activities found earlier (Greenberg and Glick, 1960 a), after administration of ACTH, are consistent with the requirement of the hypothesis of Haynes and Berthet (1957) that TPNH generation be increased *via* the G-6-P dehydrogenase reaction, and that the source of G-6-P be G-1-P derived from glycogen. An increase in phosphorylase activity leading to production of G-1-P would also be expected, and this has been borne out by subsequent studies to be reported later.

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REFERENCES

Cohen, R. B. (1961), *Endocrinology 68*, 710.
Greenberg, L. J., and Glick, D. (1958), *Endocrinology 63*, 909.
Greenberg, L. J., and Glick, D. (1960a), *J. Biol. Chem. 235*, 3028.
Greenberg, L. J., and Glick, D. (1960b), *J. Biol. Chem. 235*, 2744.
Grunbaum, B. W., Geary, J. R., Jr., and Glick, D. (1956), *J. Histochem. Cytochem. 4*, 555.
Guidotti, G., Colombo, J.-P., and Piero, P. F. (1961), *Anal. Chem. 33*, 151.
Haynes, R. C., Jr., and Berthet, L. (1957), *J. Biol. Chem. 225*, 115.
Huggett, A. St. G., and Nixon, D. A. (1957), *Lancet 2*, 368.
Hunt, T. E., and Hunt, E. (1959), *Anat. Rec. 133*, 537.
Kemp, A., and van Heijningen, A. J. M. K. (1954), *Biochem. J. 56*, 646.
Nayyar, S. N., and Glick, D. (1954), *J. Histochem. Cytochem. 4*, 284.
Nicander, L. (1957), *Acta Anat. 31*, 388.
Noble, N. L., and Papageorge, E. (1955), *Endocrinology 57*, 492.
Planel, H., and Guilhem, A. (1960), *Compt. rend. soc. biol. 154*, 1101.

Studies on Glucose Phosphorylation in Rat Liver*

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Oxidation of glucose-C^{14} in fortified whole liver homogenates was strongly dependent on glucose concentration up to 0.1 M, whereas the oxidation of both glucose-6-P-C^{14} and fructose-C^{14} was maximal at low concentrations. With an assay procedure based on glucose-6-P formation as measured by TPN^+ reduction in the presence of an excess of purified glucose-6-P dehydrogenase, it was found that the glucokinase of rat liver has an apparent K_m of 0.01 to 0.04 M and V_{max} is reached at about 0.1 M glucose. TPN^+ reduction in this system in the presence of glucose and ATP was not appreciably inhibited by 0.06 M arsenate, whereas arsenate together with excess glucose-1-P yielded a reduction rate of less than one sixth that observed with glucose and ATP. Thus glucose-1-P was ruled out as the phosphorylation product of glucose in rat liver, and it is assumed that the direct phosphorylation product is glucose-6-P. These findings, taken in conjunction with previous data, indicate that glucose utilization in rat liver is regulated by a glucokinase whose activity is highly responsive to glucose concentration within physiologic ranges.

Despite the recognition of the central role played by the liver in carbohydrate metabolism, the mechanism of glucose utilization is obscure, owing in part to a lack of information concerning the properties of the phosphorylation system (Crane and Sols, 1955) and in part to the added complication that this organ not only utilizes but also produces glucose. A large and confusing body of literature exists which indicates that both uptake and release of glucose are influenced by hormonal and nutritional factors (Cahill *et al.*, 1959; Chernick *et al.*, 1951; Levine and Fritz, 1956; Jacobs *et al.*, 1958). The comprehensive studies of Cahill *et al.* (1958b) with rat liver slices demonstrate that glucose utilization is highly dependent on the glucose concentration of the medium and suggest that the glucokinase step may be rate-controlling. When these ob-

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servations are considered in the light of the apparent free permeability of the hepatic cell to glucose (Cahill *et al.*, 1958a), it appears that the hepatic enzyme differs from other hitherto-described animal tissue hexokinases in having a relatively low affinity for glucose. In the present report, results of studies in rat liver homogenates are reported that further define the site of glucose phosphorylation as the rate-controlling and concentration-dependent step, and these findings are supported by studies of some of the properties of the isolated enzyme reaction itself.

Methods

Materials.—The labeled hexoses used in this study were obtained from Nuclear, Chicago and were diluted before use to an activity of approximately 0.05 μc per mg. Carbon-14-labeled glucose-6-P was synthesized enzymatically from the corresponding labeled glucose as follows. Yeast hexokinase (Sigma Chemical Co., type V) having an activity of 600,000 Kunitz-McDonald (1946) units/g, in an amount of 2 mg was added to 10 ml of a solution of 5 mM $NaHCO_3$, 8 mM ATP, 5 mM $MgCl_2$, and 8mM labeled glucose.

The reaction, which was followed manometrically by CO_2 evolution at 38°, was complete in about 8 minutes. Adenine nucleotides, inorganic phosphate, and sugar phosphates were precipitated by addition of barium acetate and 4 volumes of ethanol at *p*H 8.7. The precipitate was recovered by centrifugation and dissolved in a few milliliters of water by shaking with Dowex-50 ion-exchange resin in the H^+ form, and the acidic supernatant and washings were adjusted to the phenolphthalein end-point with ammonia. The solution was then passed through a 0.5 × 7.0 cm column of Dowex-1 in the Cl^- form, and, after the column was washed with a 1% glucose solution to dilute out traces of glucose-C^{14}, the hexose phosphates were eluted with 100 ml of 0.05 M NH_4Cl. The product was obtained by precipitation, as before, with barium acetate and ethanol. After washing with 95% ethanol and drying, a yield of 61% was obtained, consisting of a mixture of roughly 80% glucose-6-P and 20% fructose-6-P (the latter being due to hexose isomerase as an impurity in the yeast hexokinase). The material thus obtained was free of inorganic phosphate, adenine nucleotides, and glucose-C^{14}, and was used without further purification after conversion to the potassium salt.

Nucleoside triphosphates, glycylglycine, and Versene were obtained from the Sigma Chemical Company, and phosphorylated glycolytic intermediates and glucose-6-P dehydrogenase were products of C. F. Boehringer and Sons, Mannheim, Germany.

All rats used in these studies were adult males of "pathogen-free" strains obtained from either Carworth Farms or Charles River Breeding Laboratories. They were killed and exsanguinated by decapitation. For experiments with slices the liver was dissected, rinsed with ice-cold *p*H 7.4 phosphate buffer, and sliced into 0.5-mm sections. After blotting on filter paper, the slices were weighed to ± 5 mg and were transferred to Warburg vessels containing the substrate in 2.0 ml of a Ca-free Krebs-Ringer phosphate solution (Umbreit *et al.*, 1951) in the outer compartment and a NaOH-soaked filter paper strip in the center well. After incubation at 37° in oxygen for specified intervals the reaction was stopped by tipping in acid, and the respiratory CO_2 was recovered and counted as $BaCO_3$ with the aid of 0.7 mmole of carrier sodium carbonate as described previously (DiPietro and Weinhouse, 1959).

Homogenates were prepared essentially as described previously (DiPietro and Weinhouse, 1959). Approximately 1 g tissue was homogenized in an all-glass homogenizer of the Potter-Elvehjem type in 9.0 ml of an ice-cold solution of 0.15 M KCl and 0.01 M potassium phosphate buffer, *p*H 7.4. A 0.5-ml portion, representing 50 mg of tissue, was added together with substrate to each Warburg flask containing an incubation medium of the following composition calculated for 2.0 ml final volume: 160 mM KCl, 5 mM $MgSO_4$, 2 mM DPN^+, 0.1 mM fumarate, 0.06 mM cytochrome *c*, 7 mM potassium phosphate buffer, *p*H 7.4. Incubation and recovery of respiratory CO_2 were conducted as described for slices, except that air was used as the gas phase.

Glucose was estimated colorimetrically by the method of Seifter and Gerstenfeld (1957); a commercial glucose oxidase preparation (Glucostat reagent) obtained from the Worthington Laboratories was used.

The preparation of extracts containing glucokinase activity and the assay of this enzyme have been described by DiPietro and Weinhouse (1960), and modifications in these procedures will be discussed with the individual experiments.

Adipose Tissue Extract.—About 10 g adipose tissue, dissected from around the kidneys and testes of a normal, fed rat, was homogenized with 10 ml of buffer as described for liver. The suspension was centrifuged at 20,000 × *g* for 20 minutes and the clear aqueous layer was drawn off with a syringe. This extract was dialyzed for 24 hours against the buffer solution used for homogenization and used for hexokinase assay without further treatment. In contrast with the liver enzyme, the adipose tissue extract was relatively stable to dialysis and contained no detectable glucose-6-phosphatase.

Calculations.—All radioactivities were measured as specific activities in cpm per standard planchet, after correction to "infinite thickness." Specific and total activities were calculated by methods previously described by DiPietro and Weinhouse (1959).

The percentage of the total respiration attributable to glucose oxidation was computed from the following relationship:

Per cent respiration from added glucose =

μatoms glucose carbon converted to CO_2 ($\times$ 100)
μmoles O_2 uptake.

Results

It was pointed out first by Olson (1951) and verified later by Cahill *et al.* (1958b) that glucose oxidation in liver slices is highly dependent on the glucose level in the medium. These findings have been repeatedly verified in our own studies, as shown by a typical experiment in Table I. A 100-fold increase in glucose concentration, from 1 to 100 mM, gave a 50-fold increase in glucose oxidation, and, what is even more striking, the rate of increase was not diminished at the higher concentrations. At the highest concentration used, namely, 100 mM, 23% of the respiration was accounted for as being derived from exogenous glucose. This increase of glucose oxidation is not always accompanied by comparable increases in O_2 uptake, though a small increase is sometimes observed.

TABLE I
GLUCOSE OXIDATION BY RAT LIVER SLICES

Glucose-U-C^{14} Concentration (mM)	O_2 Uptake (μmoles)	Glucose Carbon to CO_2 (μatoms)	Respiration from Added Glucose (%)
1.0	10.0 ± 0	0.06 ± 0	0.6 ± 0
10	14.1 ± 1.9	0.59 ± 0.05	4.2 ± 0.2
30	12.6 ± 1.2	1.3 ± 0.1	10.4 ± 0.2
50	13.2 ± 0.1	1.9 ± 0	14.4 ± 0.1
100	12.7 ± 0.6	3.0 ± 0.1	23.3 ± 0.8

Each flask contained 190 ± 5 mg of pooled liver slices from normal, fed rats, suspended in 2.0 ml of saline medium containing glucose-U-C^{14}. The flasks were incubated 1 hour at 38° with O_2 in the gas phase. Other experimental details are given in the text. Each value is the average (± the deviation) of two vessels. The data in the last column are obtained as the ratio of column 3 to column 2 ($\times$ 100).

Table II shows that the same striking response to glucose concentration occurs in a liver homogenate fortified with DPN^+ and Mg ions, and thus is not dependent on an intact cell membrane. This system is considerably more active than liver slices; oxygen uptake and glucose oxidation were about as high as in slices, with about one fourth the quantity of tissue. Without marked change in total O_2 uptake, a 50-fold increase of glucose concentration, from 2 to 100 mM, caused an 8-fold increase of glucose concentration, from 2 to 100 mM, caused an 8-fold increase of glucose oxidation. At the highest glucose concentration tested, exogenous glucose oxidation accounted for 32% of the total respiration. The second experiment in Table II shows that the effect of concentration, so marked with glucose, does not occur with fructose, whose oxidation was already more than one half the maximum at 2 mM.

The uniqueness of liver with respect to the striking concentration dependence for glucose oxidation may be seen in Figure 1, where glucose oxidation in liver is compared with that in homogenates of kidney and brain. In whole kidney homogenates, the proportion of respiration attributable to exogenous glucose oxidation was at more than 75% of the maximum at 0.5 mM, and, as reported earlier (DiPietro and Weinhouse, 1959), in whole brain homogenates it was almost at the maximum rate at 0.2 mM. In liver, however, the maximum was evidently not reached even at 0.1 M glucose.

That glucose oxidation is controlled at the phosphorylation step is evident on the basis of a comparison of glucose-U-C^{14} oxidation in the liver homogenate with that of hexose-6-P-U-C^{14} as shown in Table III. At 2 mM, glucose oxidation was low as usual, whereas at the same concentration the oxidation of hexose-6-P was 8 times higher. Increasing the concentration of both substrates to 14 mM increased glucose oxidation 8-fold but depressed both the O_2 consumption and (perhaps to a somewhat lesser extent) oxidation of hexose-6-P. We suspect that this inhibition was due to an impurity in the hexose-6-P preparation manifesting itself at higher concentrations. In experiment 2, the same picture is seen with the hexose phosphates labeled in carbons 1 and 6. At 3 mM, the oxidation of hexose-6-P carbon-1 was increased 8-fold over the comparably labeled glucose, and hexose-6-P-6-C^{14} yielded almost 40 times more radioactivity than glucose-6-C^{14}. This preferential oxidation of hexose-6-P is all the more striking when one

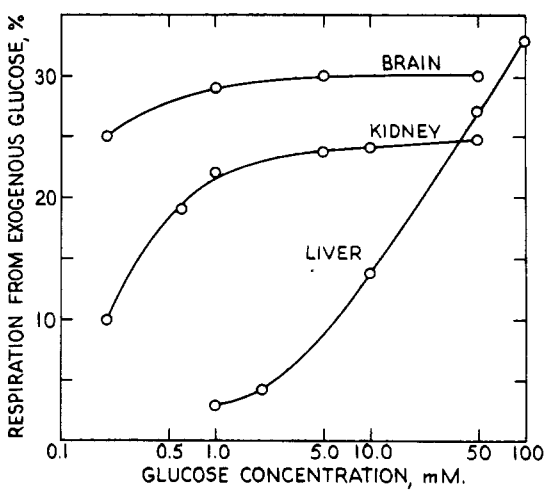

FIG. 1.—Effect of glucose concentration on respiration of tissue homogenates. Experimental conditions are described in the text. Values are given in terms of percentage of total respiration accounted for by oxidation of exogenous, uniformly C^{14}-labeled glucose, calculated as indicated in the text. Under the conditions employed, oxygen uptake in μmoles per hour was 6 for brain, 17 for kidney, and 10 for liver homogenate.

TABLE II
GLUCOSE OXIDATION BY A WHOLE HOMOGENATE OF RAT LIVER

Expt. No.	Substrate	Concentration (mM)	Oxygen Uptake (μmoles)	Glucose Carbon-14 to CO_2 (μatoms)	Respiration from Added Glucose (%)
1	Glucose	2.0	9.3 ± 0.1	0.39 ± 0.02	4.1 ± 0.4
		10	9.9 ± 0.2	1.36 ± 0.07	13.8 ± 0.5
		50	9.8 ± 0.3	2.65 ± 0.22	26.8 ± 1.5
		100	9.4 ± 0.2	3.05 ± 0.09	32.3 ± 0.8
2	Glucose	2	9.6 ± 0.2	0.34 ± 0.03	3.5 ± 0.3
		20	10.9 ± 0.4	1.9 ± 0.2	18 ± 3
		40	10.5 ± 0	2.7 ± 0	28.7 ± 0
	Fructose	2	11.2 ± 0.1	3.3 ± 0.2	29 ± 2
		20	11.9 ± 0.3	4.9 ± 0.1	41 ± 1
		40	11.3 ± 0.2	5.1 ± 0.2	44 ± 2

Each flask contained liver homogenate from a normal, fed rat equivalent to 50 mg fresh weight of liver respiring in the incubation medium described in the text. Substrate was glucose-U-C^{14} and the flasks were incubated for 1 hour in air. Each value represents the average (± standard deviation) of 3 separate vessels.

TABLE III
OXIDATION OF C^{14}-LABELED GLUCOSE AND HEXOSE-6-PHOSPHATES IN RAT LIVER HOMOGENATE

Expt. No.	Substrate	Concentration (mM)	Oxygen Uptake (μmoles)	Substrate Carbon to CO_2 (μatoms)
1	Glucose-U-C^{14}	2	7.7 ± 0.3	0.26 ± 0.03
	Hexose-6-P-U-C^{14}	2	7.5 ± 0.6	2.18 ± 0.03
	Glucose-U-C^{14}	14	7.9 ± 0.3	1.58 ± 0.01
	Hexose-6-P-U-C^{14}	14	2.9	1.66
2	Glucose-1-C^{14}	3	7.2 ± 0.2	0.078 ± 0.003
	Hexose-6-P-1-C^{14}	3	9.1 ± 0.7	0.67 ± 0
	Glucose-6-C^{14}	3	6.7 ± 0.2	0.0035 ± 0.0005
	Hexose-6-P-6-C^{14}	3	10.8 ± 0.2	0.130 ± 0

Experimental conditions as in Table II. Time of incubation was 1 hour. Each value is the average (± standard deviation) of two paired vessels.

considers that it occurred in the face of a powerful glucose-6-phosphatase activity.

Direct Studies of Hepatic Glucokinase.—The foregoing data pointed to the presence in rat liver of a glucose phosphorylation system which is far more responsive to glucose concentration in the physiologic range than any thus far reported in other tissues. While this work was in progress, however, another possible explanation of these unexpected results arose and had to be considered. Haas and Byrne (1960) and Segal (1959) demonstrated the reversibility of the action of glucose-6-phosphatase, thus raising the possibility that C^{14} from glucose enters the "pool" of glucose-6-P without net phosphorylation. Because of the high activities of this enzyme in liver, these findings raise a serious question concerning the validity of all C^{14} data pertaining to glucose utilization in this organ, and made it highly desirable to supplement the isotope tracer data with a direct study of the hepatic glucokinase. Assays of this enzyme in normal and alloxanized rats were reported by us previously (DiPietro and Weinhouse, 1960); the procedure involved the preparation of an extract freed of glucose-6-phosphatase by high-speed centrifugation according to Crane and Sols (1955) and estimation of glucose-6-P formation by spectrophotometric TPNH assay with added glucose-6-P dehydrogenase. Nearly two equivalents of TPN^+ are reduced, because of the presence in liver of 6-phosphogluconic dehydrogenase (Glock and McLean, 1953). This method proved very satisfactory for study of glucose concentration-dependence. Since it depends on measurement of product formation, the accuracy is not impaired by high substrate concentrations, and since the glucose-6-P is rapidly removed by dehydrogenation, neither product inhibition nor the competitive action of other enzymes is a problem. Under the conditions employed, the optical density changes are linear throughout the period of measurement, and the sensitivity of the spectrophotometric TPNH estimations requires the phosphorylation of only minimal quantities of glucose. At 1 mM glucose, for example, 0.4 μmole of glucose is available, whereas at the highest activity of the enzyme, with optimal substrate concentration, the O.D. change would be about 1 unit over a 10-minute period, corresponding to the total reduction of 0.06 μmole of TPN^+ and the phosphorylation of 0.03 μmole of glucose. This is equivalent to only 7.5% of the quantity added. However, because of the low activity of

the enzyme at such low glucose concentrations, utilization never approached a significant fraction of the amount present.

Typical results of many experiments in which the activity of the enzyme was measured over a wide range of glucose concentrations are shown in Figure 2. One of the drawbacks to a highly accurate estimation of glucose phosphorylation in liver extracts at low glucose concentrations is a relatively high blank, owing presumably to endogenous precursors of glucose-6-P, since the blank is about the same with or without added ATP. As shown in Figure 2, there was an initially rapid TPN^+ reduction during the first 3 or 4 minutes, then a steady rate which persisted for long periods. In practice, readings were taken every minute, and the activity was calculated from the differences in absorbancy observed between the 5th and 10th minutes. With no added glucose (some glucose was always present in the enzyme extract used) there was a "blank" value of 0.010 O.D. unit/minute, and at a glucose concentration of 0.1 M or higher, activity was maximal at 0.052 O.D. unit/minute. At 0.0025 M, activity was only slightly above the blank, and in this experiment half the maximal velocity was observed between 0.01 and 0.02 M glucose. Although a simple kinetic picture was not necessarily expected, inasmuch as at least three enzymes are involved in the estimation, nevertheless good linearity was observed in reciprocal Lineweaver-Burk plots such as is shown in the inset of Figure 2. The apparent Michaelis constant (K_m) for glucose ranged in different experiments from 0.01 to 0.04 M. With precisely the same assay procedure, a purified yeast hexokinase gave a K_m of 1×10^{-4} M, and an extract of adipose tissue also gave a K_m of 1×10^{-4} M. As shown in Figure 3, the hepatic enzyme activity was hardly measurable at a concentration of 0.001 M, where the yeast and adipose enzyme activity were already maximal. In contrast with glucose, ATP had an apparent K_m of 4×10^{-4} M, a value comparable with recorded values of 9.5×10^{-5} and 1.3×10^{-4} M for ATP for the purified yeast and brain enzymes (McDonald, 1955; Crane and Sols, 1955). That the high K_m for glucose may have been due to various inhibitors present in the crude liver extract was tested by observing the effects of addition of the liver extract to the yeast enzyme. No effect was observed either on the rate of glucose phosphorylation or on the K_m of this enzyme. In other respects, rat liver glucokinase resembles other animal tissue hexokinases. It is activated by Mg or Mn ions and is specific for ATP, no activity having been observed with UTP, ITP, or GTP. Other properties of this enzyme will be reported later.

Glucose-6-P as the Product of Glucose Phosphorylation.—It is surprising that the product of glucose phosphorylation in liver has never been conclusively identified. Although the assay proce-

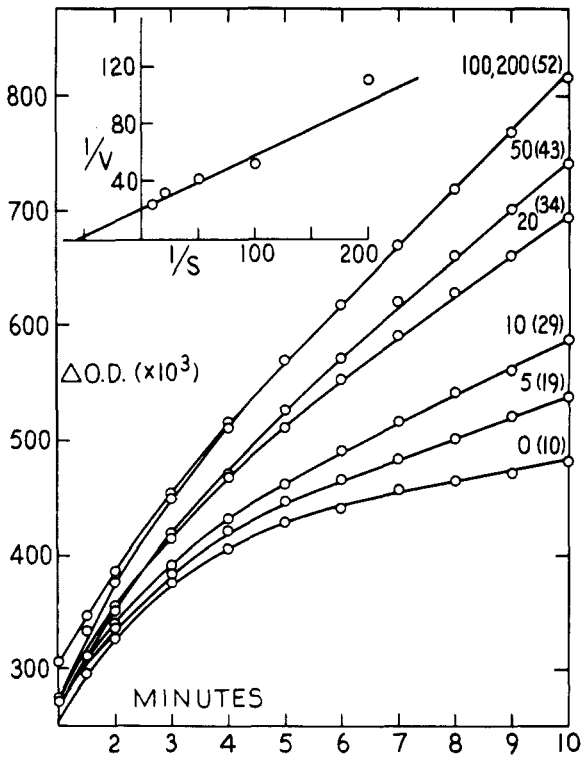

FIG. 2.—Rate of TPN^+ reduction as a function of glucose concentration in rat liver extract. Concentrations of added glucose in mM/liter are given by the numbers alongside each curve, and the numbers in parentheses are the O.D. changes ($\times 10^3$). The curve marked 0 contained all of the ingredients of the assay system, including ATP, but no added glucose. The inset shows the Lineweaver-Burk plot of these data, which yielded a K_m of 0.02 M.

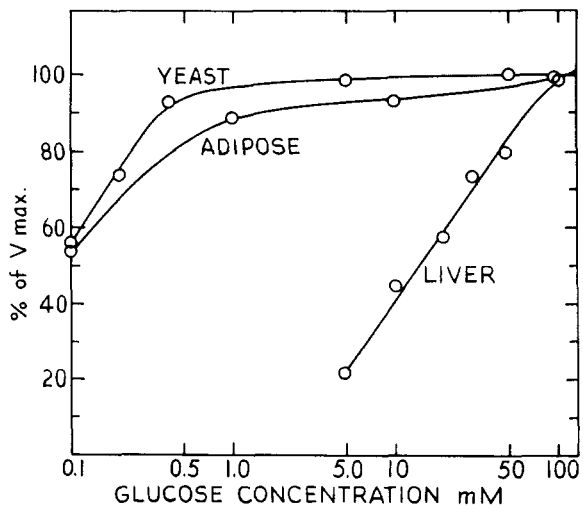

FIG. 3.—Hexokinase activities, in percentage of V_{max}, plotted as a function of glucose concentration, given in the abscissa on a logarithmic scale.

dure depends on the dehydrogenation of glucose-6-P, this does not rule out the possibility that glucose-1-P is the primary product, since this is converted to glucose-6-P by the very active phosphoglucomutase in this tissue. This possibility particularly requires consideration in view of the action of liver fructokinase in yielding fructose-1-P (Hers, 1955) and from the recent report of a glucokinase from rabbit testes which apparently yields the 1-ester (Akaeda, 1956). However, the formation of glucose-1-P in liver has been ruled out on the basis of arsenate-inhibition studies such as are shown in Table IV. In this experiment, TPN^+ reduction was measured in the glucokinase system with omissions and additions as indicated. Comparisons of lines A, B, and C show that the extract contains a very active mutase which is inhibited to the extent of 95% by addition of 0.06 M arsenate. ATP addition does not overcome the inhibition by arsenate (line E), nor does glucose addition without ATP (line K). Comparisons of lines F, G, and H show that the ATP-dependent TPN^+ reduction in the presence of glucose is inhibited only 25% by arsenate. Line I shows that glucose alone does not increase TPN^+ reduction in the presence of arsenate, and line J shows that the ATP-dependent glucose phosphorylation in the presence of arsenate is not further affected by addition of glucose-1-P. If glucose-1-P were the initial phosphorylation product, its transformation to glucose-6-P would have been almost completely inhibited. Since the ATP-dependent TPN^+ reduction was inhibited only 25%, glucose-1-P formation can be ruled out.[1]

TABLE IV

ELIMINATION OF GLUCOSE-1-P AS THE PRODUCT OF GLUCOSE PHOSPHORYLATION IN RAT LIVER

	Additions				
Cuvet	ATP	Glucose	Glucose-1-P	Arsenate	Δ O.D./Min.
A	0	0	0	0	0
B	0	0	+	+	0.010
C	0	0	+	0	0.30
D	0	0	0	+	0.002
E	+	0	+	+	0.010
F	0	+	0	0	0.006
G	+	+	0	0	0.065
H	+	+	0	+	0.050
I	0	+	0	+	0.010
J	+	+	+	+	0.050
K	0	+	+	+	0.011

Each microcuvet contained the following ingredients in such amount as to yield the indicated final concentrations in 0.40 ml glycylglycine buffer, *p*H 7.4, 44 mM: TPN^+, 0.75 mM; $MgCl_2$, 7.5 mM; glucose-6-P dehydrogenase, 0.02 Kornberg units. The substances designated in the table were present in the following final concentrations. ATP, 3 mM; glucose, 0.1 M; glucose-1-P, 0.1 mM; arsenate, 0.06 M. The reaction was started by adding 10 μl of enzyme extract, thus bringing the total volume up to 0.40 ml, and the O.D. changes were recorded each minute for at least 10 minutes. The velocity shown in the last column represents the slopes of the curves over the 5 to 10 minute interval, when the rates were strictly linear.

DISCUSSION

A direct relationship between hepatic glucose inflow and outflow and the blood glucose level seems well established. It was originally pointed out by Cori and Cori (1929–30) that glycogen synthesis in rat liver requires a high blood glucose concentration. Soskin *et al.* (1938) showed that livers of dogs release glucose at low blood levels and take up glucose at high levels. Cahill *et al.* (1958a) established that the hepatic cell membrane is freely permeable to glucose (and other sugars), and found that the level at which glucose output ceases and inflow begins is at approximately 150 mg per 100 ml (0.008 M). These findings were amplified by a variety of studies with liver slices *in vitro*, already mentioned, in which glucose utilization for a variety of processes was shown to be highly dependent on glucose concentration at physiologic ranges (Cahill *et al.*, 1958b). On the basis of the effect of glucose concentration on the incorporation of glucose-U-C^{14} into glycogen and CO_2 in rat liver slices, Spiro (1959) calculated an average K_m for glucose utilization of 5.9×10^{-2} M. Cahill *et al.* (1959) inferred on the basis of these findings that the interplay between hepatic glucokinase and glucose-6-phosphatase must exert a controlling influence on glucose utilization in this organ.

The present study with liver homogenates has affirmed these inferences concerning the rate-controlling role of glucokinase in the utilization of glucose by rat liver, and the studies of the isolated phosphorylation reaction leave no further doubt that this is the site of the high concentration dependence. Thus this enzyme plays a role which apparently is not duplicated in other animal tissues thus far studied. Muscle cells contain a hexokinase with a high affinity for glucose

[1] During the course of these studies an alternate route of glucose phosphorylation in rat liver, possibly accounting for the high dependence on glucose concentration, was considered. This involves the direct oxidation of glucose, via glucose dehydrogenase (Harrison, 1931; Strecker and Korkes, 1952), followed by the successive actions of gluconokinase (Leder, 1957) and glucose-6-P dehydrogenase acting in reverse to yield glucose-6-P. However, two observations clearly ruled out this pathway as a quantitatively important one for glucose utilization in rat liver. First, glucose dehydrogenase, when assayed by the method of Strecker and Korkes (1952) was not found in appreciable amounts in rat liver; second, "trapping" experiments were conducted in liver homogenates in which glucose-C^{14} and unlabeled gluconate were incubated together, and the remaining gluconate was isolated by adsorption and elution on an ion-exchange column. The resultant gluconate had only traces of radioactivity.

($K_m = 5 \times 10^{-4}$ M) (Morgan *et al.*, 1961), and utilization would normally proceed at maximal rates were it not for a permeability barrier at the cell membrane, responsive to such factors as insulin, hyperglycemia, anoxia, and work (Levine *et al.*, 1950; Kipnis *et al.*, 1959; Morgan *et al.*, 1961; Goldstein, 1961). Although there are no published reports on the properties of adipose tissue hexokinase, there is considerable evidence that there is powerful hormonal control of glucose utilization by this tissue (Winegrad and Renold, 1958; Wertheimer and Shafrir, 1960), and preliminary studies by us, already mentioned, confirm that the hexokinase of adipose tissue is similar to that of muscle in its affinity for glucose. It is interesting that the utilization of glucose by the rat epididymal fat pad is also enhanced by increasing the glucose concentration of the medium (Jeanrenaud and Renold, 1959; Leonards and Landau, 1960) and in this respect resembles liver. However, the relatively high affinity of adipose hexokinase for glucose suggests that in this tissue the effect is due not to a variable phosphorylation rate but more probably to permeability. Brain also contains a hexokinase with a high glucose affinity ($K_m = 8 \times 10^{-6}$ M) (Sols and Crane, 1959), and, since this tissue is apparently insensitive to insulin, glucose utilization presumably occurs at maximal rate as long as it is available in the body fluids (McIlwain, 1955).

Previous reports dealing with specific values of the K_m for hepatic glucose phosphorylation have been made by Spiro (1959) and by Lange and Kohn (1961). On the basis of the effects of glucose concentration on its incorporation into glycogen and CO_2 in rat liver slices *in vitro*, the former reported a K_m of 0.067. On the other hand the latter, who made a survey of the hexokinases of various rat tissues, reported a value of 4×10^{-5} M. These investigators used a titrimetric method based on acid production. They did not comment on the apparent discrepancy between their results and the high concentration dependence for glucose phosphorylation inferred from the liver slice data.

Neither examination of the published data nor a private conversation with these investigators has revealed a reason for this discrepancy. There is a possibility that we are dealing with different enzymes, but this seems unlikely, since the only difference in the preparation is that we centrifuged the supernatant solution at $100,000 \times g$ to remove the microsomes and the glucose-6-phosphatase, whereas Lange and Kohn centrifuged at $20,000 \times g$ and relied on the addition of fluoride to inhibit glucose-6-phosphatase. We have established that the microsome fraction thus removed by us is extremely high in glucose-6-phosphatase activity, but has essentially no glucokinase activity. Another unexplainable discrepancy is that Lange and Kohn observed specific activities of approximately 4 μmoles glucose phosphorylated per g liver per minute, whereas our optimal values were only of the order of 0.4 to 0.5 μmole per g liver per minute. Specific activities similar to ours were also reported by Vaughan *et al.* (1960), and values of this order of magnitude, obtained at 25°, compare favorably with a maximal rate of glucose phosphorylation in the perfused rat heart of 14.5 mg per g per hour (1.3 μmole per g per minute) at 37° (Morgan *et al.*, 1961). They are considerably higher than the rates of 0.1 to 0.2 μmole per g liver per minute obtained by Slein *et al.* (1950) and by Long (1952), who measured glucose disappearance in the presence of fluoride to minimize glucose-6-phosphatase activity. Long pointed out that the observed rate of glucose utilization was one third of that required to account for glycogen synthesis *in vivo*. These low values are not unexpected in the light of our studies, since the substrate concentrations of 1 to 4×10^{-3} M that they used are considerably below those required to saturate the enzyme. We pointed out in our previous report (DiPietro and Weinhouse, 1960) that the V_{max} observed by us was sufficient to account for a liver glycogen deposition of approximately 1% per hour at 37°, thus accounting for glycogen deposition in rat liver *in vivo* during periods of high blood glucose levels.[2]

The present studies, taken in conjunction with our previous results and those of other investigators with liver slices, point to glucokinase as the chief controlling factor in the hepatic uptake of glucose, and further suggest that many of the dietary and hormonal effects on hepatic glucose uptake may be attributable to the effects of these factors on the activity of this enzyme. These and other properties of hepatic glucokinase are under further investigation.

References

Akaeda, I. (1956), *J. Biochem. Tokyo 43*, 645.

Cahill, G. F., Jr., Ashmore, J., Earle, A. S., and Zottu, S. (1958a), *Am. J. Physiol. 192*, 491.

Cahill, G. F., Ashmore, J., Renold, A. E., and Hastings, A. B. (1959), *Am. J. Med. 26*, 264.

Cahill, G. F., Jr., Hastings, A. B., Ashmore, J., and Zottu, S. (1958b), *J. Biol. Chem. 230*, 125.

[2] We previously reported (DiPietro and Weinhouse, 1960) that hepatic glucokinase is lowered in fasting and diabetes. During the course of this work, our findings have been supported and extended by several contributions appearing in the Russian literature. Ilyin and Shanygina (1960a) and Shanygina (1959), using an assay method based on glucose disappearance, reported a decrease of rabbit liver hexokinase in starvation and alloxan diabetes that was restored by prior insulin injection. More recently, Ilyin and Shanygina (1960b) reported that rat liver hexokinase was suppressed by cortisone and that this effect also was overcome by prior insulin injection. Since a whole homogenate was used for enzyme assay in these studies, the effect of glucose-6-phosphatase is not clear, although the authors state in a private communication that this enzyme did not interfere.

Chernick, T. T., Chaikoff, I. L., and Abraham, S. (1951), *J. Biol. Chem. 193*, 793.
Cori, C. F., and Cori, G. T. (1929–30), *J. Biol. Chem. 85*, 275.
Crane, R. K., and Sols, A. (1955), *in* Methods in Enzymology, vol. I, Colowick, S. P., and Kaplan, N. O., editors, New York, Academic Press, Inc., p. 283.
DiPietro, D. L., and Weinhouse, S. (1959), *Arch. Biochem. Biophys. 80*, 268.
DiPietro, D. L., and Weinhouse, S. (1960), *J. Biol. Chem. 235*, 2542.
Glock, G. E., and McLean, P. (1953), *Biochem. J. 55*, 400.
Goldstein, M. J. (1961), *Am. J. Physiol. 200*, 67.
Haas, L. F., and Byrne, W. L. (1960), *Science 131*, 991.
Harrison, D. C. (1931), *Biochem. J. 25*, 1016.
Hers, H. G. (1955), *in* Methods in Enzymology, vol. I, Colowick, S. P., and Kaplan, N. O., editors, New York, Academic Press, Inc., p. 286.
Ilyin, V. S., and Shanygina, K. I. (1960a), *Problems in Med. Chem., USSR 6*, 288.
Ilyin, V. S., and Shanygina, K. I. (1960b), *Problems in Med. Chem., USSR, 6*, 291.
Jacobs, G., Reichard, G. A., Jr., Goodman, E H., Jr., Friedmann, B., and Weinhouse, S. (1958), *Diabetes 7*, 358.
Jeanrenaud, B., and Renold, A. E. (1959), *J. Biol. Chem. 234*, 3082.
Kipnis, D. M., Helmreich, E., and Cori, C. F. (1959), *J. Biol. Chem. 234*, 165.
Kunitz, M., and McDonald, M. R. (1946), *J. Gen. Physiol. 29*, 393.
Lange, C. F., and Kohn, P. (1961), *J. Biol. Chem. 236*, 1.
Leder, I. G. (1957), *J. Biol. Chem. 225*, 125.
Leonards, J. R., and Landau, B. R. (1960), *Arch. Biochem. Biophys. 91*, 194.
Levine, R., and Fritz, I. B. (1956), *Diabetes 5*, 209.
Levine, R., Goldstein, M., Huddlestun, B., and Klein, S. (1950), *Am. J. Physiol. 163*, 70.
Long, C. (1952), *Biochem. J. 50*, 407.
McDonald, M. R. (1955), *in* Methods in Enzymology, vol. I, Colowick, S. P., and Kaplan, N. O., editors, New York, Academic Press, Inc., p. 274.
McIlwain, H. (1955), Biochemistry and the Central Nervous System, Boston, Little Brown and Co.
Morgan, H. E., Henderson, M. J., Regen, D. M., and Park, C. R. (1961), *J. Biol. Chem. 236*, 253.
Olson, R. E. (1951), *Cancer Research 11*, 571.
Segal, H. L. (1959), *J. Am. Chem. Soc. 81*, 4047.
Seifter, A., and Gerstenfeld, S. (1957), *J. Lab. Clin. Med. 51*, 448.
Shanygina, K. I. (1959), Proc. Inst. Exp. Med. AMN USSR, Leningrad.
Slein, M. W., Cori, G. T., and Cori, C. F. (1950), *J. Biol. Chem. 186*, 763.
Sols, A., and Crane, R. K. (1954), *J. Biol. Chem. 210*, 581.
Soskin, S., Essex, H. E., Herrick, J. P., and Mann, F. C. (1938), *Am. J. Physiol. 124*, 558.
Spiro, R. (1959), *J. Biol. Chem. 233*, 546.
Strecker, H. J., and Korkes, S. (1952), *J. Biol. Chem. 196*, 769.
Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1951), Manometric Techniques and Tissue Metabolism, ed. 2, Minneapolis, Burgess Publishing Co.
Vaughan, D. A., Hannon, J. P., and Vaughan, L. N. (1960), *Am. J. Physiol. 199*, 1041.
Wertheimer, E., and Shafrir, E. (1960), *Recent Progr. Hormone Research 16*, 467.
Winegrad, A. I., and Renold, A. E. (1958), *J. Biol. Chem. 233*, 267.