation.

3. The simple relation between the uptake of glucose/g. of tissue and the weight of tissue/flask makes it possible to predict by calculation the uptake of glucose by different amounts of tissue; the stimulating effect of insulin may only be assayed from a comparison of the glucose uptake in the absence and in the presence of added insulin, if the mean weight of tissue/flask is the same in the two sets of experiments. Differences between the size of the insulin effect measured with thin or thick preparations were not statistically significant.

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The N-Terminal Amino Acid Residues of Gelatin

1. INTACT GELATINS

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During the past five or six years there has been a considerable revival of interest in the biochemistry and physical chemistry of collagen and gelatin (Randall, 1953; Pouradier & Venet, 1950, 1952; also others). Gelatin may be defined as the protein derived from collagen by irreversible procedures which render the collagen water-soluble. The irreversibility differentiates gelatin from 'soluble collagen'. Gelatin is not homogeneous within any one sample, and different samples differ in important properties and structural characteristics (e.g. molecular weights, molecular-weight distribution, isoelectric point, viscosity of solutions, rigidity of gels). The possibility arises that these differences may be due in part at least to the method of manufacture, of which there are two main procedures, the acid process and the alkali process (Ward, 1951; Ames, 1952) or to the type of collagenous tissue used. There are three principal sources of collagen used commercially: skin, bone and sinew. This fact was not always appreciated in the past by those unfamiliar with the industrial process and has often led to difficulties in correlating results of different workers engaged on similar research.

The nature of the bonds broken when collagen is converted into gelatin is still a matter of speculation, and the problem is evidently connected with theories of the forces which bind individual protein chains into collagen fibrils. Recent views put forward include hydrogen bonding (Gustavson, 1950), the presence of chondroitin sulphuric acid (Jackson, 1953) and a $-\text{CO}-\text{NH}-\text{CO}-$ linkage (Ames, 1952). Bowes & Moss (1951), using Sanger's 1-fluoro-2,4-dinitrobenzene (FDNB) technique, have shown that, while native collagen appears to have no N-terminal end groups, gelatin gives small amounts of

N-terminal aspartic acid, glutamic acid, glycine and threonine. It appears, therefore, that the rupture of peptide bonds may be involved in the conversion of collagen into gelatin.

The purpose of the present investigation was to ascertain any gross differences in N-terminal residues between gelatins with different collagenous precursors or with the process of manufacture used.

The N-terminal-residue method gives useful approximations for the number-average molecular weights of gelatin samples, based on the assumption that the protein comprises single polypeptide chains each with one N-terminal residue.

As Sanger's end-group technique has been applied to an increasing number of proteins, certain workers have reported that not all of the ϵ -amino groups of lysine and hydroxylysine residues are available to react with FDNB, although in some instances these groups are available to nitrous acid (Salo, 1950) or keten (Porter, 1948). Porter found that more than one-third of ϵ -amino groups of β -lactoglobulin and of several serum globulins failed to react with FDNB when these proteins were in the native state. On denaturation all the groups became available to the reagent. It was suggested, therefore, that the configuration of the polypeptide chain in the native protein could not accommodate the bulky 2,4-dinitrophenyl (DNP) group. A similar phenomenon has been reported with collagen and gelatin, although there is disagreement over the extent of availability of ϵ -amino groups; Bowes & Moss (1951) found about 50% availability with native collagen and gelatin, while Sykes (1952) reported about 75–80% availability with hide powder. It was therefore of interest to examine DNP-gelatins for their content of N $^{\epsilon}$ -DNP-lysine.