J. Physiol. (1956), 131, 526–541

THE ACTION OF INSULIN ON THE PENETRATION OF SUGARS INTO THE PERFUSED HEART*

By R. B. FISHER and D. B. LINDSAY†
Department of Biochemistry, University of Oxford

(*Received* 19 *May* 1955)

Levine and his colleagues (Levine, Goldstein, Huddlestun & Klein, 1950; Goldstein, Henry, Huddlestun & Levine, 1953) have shown that insulin can increase the volume of distribution of galactose and of configurationally related sugars in the body fluids of eviscerated dogs and rats. Their work suggests that about half the body water is inaccessible to these sugars in the absence of circulating insulin, whereas the whole of the body water becomes accessible to them when insulin is administered. Since the sugars which exhibit this response to insulin have the same configuration of substituents on carbon atoms 1 to 3 as has glucose, there is ground for supposing that the behaviour of the sugars studied may bear on the physiological role of insulin.

The effect of insulin on the volume of distribution of glucose has been studied by Drury & Wick (1951) using ^{14}C-labelled glucose. Insulin has no detectable effect but, as these authors point out, this is not inconsistent with the conception that insulin acts by increasing the rate of access of glucose to the interior of cells: until the rate of access is increased to the point that it is no longer the rate-determining step in intracellular glucose metabolism there will be no appreciable rise in intracellular glucose content and therefore no increase in the volume of distribution of glucose.

Tests of the Levine hypothesis have been made by Haft, Mirsky & Perisutti (1953) and by Park (1953), using isolated rat diaphragm. Haft *et al.* (1953) showed that insulin increases the rate of entry of galactose into the tissue as well as the rate of loss of glucose from the medium, but there is nothing to indicate whether these two effects are aspects of the same fundamental action of insulin. Park (1953) has examined the effects of high concentration of glucose and insulin on the glucose content of the diaphragm, and has found that the intracellular glucose content appears to rise in these conditions. This

* A preliminary account of some of this work has appeared in the Proceedings of the Physiological Society (*J. Physiol.* 1954, **124**, 20–21*P*).

† Present address: A.R.C. Institute of Animal Physiology, Babraham, near Cambridge.

finding is the only one directly suggesting that insulin may exert its effect on glucose metabolism by increasing the access of glucose to the interior of cells: but it can only be harmonized with the failure of Drury & Wick (1951) to find an increase in total body glucose space after insulin by supposing diaphragm to be unrepresentative of skeletal muscle.

The present work makes use of the surviving rat heart preparation of Bleehen & Fisher (1954) to test the relation between the effects of insulin on galactose penetration into the heart muscle cells and its effect on glucose utilization within them. If the two effects are dependent on a common action of insulin on penetration of sugars into cells it is to be expected that:

(1) The concentrations of insulin below which there is no detectable effect and above which there is no increase in effect will be the same for galactose penetration and glucose utilization.

(2) Galactose penetration will be diminished in the presence of glucose, and glucose utilization will be diminished in the presence of galactose, owing to competition of the sugars for the available insulin or for the common insulin-affected step in the two processes.

(3) Insulin will increase in some degree the intracellular concentration of glucose as well as that of galactose.

The work to be described is concerned with testing these expectations.

METHODS

Analytical procedures

Glucose was usually estimated by the method of Hagedorn & Jensen (1923) in cadmium hydroxide filtrates (Fujita & Iwatake, 1931), as the difference between total reducing substance and that remaining after yeast fermentation by the procedure of Bleehen & Fisher (1954). In view of the possibility that this procedure might estimate reducing phosphoric esters as glucose some trials have been made with preparations of glucose-6-phosphate and fructose-6-phosphate kindly provided by Dr R. Hems and Dr P. W. Kent. Using concentrations of these esters sufficient to give apparent glucose concentrations equal to those found in heart extracts, it was found that only about 5% of the reducing substance appeared in cadmium hydroxide filtrates and that the reducing power of these filtrates was not lessened by yeast fermentation in our conditions. Some trials were also made of treatment of heart extracts with $ZnSO_4$ and $Ba(OH)_2$. This is more effective than cadmium hydroxide in removing glucose-6-phosphate and fructose-6-phosphate, but in parallel determinations on heart extracts the two precipitation procedures gave closely similar estimates of fermentable sugar. We therefore believe that the majority, if not all, of what we designate 'glucose' is truly glucose. In some experiments, in which a high concentration of galactose was present, glucose was estimated manometrically, using 'notatin' (Keilin & Hartree, 1948) and measuring the oxygen absorption during the oxidation of glucose to gluconic acid. The 'notatin' was a crude concentrate of medium in which *Penicillium notatum* has been grown (for which we are indebted to Dr N. G. Heatley), and was made by tenfold concentration of the medium *in vacuo* followed by precipitation at 4° with an equal volume of acetone. The precipitate was redissolved in a small volume of water and reprecipitated with acetone. After two further precipitations the acetone was removed *in vacuo* and the residue stored in the cold room. An aliquot of this, which could oxidize 5 mg of glucose in 3 ml. solution within 20 min, was used for each estimation. The preparation retained its activity for several weeks at 0°. Tests showed that

heart tissue perfused with glucose-free medium yielded no apparent glucose, and that galactose was not oxidized and did not inhibit the oxidation of glucose. These estimations were performed on cadmium hydroxide filtrates, and are not therefore likely to have been perturbed by the presence of phosphoric esters of glucose, even if the crude notatin contained enzymes capable of hydrolysing them.

Galactose was estimated by the Hagedorn & Jensen (1923) procedure applied to cadmium hydroxide filtrates from which the glucose had been removed by yeast fermentation, as described by Bleehen & Fisher (1954). A correction was applied for non-galactose non-fermentable substances. This is described in the Results section.

Sorbitol was estimated by oxidation with sodium metaperiodate ($NaIO_4$, $3H_2O$: Hopkins & Williams) and titration of the acid formed. The theoretical yield of acid is 4 equivalents per mole of sorbitol, but this yield is obtained only when the metaperiodate concentration is very high, and in these circumstances all but the freshest metaperiodate solutions give a considerable blank titration. It has been found that the yield of acid is linearly related to the logarithm of the metaperiodate concentration, increasing by about 0·3 equivalent per mole for a tenfold increase in metaperiodate concentration. For the present work, in which 0·1–0·5 mg of sorbitol in 10 ml. was to be estimated, 1 ml. of 0·03 M metaperiodate was added to each 10 ml. aliquot. The blank titration was negligible.

The procedure was: (1) Brom-cresol purple was added to 10 ml. solution of sorbitol, which was titrated to the turning point of the indicator, stirring with CO_2-free air. (2) 1 ml. 0·03 M metaperiodate was added, followed by 1 ml. ethylene glycol. The interval between these additions is not critical, since the reaction of sorbitol with metaperiodate is very rapid. The ethylene glycol removes excess metaperiodate. (3) More brom-cresol purple was added. The metaperiodate attacks the indicator. (4) The solution was titrated with N/200-NaOH whilst it was being stirred with CO_2-free air. We have found it convenient to add to the solution a small amount of a carbonic anhydrase preparation, made by Meldrum & Roughton's (1933–4) alcohol-chloroform method. In its presence the removal of CO_2 by the CO_2-free air is sufficiently rapid to obviate the need for CO_2-free NaOH. In these conditions 1 mg of sorbitol yields acid equivalent to 3·2 ml. N/200-NaOH.

Lactate was estimated by the method of Barker & Summerson (1941).

Glycogen was estimated by the method of Good, Kramer & Somogyi (1933).

Inulin and *chloride* were estimated as described by Bleehen & Fisher (1954).

Perfusion procedure

Closed circuit perfusions of rat hearts were set up as described by Bleehen & Fisher (1954), with one modification. When the heart was removed from the animal it was placed in a dish of medium at room temperature, not at perfusion temperature, and it was then attached to a cannula connected to a reservoir containing saline at room temperature. After washing out the heart with this saline, the cannula was then transferred to a closed circuit apparatus containing oxygenated perfusion medium at 38° C.

RESULTS

The utilization of galactose. Hearts were perfused with medium containing galactose (150 mg/100 ml.) and insulin (2 mu./ml.). Galactose utilization was determined by the method described by Bleehen & Fisher (1945) for glucose utilization. Four hearts gave an estimated mean utilization of $1·9 \pm 1·5$ mg/g dry wt./hr. The mean utilization of glucose in similar conditions is $44 \pm 2·6$ mg/g dry wt./hr.

Penetration of galactose in absence of insulin. Table 1 shows the time-course of galactose penetration into the heart. Hearts were perfused with medium containing 1000 mg galactose and 150 mg glucose per 100 ml. for different

lengths of time and the total non-fermentable reducing substance of heart and perfusate was then determined. The ratio of total cardiac non-fermentable reducing substance per unit weight of heart to the amount per unit volume of perfusate was taken as 'apparent galactose space'. The 'corrected galactose space' is determined by subtracting from the cardiac reducing substance the amount of non-fermentable reducing substance found in unit weight of heart perfused with media similar in all respects, excepting that they are free of galactose. Table 2 shows that this correction is not appreciably dependent on the nature of the medium perfusing the heart.

TABLE 1. The time course of penetration of galactose into the rat heart during perfusion with medium containing 150 mg glucose and 1000 mg galactose per 100 ml.

Perfusion time (min)	No. of expts.	Galactose space (μl./g)	
		Crude	Corrected
17	1	597	525
30	6	657±15	583
60	7	686±27	620
90	3	727	654

TABLE 2. Non-fermentable reducing substances in perfused hearts after 30 min periods of perfusion with various media

Glucose (mg/100 ml.)	Sorbitol (mg/100 ml.)	Insulin (m-u./ml.)	No. of expts.	Non-fermentable reducing substances, as galactose (mg/g fresh heart)
600	—	—	3	0·77
600	—	2	6	0·83
600	1000	—	6	0·81
600	1000	2	6	0·78
150	1000	—	6	0·66
150	1000	2	6	0·82
Mean of all determinations:			33	0·77±0·02

The total water of perfused hearts is around 800 μl./g. The data of Table 1 therefore indicate that a large fraction of the heart water is penetrated by galactose in these conditions in as short a time as 17 min. After this time further penetration occurs slowly.

Effect of insulin on the penetration of galactose. A perfusion time of 30 min was chosen for the study of the effect of insulin, because Table 1 shows that in the absence of insulin further penetration of galactose is occurring slowly at this time, although a fair proportion of heart water is still not penetrated by galactose. Experiments were first made with the same concentrations of sugar as used in the experiments just described, and with various concentrations of insulin in the perfusate. The results are given in col. 3 of Table 3. There is a progressive increase in galactose space with increase in insulin concentration up to 2 m-u./ml. the concentration found by Bleehen & Fisher (1954) to have maximal effect on glucose utilization.

Effect of change in concentration of glucose and galactose on the penetration of galactose. The first line of Table 3 shows: (1) that in the absence of glucose the

extent of galactose penetration in the standard period of 30 min diminishes with increase in galactose concentration; and (2) that the presence of glucose in the medium at either of the galactose concentrations used markedly diminishes the extent of penetration of galactose. It may also be seen that by making the ratio of glucose to galactose in the medium sufficiently high the relation of the rates of penetration of galactose at the two concentrations chosen can be reversed.

TABLE 3. The effect of insulin on the penetration of galactose into hearts perfused with media containing different concentrations of galactose and glucose

	Perfusion medium			
Galactose (mg/100 ml.) ...	300	1000	1000	300
Glucose (mg/100 ml.) ...	nil	nil	150	600
	Galactose space (μl./g fresh heart)			
No insulin	783 (3)	672±4 (8)	608±9 (13)	515±15 (7)
0·02 m-u./ml. insulin	—	685±4 (5)	623±6 (9)	—
0·2 m-u./ml. insulin	—	713±13 (4)	639±7 (4)	—
2·0 m-u./ml. insulin	801 (3)	748±6 (6)	684±9 (8)	618±16 (6)
20·0 m-u./ml. insulin	—	—	687±10 (11)	649±15 (6)

The figures in parentheses indicate the numbers of experiments: the estimates of error are standard errors of means.

The effects of insulin on galactose penetration have been examined in these different conditions. It is found to increase penetration in all these conditions, except when the medium contains galactose alone in a concentration of 300 mg/100 ml.; and in these conditions galactose appears to come into equilibrium with the whole of the heart water in 30 min, even in the absence of insulin. The galactose spaces at the end of 30 min are ranked in the same order when there is 2 m-u./ml. of insulin in the medium as when there is no insulin, and more detailed inspection of Table 3 indicates that the effect of insulin is independent of the presence of glucose.

Effect of galactose on glucose utilization. The finding of interaction between glucose and galactose, described above, suggested that galactose might interfere with the utilization of glucose. Determinations of glucose utilization in the presence of 150 mg/100 ml. of glucose and of 2 m-u./ml. of insulin were therefore made in the presence and absence of 100 mg galactose per 100 ml. The general procedure was that described by Bleehen & Fisher (1954), but the glucose was determined manometrically with the aid of notatin as described under 'Methods'.

Table 4 gives the results of these experiments. Experiments were performed with two different calcium concentrations in the medium because it has been found in unpublished work that calcium concentration markedly altered the glucose utilization. The fact that galactose appears to depress glucose utiliza-

tion at both the calcium concentrations used suggests that its effect is not an accidental one peculiar to a particular ionic composition of the medium.

Penetration of inulin and sorbitol into the heart. The relations between the glucose utilization effect and the galactose penetration effect of insulin disclosed by these experiments suggested that insulin might increase the penetration of glucose into the intracellular water of the heart. In view of the rate of consumption of glucose by the heart it appeared that this might be difficult to establish unless the amount of glucose in the extracellular water could be measured with some precision. Experiments were therefore made to determine the extent of penetration of inulin and of sorbitol into the heart with a view to using them for this purpose. In six 30 min experiments with perfusate containing 150 mg glucose per 100 ml. the mean inulin space was $305 \pm 10\,\mu$l./g heart and in six similar experiments with sorbitol the space was $378 \pm 6\,\mu$l./g.

TABLE 4. The effect of galactose on the rate of utilization of glucose by the rat heart perfused with medium containing 150 mg glucose per 100 ml. and insulin 2 mU/ml. The period of perfusion was 1 hr. Eight experiments in each set

	Glucose utilization (mg/g dry wt./hr) in presence of	
Ca concn. in perfusate	No galactose	Galactose 1000 mg/100 ml.
0·63 mM	39·8 ± 3·51	33·4 ± 2·70
1·26 mM	46·5 ± 2·80	36·8 ± 2·68
Means	43·1	35·1
Difference	8·0 ± 2·92 ($P = 0·01$)	

Thus inulin penetrates into a smaller fraction of heart water than does sorbitol. Presumably it penetrates more slowly or not at all into some subfraction of the extracellular water. This could be the water of the connective tissue elements. The fraction of the heart water penetrated is consistent with the view that the sorbitol space is equal to the extracellular space. Other series of determinations of sorbitol space have been made and are shown in Table 5. This space is unaltered when the glucose concentration is raised to 600 mg/100 ml.; but it is reduced to about $350\,\mu$l. in the presence of insulin. This finding is entirely consistent with the assumption of confinement of sorbitol to the extracellular space, since the increased metabolic rate of the tissue in the presence of insulin could well give rise to increase in total intracellular osmotically active material, with consequent increase in the proportion of intracellular to extracellular water in the tissue.

Effect of insulin on glucose penetration into the heart. Hearts were perfused for 30 min with medium containing 150 or 600 mg/100 ml. of glucose and 1000 mg/100 ml. of sorbitol in the presence and absence of 2 m-u./ml. of insulin, and the glucose and sorbitol spaces were determined. The results are collected in Table 5, and it is seen that, contrary to expectation, the effect of insulin on the total glucose space is marked. The effect on the difference between glucose

space and sorbitol space is even more striking. There can be no doubt, from these findings, that insulin increases the rate of penetration of glucose into the cells of the heart.

The fate of the glucose utilized by the perfused heart. In order to interpret these findings it was desirable to know what was the fate of the glucose which disappeared from the medium in these experiments. Estimations of glycogen content were therefore made on hearts taken immediately after washing out the vessels with cold perfusate, and on hearts after 15 and 75 min periods of

TABLE 5. The effect of insulin on the glucose space of the perfused rat heart (30 min periods of perfusion)

Glucose (mg/100 ml.)	150	150	600	600
Insulin (m-u./ml.)	—	2	—	2
No. of expts.	6	6	6	6
Mean glucose space (μl./g heart)	457±16	576±24	426±21	596±9
Mean sorbitol space (μl./g heart)	378±6	351±10	383±18	359±15
Difference	79±15	224±28	43±11	237±17

TABLE 6. The effect of perfusion with medium containing 150 mg/100 ml. of glucose on the glycogen content of the heart and the lactate content of the perfusion medium

Perfusion time	Zero	15 min		75 min	
Insulin concentration	Zero	Zero	2 m-u./ml.	Zero	2 m-u./ml.
Mean glycogen content of heart (mg/100 g)	246±33 (6)	429±39 (6)	433±39 (6)	352±20 (6)	567±20 (6)
Mean lactate content of medium (mg/100 ml.)	—	10·0 (3)	5·8 (3)	10·0 (3)	6·8 (3)

perfusion at 38° C with 150 mg/100 ml. glucose. The lactate content of the medium was also measured at 15 and 75 min. The results are collected in Table 6. It is seen that the glycogen content rises equally sharply in the first 15 min in the presence and absence of insulin. It continues to rise in the next hour in the presence of insulin, and falls slightly in its absence. The lactate concentration is very low in all circumstances, and of the order of the lowest reported blood lactate concentrations. It does not change appreciably in the interval between 15 and 75 min, but the lactate concentrations in the presence of insulin are lower than those in its absence. It appears that the major part of the extra glucose disappearance brought about by insulin is due to glucose oxidation, little or none is due to glycolysis, and an appreciable fraction is due to glycogen formation.

The effect of 'pre-perfusion' on the penetration of galactose into the heart. The rate of glycogen formation observed in the first 15 min of perfusion with glucose in the absence of insulin is approximately equivalent to the maximal rate of glucose utilization observed by Bleehen & Fisher (1954) in the presence of insulin. This would be explicable in the light of the findings of these workers concerning the time of decay of insulin action in the perfused heart if the heart extracellular fluid contained insulin at the time of beginning perfusion, since the mean effective concentration of insulin over the first 15 min would be

expected to be approximately one-third the initial concentration. Correspondingly, the mean effective insulin concentration over the first 30 min would be one-tenth of the initial concentration. In the galactose penetration experiments reported earlier in this paper perfusion with galactose was begun as soon as the heart had been washed out with cold medium. In the 30 min period of perfusion used any insulin present at the beginning would therefore be expected to exert an effect equivalent to the persistence throughout the period of about one-tenth of the initial concentration. Since the observations of glycogen deposition (Table 6) suggested that the initial insulin concentration might be quite large, a series of experiments was made in which hearts were perfused for 39 min with medium containing 600 mg/100 ml. glucose but no galactose, and were then transferred for 30 min to medium containing the same concentration of glucose and 300 mg/100 ml. of galactose. The mean galactose space found in a set of six hearts was 461 ± 26 μl./g heart compared with 515 ± 15 for seven hearts perfused with similar medium for the same time, but without the period of pre-perfusion. Although the difference between these series could have arisen once by chance in from ten to twenty such comparisons, it supports the indication afforded by the glycogen determinations that there is residual insulin in the heart at the time of beginning of perfusion.

DISCUSSION

In this work on the isolated rat heart it has been possible to demonstrate correspondence and interactions between insulin effects on the behaviour of glucose and galactose which so strengthen the hypothesis of Levine *et al.* (1950) that these effects have a common basis as to make it almost certain that a major physiological action of insulin, in this tissue at least, is to facilitate the entry of glucose into the muscle cells.

The nature of the glucose metabolism of the perfused heart. In view of the artificial nature of the preparation it is of importance that the glucose disappearing from the perfusate seems to be metabolized according to a normal pattern. By far the greatest part disappears completely and is presumably oxidized completely. Some is converted to glycogen and this process as well as the presumed oxidation is accelerated by insulin: which therefore must be supposed in this preparation, as in others, to affect some early step in glucose metabolism common to anabolic and catabolic pathways. The lactate concentration in the perfusate is low at all times, and is of the same order as resting blood lactate *in vivo*. There is no evidence of increase in this concentration during perfusion, so that glycolysis presumably does not occur. It may be indicative of some secondary action of insulin that the lactate concentration in the presence of insulin is systematically slightly lower than in the absence of insulin.

Penetration of galactose into the heart in the absence of insulin. When the galactose space of the heart is compared with the sorbitol space after 30 min perfusion, the galactose space is always the larger. The mean sorbitol space in the absence of insulin is 38 ml./100 g perfused heart. That this space is probably equal to the extracellular space is indicated by the following considerations. The water content of hearts was determined after perfusion with the different media used in this work. The mean water content after 30 min perfusion was found to be 4·1 g H_2O/g solid, and there were no significant differences between different series. The mean water content of unperfused hearts is 3·3 g/g solid (Bleehen & Fisher, 1954). The extra water accumulating during perfusion therefore corresponds to 0·8 g/g solid, or per 5·1 g of perfused heart, i.e. 15·6 g extra water per 100 g perfused heart. If it is the case that the extra water is extracellular, this means that a sorbitol space of 38 ml./100 g perfused heart corresponds to a sorbitol space of (38 − 15·6) ml./(100 − 15·6) g of unperfused heart, i.e. between 26 and 27 ml./100 g. Lemley & Meneely (1952) found a sodium space in rat heart *in vivo* of 28 ml./100 g. Manery & Bale (1941) found a sodium space of 32 and a chloride space of 25 ml./100 g. It is therefore justifiable to consider the sorbitol space as corresponding closely to the extracellular water content.

Our measurements of the water content of hearts perfused with media of different compositions show that after 30 min the total water content is very close to 800 μl. H_2O/g tissue. Thus the perfused rat heart may be taken to contain 380 μl. of extracellular water (sorbitol space) and 420 μl. of intracellular water per g. When the heart is perfused for 30 min with 300 mg galactose per 100 ml. (Table 3) and the galactose space is 780 μl./g, this implies a penetration of (780–380) μl. of intracellular water in every g of heart, out of the total of 420 μl., corresponding to 95% penetration of the intracellular water. When the heart is perfused with 1000 mg galactose/100 ml. for 30 min the figure for percentage penetration of intracellular water is only 69%. Thus the rate of penetration of galactose is not proportional to the concentration in the medium. This would be expected if there were a 'facilitated transfer' of galactose across the cell membrane of the kind described by Widdas (1954): in this type of process, transfer is regarded as dependent on combination with a specific carrier molecule present in the membrane in limited amount, so that there is an upper limit to the rate of transfer and so that a non-linear relation exists between external concentration and rate of transfer into the cell.

Penetration of galactose in the presence of glucose. Further evidence in support of 'facilitated transfer' is afforded by the effect of the presence of glucose on the rate of penetration of galactose. The data of the first line of Table 3 show that addition of glucose to the medium markedly depresses the rate of entry of galactose into the heart. In 30 min perfusions in the presence

of 150 mg glucose/100 ml. the penetration of galactose (1000 mg/100 ml.) into the intracellular water is reduced from 69 to 54%. In the presence of 600 mg glucose/100 ml. the penetration of galactose (300 mg/100 ml.) is reduced from 95 to 32%. These effects might be due to some general metabolic effect of glucose on the properties of the cell membrane, but as there is also evidence (Table 4) of a reciprocal effect of galactose on glucose utilization it seems more reasonable to interpret these findings as due to competition for a common carrier.

Penetration of galactose in the presence of insulin. Bleehen & Fisher (1954) were unable to detect any effect of insulin on the glucose utilization of the perfused rat heart at concentrations less than 0·06 m-u./ml. They found that the glucose utilization increased with increasing insulin concentration up to 2 m-u./ml., but that further increase in insulin concentration produced no further increase in utilization. Table 3 shows that, both in the presence and absence of glucose, insulin increases the penetration of galactose into the heart; that there is a marginal effect when the insulin added to the perfusate is 0·02 m-u./ml. and that there is no further effect when the concentration is raised above 2 m-u./ml. Thus the range of concentrations over which insulin affects galactose penetration corresponds closely with the range over which it affects glucose utilization: there is no bar to the supposition that the two insulin effects have a common basis.

However, as has already been pointed out, there is a difference in procedure between the glucose utilization experiments and the galactose penetration experiments which bears on the present comparison. Owing to the time required to wash pre-existing insulin out of the heart the effective residual insulin concentration in the experiments without added insulin will be higher in the galactose penetration experiments (which began immediately the perfusion was set up) than in the glucose utilization experiments (in which a 15 min equilibration period preceded the determination of glucose utilization rate). The difference in amounts of persisting insulin cannot be estimated, but this difference between the experiments means that more attention has to be paid to correspondence in the effects of the higher concentrations of insulin than to those of lower concentrations.

It has already been noted that the intracellular water is 95% permeated by galactose after 30 min perfusion with medium containing 300 mg/100 ml. When 2 m-u./ml. insulin is added to such medium there is no significant change in the degree of galactose penetration. This appears to preclude the possibility that insulin stimulates some active transport mechanism. Neither in this instance nor in any other have we observed galactose contents of hearts corresponding to higher concentrations of galactose in intracellular water than in extracellular water.

A point of interest in connexion with galactose penetration is its speed. The

experiments with 1000 mg/100 ml. galactose and 2 mU/ml. insulin (Table 3) give the highest rate of galactose penetration: and, as the data of Table 1 show, this must be an underestimate of the rate at which it can penetrate since the mean rate is clearly much lower than the initial rate. In this instance, taking the extracellular water as equal to 370 μl./g (the mean of the range of sorbitol spaces found in all perfused hearts), and the galactose space as 750 μl./g, we have an estimate of intracellular galactose content equivalent to equilibration of the perfusate with 380 μl. of water/g heart which is equivalent at the concentration of galactose used to the penetration of 3·8 mg galactose/g wet heart in 30 min, equivalent to 38 mg/g dry wt./hr. The maximal rate of glucose utilization observed by Bleehen & Fisher (1954) was 78 mg/g dry wt./hr, in hearts with incompetent aortic valves perfused with insulin-containing medium. Both these estimates are certainly underestimates of the maximal rate at which the sugars can be transported into the cells. They serve to indicate, however, that there is nothing inherently improbable in the assumption that the two sugars are transported by the same mechanism, which should have the same maximal capacity for transport of both of them.

This has some bearing on the persistent notion that hexokinase may be specifically concerned in insulin action. Rosenberg & Wilbrandt (1952) appear to suggest that phosphorylation of sugars by hexokinase in the cell membrane may be the specific step which confers on sugar transport the properties of a 'carrier-borne' process. Sols & Crane (1954*a*) have studied the kinetics of brain hexokinase and found, *inter alia*, that the maximal rate of reaction with galactose is only one-fiftieth of that with glucose. In a preliminary note Sols & Crane (1954*b*) report that the activity of heart muscle hexokinase from several species is essentially similar to brain hexokinase in affinities for substrates and in relative phosphorylation rates. It is therefore difficult to reconcile the similarity of order of magnitude of rates of glucose and galactose penetration into heart muscle cells, which is shown by the present work, with the notion that hexokinase action is the rate-limiting reaction in sugar transport, which it would have to be if insulin increased the rate of sugar transport by action on hexokinase.

Penetration of glucose into the heart. If it be true that a major or the only physiological effect of insulin on the heart is to increase the ease of penetration of glucose, the increase in glucose utilization brought about by insulin should be a consequence of increased intracellular glucose concentration: whereas if the major effect of insulin were a stimulation of some intracellular catalysis, insulin should produce a fall in intracellular concentration. Using sorbitol space as a basis for estimating intracellular water and glucose, as in the galactose experiments, we have found that perfusion for 39 min with medium containing 2 mU/ml. of insulin causes an increase in mean intracellular glucose concentration from 27 to 74 mg/100 ml. when the external concentration is

150 mg/100 ml., and from 59 to 322 mg/100 ml. when the external concentration is 600 mg/100 ml. These figures are derived from the data of Table 5. These results put it beyond doubt that the major effect of insulin on glucose metabolism in this tissue is to increase the ease of penetration of the sugar into the cells.

This does not mean that the rate of glucose utilization is necessarily related in any simple fashion to the observed mean concentrations of glucose. The evidence suggests (Long, 1952; Wiebelhaus & Lardy, 1949; Sols & Crane 1954*a*) that hexokinase is saturated at very low glucose concentrations. Sols & Crane (1954*a*) estimate the K_m for the glucose-hexokinase reaction to be 8×10^{-6} M, which corresponds to 90% saturation at 1·3 mg glucose/100 ml. At any appreciable mean glucose content of the cell, therefore, the relation between glucose content and rate of glucose utilization will be more closely determined by the proportion of the cell volume in which the concentration of glucose exceeds this concentration than by the amount of glucose in this volume. If glucose distribution in the intracellular water were solely determined by diffusion this would lead to a unique relation between glucose content and rate of glucose utilization. But if specific biological activities such as protoplasmic streaming or other intracellular displacements accompanying contractile activity can occur, different patterns of distribution of glucose could conceivably occur at different times in association with similar rates of glucose utilization; i.e. such that the fraction of the total hexokinase exposed to glucose concentrations equal to or greater than that needed to saturate it to a high degree was the same for the different patterns of glucose distribution.

We do not propose to lay particular stress on the point that the estimated intracellular glucose concentrations in the presence of 2 m-u./ml. insulin are widely different at the two different perfusate glucose concentrations used (150 and 600 mg/100 ml.), although Bleehen & Fisher (1954) found that the rates of glucose utilization in these conditions were very similar (44 and 48 mg/g dry wt./hr respectively). Another factor which confirms the unprofitability of discussion of quantitative aspects of this relation is that 'intracellular water' is undoubtedly made up of several compartments. Glucose may gain access to these with different degrees of ease, though it may be the case that hexokinase is distributed throughout the cytoplasm (Sols & Crane, 1954*b*). One of these compartments is the 'membrane' across which the specific transfer takes place, and there is no basis for estimating its relative mass. Other obvious subdivisions are intramitochondrial and intranuclear water.

The crucially important finding is that insulin increases the intracellular concentration of glucose, whereas if its preponderant action were to activate some rate-limiting process in the chemical transformation of glucose it would be expected to reduce the intracellular glucose concentration.

The effect of galactose on the utilization of glucose by the heart. The data available show a definite diminution in the rate of glucose utilization when galactose is added to the perfusate. We have shown that there is no galactose utilization by the heart, so that it is improbable that any derivative of galactose is interfering with glucose utilization. Sols & Crane (1954*a*) have shown that for brain hexokinase the K_m of the galactose-hexokinase reaction is 10^4 times that of the glucose-hexokinase reaction, so that galactose cannot be expected to compete detectably with glucose for hexokinase. Thus, the competition between glucose and galactose for transport into the cell is not due to metabolic interaction between the two, or to competition for a carrier mechanism based on hexokinase.

The relation of the hexose carrier mechanism to insulin. The observations that glucose interferes with galactose entry into heart cells, that galactose interferes with glucose utilization, and that the same range of concentrations of insulin accelerates both processes, provide good ground for supposing that insulin acts by influencing a specific hexose carrier mechanism. Insulin does not seem, however, to be equally important in determining the rate of entry of glucose and galactose into different types of cell. Although glucose can enter skeletal muscle in the absence of added insulin in the environment of its cells (Lundsgaard, Nielsen & Ørskov, 1939*a*, *b*) the concentration of glucose inside skeletal muscle cells is normally very low indeed (Cori, Closs & Cori, 1933; Park 1953). The evidence of Levine and his colleagues (Levine *et al.* 1950), already cited, also indicates that in the absence of insulin some large mass of tissue, which by virtue of its size cannot be other than the skeletal musculature, is almost or entirely impermeable to galactose. It would seem that the rate of entry of galactose and glucose into skeletal muscle may be highly dependent on the presence of insulin.

In the present experiments on the rat heart, it is clear that the entry of glucose and galactose into cardiac muscle cells can occur quite readily in the absence of added insulin. In view of the demonstration by Bleehen & Fisher (1954), of the speed with which the action of residual insulin in the perfused heart decays, it appears safe to say that the two sugars can penetrate cardiac muscle cells in the absence of insulin. Bleehen & Fisher (1954) have given reasons for believing that the evidence (Stadie, Haugaard, Marsh & Hills, 1949; Stadie, Haugaard & Vaughan, 1952) does not support the view that insulin remains for a considerable period combined with muscle in an active form, except when an enormous excess of insulin is provided. The conclusions of Stadie *et al.* (1952) are based on the assumption that all the isotopic label recovered from tissues is present attached to insulin. The work of Elgee, Williams & Lee (1954) has shown that isotopically labelled insulin is rapidly converted into forms in which the isotopic label is no longer precipitable with the tissue protein, as is insulin. The rapid destruction of insulin found by these

workers is much more in conformity with the conclusions of Bleehen & Fisher (1954) than with those of Stadie *et al.* (1952). It may be taken, then, that the sugars penetrate cardiac muscle cells in the absence of insulin, but penetrate more rapidly in its presence.

In the human erythrocyte, for which there is the most complete demonstration of a specific carrier mechanism for hexoses, Widdas (1954) has shown that at low concentrations of glucose the rate of equilibrium between cell water and medium is exceedingly rapid. It seems unlikely that insulin could increase it appreciably.

These considerations suggest that insulin is not the hexose carrier. Insulin might act by combining with pre-existing free carrier molecules to produce a complex with higher affinity for glucose. The apparent large differences in permeability of skeletal muscle and heart muscle to galactose in the absence of insulin, taken together with their high permeability to this sugar in the presence of insulin, would require the assumption, on this hypothesis, that the carriers in the two different cell membranes had very different intrinsic capacities to transport glucose, but became similarly active in the presence of insulin.

An alternative explanation of the mode of action of insulin depends on the assumption that cell membranes exhibiting specific transport possess, in addition to carrier molecules, some second constituent, present in variable proportions, capable of combining with carrier to form an inactive complex. If insulin were capable of combining with this second constituent, thereby freeing carrier, the observed apparent independence of the two properties, possession of specific carrier and sensitivity to insulin, would be accounted for.

There is no evidence to decide between these two hypotheses. We are inclined to accept the second hypothesis provisionally, since it is on the whole simpler. It does, however, possess a feature that may be regarded as a disadvantage: it requires one to assume the occurrence in the membranes of insulin-sensitive cells of a constituent, the carrier-inhibitor, whose only feature is that it acts to the disadvantage of its possessor.

The toxicity of galactose. The depressant effect of galactose on glucose utilization could conceivably account for the toxic effects of galactose. Guha (1931 *a*, *b*) described rapid decline in body weight and death in about 2 weeks in rats on an otherwise adequate diet in which galactose was substituted for other carbohydrates, glucose, sucrose or maltose. Mice fed on diets in which galactose was the sole carbohydrate died in a week, having lost 30 % of their body weight. It is possible that the condition of galactosaemia, of which there are recent accounts by Bray, Isaac & Watkins (1952), Hudson, Ireland, Ockenden & White-Jones (1954) and Fox, Fyfe & Mollison (1954) may also be due to this action of galactose.

The effect of galactose on glucose utilization in the rat heart is small (Table 4), but this may be due to the fact that glucose can penetrate into this organ in the presence of insulin much more rapidly than it can be utilized by it. Cori *et al.* (1933) showed that the glucose content of skeletal muscle is in much smaller ratio to the glucose content of plasma than is the case in cardiac muscle; it is therefore possible that in this tissue transport of glucose across the cell membrane may limit much more strictly the rate of glucose utilization than it does in the heart. If this be so, then, in this tissue and in any other with similar properties, inhibition of glucose transport by competition with galactose for a carrier could have serious consequences, which would not be readily overcome, since intracellular galactose might be expected to interfere with the carrier mechanism as readily as extracellular galactose.

SUMMARY

1. The perfused rat heart does not utilize galactose to an appreciable extent, although this sugar penetrates into the intracellular water.

2. The rate of penetration of galactose is non-linearly related to the external concentration, is diminished by glucose and is increased by insulin (0·20 – 2·0 mU/ml.).

3. The rate of glucose utilization by the heart is diminished by galactose.

4. Inulin and sorbitol are confined to the extracellular water of the heart. Comparison of glucose and sorbitol spaces shows that insulin increases the intracellular glucose concentration.

5. The primary action of insulin on the heart appears to be to increase the ease of penetration of the intracellular water by glucose and related sugars.

We wish to express our thanks to the Agricultural Research Council for a training grant to D.B.L. during part of this work.

REFERENCES

Barker, S. B. & Summerson, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535–554.

Bleehen, N. M. & Fisher, R. B. (1954). The action of insulin in the isolated rat heart. *J. Physiol.* **123**, 260–276.

Bray, P. T., Isaac, R. J. & Watkins, A. G. (1952). Galactosaemia. *Arch. Dis. Childh.* **27**, 341–347.

Cori, G. T., Closs, C. O. & Cori, C. F. (1933). Fermentable sugar in heart and skeletal muscle. *J. biol. Chem.* **103**, 13–24.

Drury, D. R. & Wick, A. N. (1951). Insulin and the volume of distribution of glucose. *Amer. J. Physiol.* **166**, 159–164.

Elgee, N. J., Williams, R. H. & Lee, N. D. (1954). Distribution and degradation studies with insulin-^{131} I. *J. clin. Invest.* **33**, 1252–1260.

Fox, E. G., Fyfe, W. M. & Mollison, A. W. (1954). Galactose diabetes. *Brit. med. J.* **1**, 245–247.

Fujita, A. & Iwatake, D. (1931). Bestimmung des echten Blutzuckers ohne Hefe. *Biochem. Z.* **242**, 43–60.

Goldstein, M. S., Henry, W. L., Huddlestun, B. & Levine, R. (1953). Action of insulin on transfer of sugars across cell barriers: common chemical configuration of substances responsive to action of the hormone. *Amer. J. Physiol.* **173**, 207–211.

Good, C. A., Kramer, H. & Somogyi, M. (1933). The determination of glycogen. *J. biol. Chem.* **100**, 485–491.

GUHA, B. C. (1931*a*). The physiological function of vitamin B_1. *Biochem. J.* **25**, 1367–1384.

GUHA, B. C. (1931*b*). On galactose as the dietary carbohydrate. *Biochem. J.* **25**, 1385–1390.

HAFT, D., MIRSKY, I. A. & PERISUTTI, G. (1953). Influence of insulin on uptake of monosaccharides by rat diaphragm. *Proc. Soc. exp. Biol., N.Y.*, **83**, 60–62.

HAGEDORN, H. C. & JENSEN, B. N. (1923). Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. *Biochem. Z.* **135**, 46–48.

HUDSON, F. P., IRELAND, J. T., OCKENDEN, B. G. & WHITE-JONES, R. H. (1954). Diagnosis and treatment of galactosaemia. *Brit. med. J.* **1**, 242–245.

KEILIN, D. & HARTREE, E. F. (1948). The use of glucose oxidase (notatin) for the determination of glucose in biological systems and for the study of glucose-producing systems by manometric methods. *Biochem. J.* **42**, 230–238.

LEMLEY, J. M. & MENEELY, G. R. (1952). Distribution of tissue fluid in hearts of rats subjected to anoxia. *Amer. J. Physiol.* **169**, 61–65.

LEVINE, R., GOLDSTEIN, M. S., HUDDLESTUN, B. & KLEIN, J. P. (1950). Action of insulin on the 'permeability' of cells to free hexoses, as studied by its effect on the distribution of galactose. *Amer. J. Physiol.* **163**, 70–76.

LONG, C. (1952). Studies involving enzyme phosphorylation. I. The hexokinase activity of rat tissues. *Biochem. J.* **50**, 407–415.

LUNDSGAARD, E., NIELSEN, N. A. & ØRSKOV, S. L. (1939*a*). On the possibility of demonstrating an effect of insulin on isolated mammalian liver. *Skand. Arch. Physiol.* **81**, 11–19.

LUNDSGAARD, E., NIELSEN, N. A. & ØRSKOV, S. L. (1939*b*). On the utilization of glucose and the formation of lactic acid in the isolated hind-limb preparation. *Skand. Arch. Physiol.* **81**, 20–28.

MANERY, J. F. & BALE, W. F. (1941). Penetration of radioactive sodium and phosphorus into extra- and intra-cellular phases of tissue. *Amer. J. Physiol.* **132**, 215–231.

MELDRUM, N. U. & ROUGHTON, F. J. W. (1933–4). Carbonic anhydrase, its preparation and properties. *J. Physiol.* **80**, 113–142.

PARK, C. R. (1953). The action of insulin on glucose uptake of muscle. *J. clin. Invest.* **32**, 593.

ROSENBERG, TH. & WILBRANDT, W. (1952). Enzymatic processes in cell membrane penetration. *Int. Rev. Cytol.* **1**, 65–92.

SOLS, A. & CRANE, R. K. (1954*a*). Substrate specificity of brain hexokinase. *J. biol. Chem.* **210**, 581–595.

SOLS, A. & CRANE, R. K. (1945*b*). Heart muscle hexokinase. *Fed. Proc.* **13**, 301.

STADIE, W. C., HAUGAARD, N., MARSH, J. B. & HILLS, A. G. (1949). The chemical combination of insulin with muscle of normal rat. *Amer. J. med. Sci.* **218**, 265–274.

STADIE, W. C., HAUGAARD, N. & VAUGHAN, M. (1952). Insulin binding with isotopically labelled insulin. *J. biol. Chem.* **199**, 729–739.

WIDDAS, W. F. (1954). Facilitated transfer of hexoses across the human erythrocyte membrane. *J. Physiol.* **125**, 163–180.

WIEBELHAUS, V. D. & LARDY, H. A. (1949). Phosphorylation of hexoses by brain hexokinase. *Arch. Biochem.* **21**, 321–329.