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GLUCOSE UTILIZATION IN THE HUMAN EPIDERMIS: ITS CONTROL BY HEXOKINASE*

KENNETH M. HALPRIN, M.D. AND AKIRA OHKAWARA, M.D.

Glucose transport into the epidermal cell does not seem to be hindered by its passage through the cell membrane. The evidence for this consists of the finding of free glucose within the epidermis by Schragger (1). In addition, insulin which would be expected to affect the process of membrane transport if this posed a barrier to glucose entry has no effect on glucose entry into epidermis (2).

An appreciable intracellular concentration of free glucose would not be possible if the enzyme responsible for phosphorylating glucose, *i.e.* hexokinase, were able to utilize all of the glucose being presented to it. The finding of free glucose within the epidermis therefore implies that hexokinase activity is limiting the amount of glucose which is phosphorylated to glucose-6-phosphate. All subsequent metabolic processes (glycogen formation, glycolysis, Krebs cycle, pentose shunt, etc.) require glucose-6-phosphate as their beginning material. By limiting the amount of glucose-6-phosphate which is formed from free glucose, the hexokinase reaction is acting to control the amount of carbohydrate substrate which is available to the cell for all these other processes. It constitutes a block in carbohydrate metabolism which affects the entire economy of the cell.

There are two quite different ways to assess the activity of this reaction. One is to measure the disappearance of glucose from a glucose containing physiologic medium when an epidermal slice is allowed to float upon the surface. The cell is using all the glucose which disappears. This glucose must be phosphorylated by hexokinase to be used and the rate of disappearance of glucose is a direct measure of the hexokinase reaction. These preparations are presumably fairly close to the actual *in vivo* situation. Measurements of this kind indicate a rate of 0.20–0.4 mμm (millimicromole) of glucose used per minute per milligram of fresh epidermis (3–5).

The second method is to homogenize the tissue and to measure hexokinase activity in the homogenate. These *in vitro* assays are done under optimal conditions of high substrate concentrations and the absence of inhibitors. They measure the maximal rate of substrate turnover the amount of enzyme present within the tissue is capable of producing. We find a rate of 3.0 mμm of glucose turned into glucose-6-phosphate per minute per milligram of fresh epidermis at 37°C.

It is quite obvious from the discrepancy in the figures given by the two methods that conditions within the epidermal slice (and also presumably *in vivo*) do not correspond with the optimal conditions used in the *in vitro* assay. *In vivo* although the amount of enzyme present in 1 milligram of epidermis is enough to phosphorylate 3.0 mμm of glucose per minute it is prevented from doing so either by a lack of adequate substrate or by the presence of inhibitors.

This study was done to define the conditions *in vivo* which prevent hexokinase from acting at its maximal rate. We have quantitated the amount of the various substrates and inhibitors present in the epidermis and studied the effects such concentrations might be expected to have on human epidermal hexokinase.

MATERIALS

Epidermis from the back of normal male students aged 20–25 was removed by the use of an electrokeratome (6). Samples weighed 30–100 milligrams and contained from 0–25% dermal contamination. Anesthesia was not used.

Epidermis to be used for substrate or inhibitor concentration determinations was frozen in liquid N_2 within 5–10 seconds of removal. It was later weighed and homogenized in cold 0.6 N perchloric acid while still frozen. The perchloric acid extract was neutralized with 2 N KOH and the insoluble potassium perchlorate removed by centrifugation.

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* From the Department of Dermatology, The University of Oregon Medical School, Portland, Oregon.

The resulting clear extract was used for all substrate and inhibitor concentration determinations and is referred to hereafter as the "acid extract".

Epidermis to be used for enzyme kinetic studies was weighed and homogenized in 10 × its volume of cold .05 M "tris" buffer pH 7.4 containing .003 M Mg Cl_2 and .0005 M EDTA. This is referred to hereafter as "epidermal homogenate".

METHODS

All analyses and reactions were done fluorometrically using a Farrand model 2A fluorometer with a microammeter attachment which recorded directly into a potentiometric recorder. These analyses depend upon the fluorescence produced by the reduced pyridine nucleotides TPNH and DPNH. These nucleotides act as cofactors in many oxidation-reduction reactions and serve as useful indicators. Their oxidized forms do not absorb or fluoresce while the reduced forms absorb light of 340 mμ wavelength and then fluoresce at approximately 460 mμ. By the use of suitable filers this fluorescent wavelength can be isolated and its magnitude is a direct measure of the amount of reduced nucleotide in the solution. For a fuller discussion of this method see Lowry (7). All assays were done at 25° C.

Glucose-6-phosphate assay.—Two μl (microliters) of glucose-6-phosphate dehydrogenase (0.1 unit) and 10 μl. of TPN (0.1 μm) are added to 1 cc of tris buffer pH 7.4 and the fluorescence determined. 10 μl of "acid extract" are then added and the increase in fluorescence is noted. This increase is due to the production of TPNH during the oxidation of glucose-6-phosphate to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. The instrument must be calibrated so that the fluorescence reading can be converted into μm of TPNH or DPNH present. The amount of TPNH found is a direct measure of the amount of glucose-6-phosphate originally added to the cuvette.

Glucose assay.—Ten μl. of TPN (0.1 μm), 2 μl. of glucose-6-phosphate dehydrogenase (0.1 μl.) 10 μl of ATP (0.7 μm) and 2 μl. of hexokinase (0.1 unit) are added to 1 cc of tris buffer pH 7.4 containing .003 M $MgCl_2$ and .0005 M EDTA. The fluorescence is determined initially and after the addition of 10 μl. of "acid extract". The glucose in the extract is turned into glucose-6-phosphate by the action of hexokinase. Glucose-6-phosphate formed is immediately turned into 6-phosphogluconate by glucose-6-phosphate dehydrogenase with the simultaneous reduction of TPN to TPNH. The amount of TPNH formed is a direct measure of the amount of glucose originally in the "acid extract".

ATP assay.—This assay is exactly like the glucose assay with the single change of substituting 10 μl of glucose (0.5 μm) for the ATP.

ADP assay.—Phosphoenolpyruvate (0.7 μm) and pyruvic kinase (0.1 unit) are added to 10 μl of "acid extract" in 1 cc of buffer. The ADP is changed to ATP by this reaction and an equivalent amount of pyruvate is formed from the phosphoenolpyruvate. The pyruvate so formed is determined by adding 10 μl of DPNH (0.7 μm) and 2 μl. of lactic dehydrogenase to the tube. This enzyme converts pyruvate to lactate with the simultaneous oxidation of an equivalent amount of DPNH and this decrease in fluorescence is measured.

Hexokinase assay.—Ten μl of "homogenate" are added to 1 cc of buffer containing .003 M $MgCl_2$, .0005 M EDTA, glucose-6-phosphate dehydrogenase (.01 unit) TPN (0.1 μm) and ATP (0.7 μm). The reaction is started by adding glucose (0.5 μm) and the rate of glucose-6-phosphate formation (actually TPNH formation) is followed on the recorder. The slope of the increase is a measure of the hexokinase activity under maximal substrate conditions.

RESULTS AND DISCUSSION

Effect of glucose concentration on hexokinase activity.—Figure 1 shows the change in hexokinase activity which occurs with various glucose concentrations. It can be seen that maximal activity is obtained at a concentration of approximately 0.5 μm/cc. Figure 2 is a plot of l/substrate concentration vs. l/velocity. The Km (substrate concentration giving ½ the maximal velocity) for glucose derived from this plot is 4.1×10^{-5} M.

Table 1 shows the concentration of glucose as determined in human epidermis. These values are above the concentrations which are

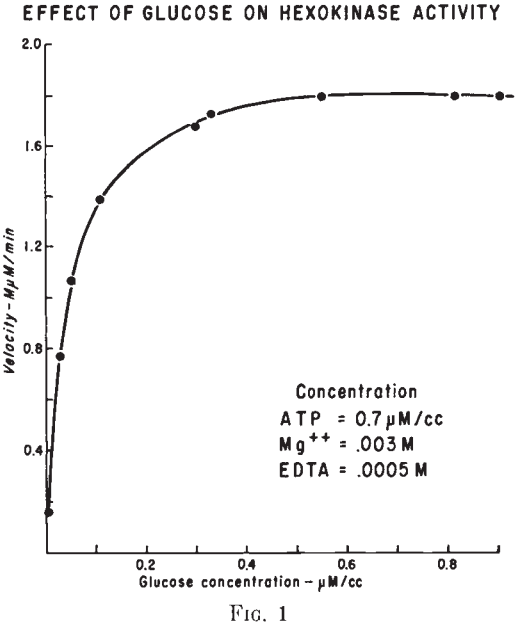

FIG. 1

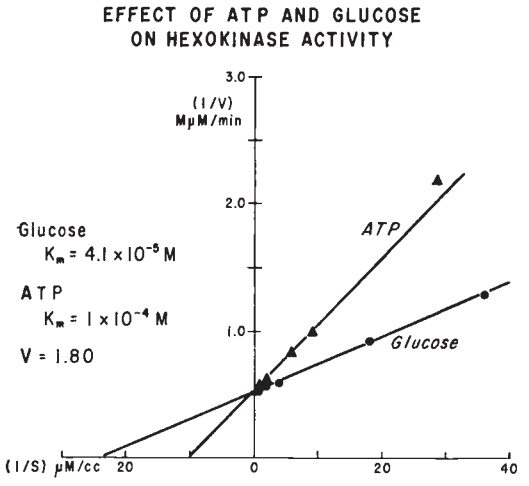

FIG. 2

TABLE 1

Substance	Concentration		% of maximal *in vitro* activity
	mμm/mg epidermis	μm/cc intracellular H_2O	
Glucose (11)*	1.25 ± 0.36	1.88	100
ATP (9)	0.28 ± 0.07	0.42	80
Glucose-6-phosphate (8)	<0.1	0.1	? See text
ADP (7)	1.6 ± 0.5	2.4	28
Phosphate (2)	11.8 ± 1.3	17.7	70
All of above conditions added together			25

* The number in parenthesis refers to the number of subjects.

needed to give maximal rates of hexokinase activity as seen in Figure 1. Depression of hexokinase activity in vivo cannot be ascribed to a lack of adequate glucose.

Effect of ATP concentration on hexokinase activity—Figure 3 shows the change in hexokinase activity which occurs with various ATP concentrations. Maximal activity requires a concentration of 0.7 μm/cc. Km for ATP = 1×10^{-4} M as seen in Figure 2.

The epidermal concentration of ATP as seen in Table 1 is 0.28 mμm/mg. This is equivalent to a fluid concentration of ATP of 0.42-μm/cc. This concentration according to Figure 3 would reduce the activity of hexokinase to 1.45

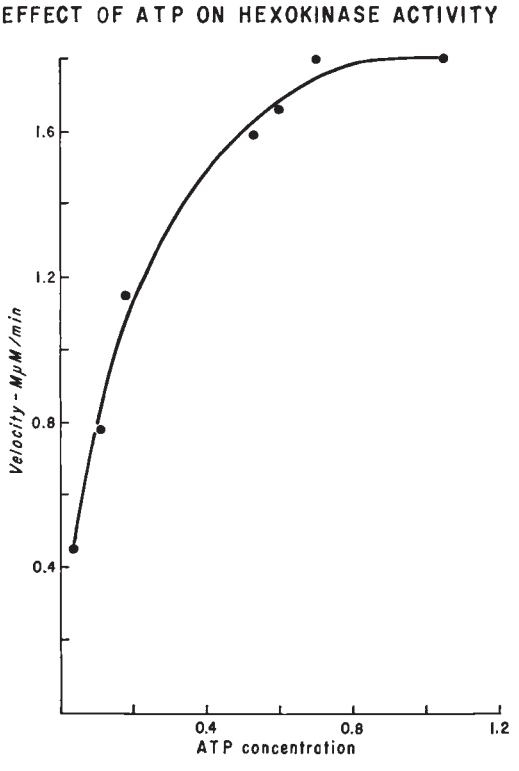

FIG. 3

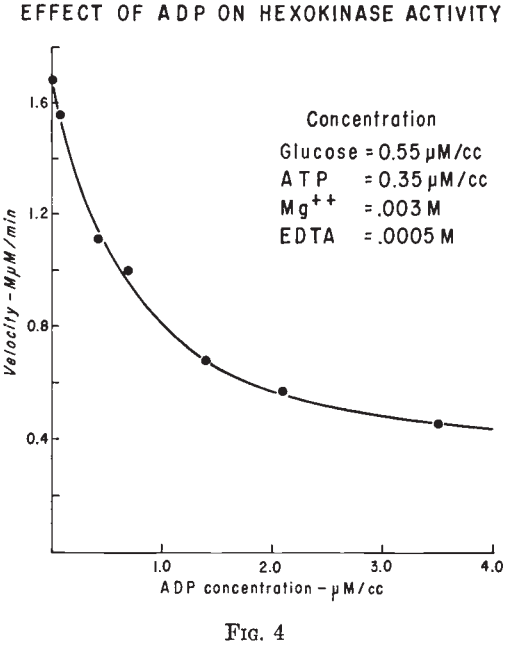

FIG. 4

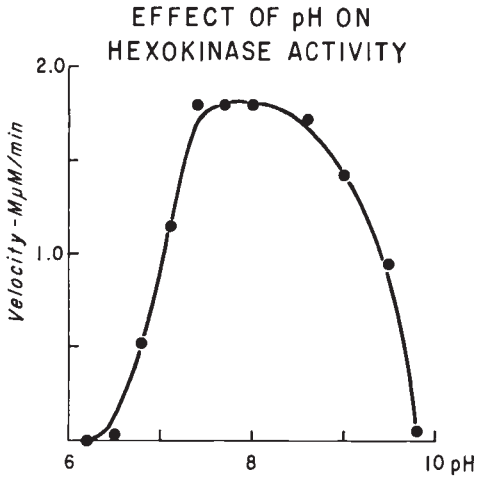

FIG. 5

mμm/min/mg from its maximum of 1.80 mμm/min/mg. This reduction is significant and may explain a part of the reduced activity seen *in vivo*. However, the rate is still 80% of maximal and is therefore not the principal controlling factor.

Effect of Glucose-6-phosphate concentration on hexokinse activity.—Unfortunately our assay system precludes dynamic measurements of the effect of this compound. However, the epidermal concentration of glucose-6-phosphate as seen in Table 1 is very low. Brain hexokinase is inhibited 50% by a glucose-6-phosphate concentration of 0.4 μm/cc (8). If epidermal hexokinase is similar to the brain enzyme the measured concentration of glucose-6-phosphate would have very little effect. This point, however, cannot be directly answered as yet.

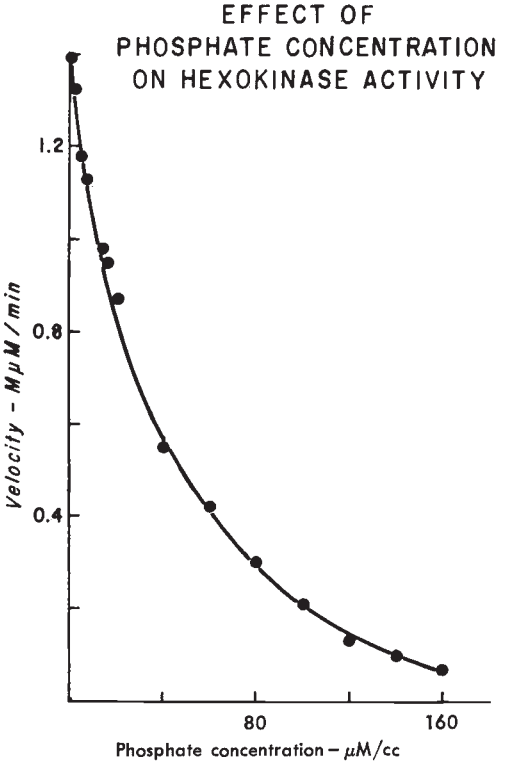

FIG. 7

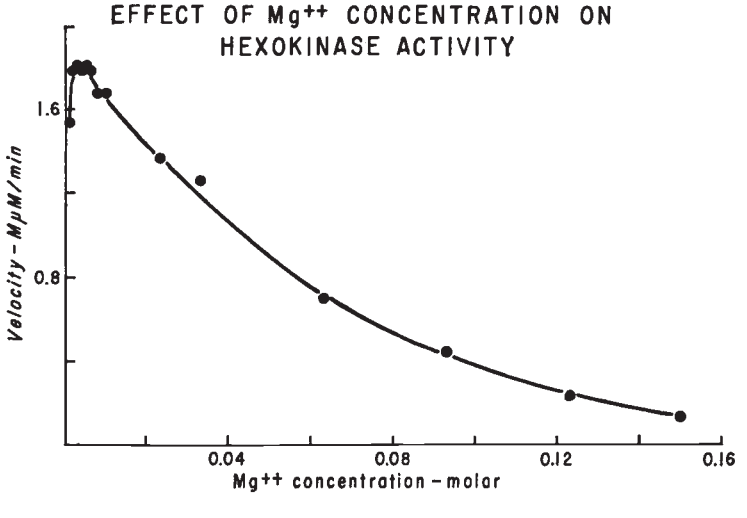

FIG. 6

Effect of ADP concentration on hexokinase activity.—Figure 4 demonstrates the effect of changing ADP concentrations. 50% inhibition occurs at a concentration of 1.0 μm/cc which is equivalent to a Ki (concentration at which velocity is reduced from its maximal by 50%) of 1×10^{-4} M. As seen in Table 1, the tissue concentration of ADP is 1.6 mμm/mg or 2.4 μm/cc when considering the intracellular fluid concentration. This concentration will reduce the hexokinase reaction to approximately 28% of its maximal value and is sufficient in itself to explain most of the *in vivo* inhibition.

Effect of pH on hexokinase activity.—Figure 5 shows the pH activity curve of hexokinase. A drop in pH from 7.4 to 7.0 would result in a 50% inhibition. It is difficult to estimate whether such an effect would occur *in vivo*, but it would certainly not be an efficient control mechanism.

Effect of Mg^{++} concentration on hexokinase activity—Maximal activity of the enzyme is obtained at magnesium concentrations of 0.002–0.007 M as seen in Figure 6. It seems unlikely that wide variations of the intracellular ionic strength would occur, but these have not been measured.

Effect of PO_4^{3-} concentration on hexokinase activity.—Phosphate is a known inhibition of hexokinase as seen in Figure 7. A concentration of 15 μm/cc (1.5×10^{-2} M) results in 25% inhibition. Measurement of phosphate in human epidermis gives values of 17.7 μm/cc and this may cause a significant reduction. It is difficult, however, to imagine very wide variations occurring in the concentration of this compound, but such a controlling factor must be considered.

Other pathways of glucose utilization.—In other organisms and tissues the direct oxidation of glucose to gluconic acid or its reduction to sorbitol are known to occur. We have looked for these pathways in epidermis and have been unable to demonstrate either the direct oxidation or a reduction to sorbitol.

Effect of insulin on hexokinase activity.—No effect could be demonstrated at concentrations of 1 unit/cc.

Total inhibitory effects.—Adding the various substrates and inhibitors together in physiologic concentrations might be expected to give additive effects which would reduce the activity of hexokinase to even lower levels than the 28% of maximal velocity covered by the ADP concentration above. However, when this is done, the total inhibition of hexokinase activity is still only 25% of maximal. Evidently the inhibitory effects are not additive and perhaps phosphate and ADP act at the same site.

SUMMARY AND CONCLUSIONS

By comparing the *in vivo* concentrations of various substrates and inhibitors of hexokinase activity with their demonstrable effects as shown *in vitro*, it has been possible to demonstrate the factors which account for the *in vivo* inhibition of hexokinase. This inhibition is due to the low concentration of ATP and especially the high concentration of ADP and inorganic phosphate.

Since hexokinase acts as a gate to allow the free flow of glucose into the various metabolic channels of the cell, ADP and perhaps inorganic phosphate are in effect controlling the entire cellular economy. Any process which lowers the ADP or the phosphate concentration will cause a direct rise in the amount of glucose being utilized by the cell. Increased glycolysis and Krebs cycle functioning which utilize ADP and manufacture ATP will therefore increase the glucose utilization by the cell while conversely an increase in synthetic processes which break ATP down into ADP will tend to lower the utilization of glucose.

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