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The Metabolism of Glucose 6-Phosphate by Mammalian Cerebral Cortex *in vitro*

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1. The metabolism of glucose 6-phosphate in rat cerebral-cortex slices *in vitro* was compared with that of glucose. It was found that a glucose 6-phosphate concentration of 25mM was required to achieve maximal oxygen uptake rates and ATP concentrations, whereas only 2mM-glucose was required. 2. When 25mM-[U-^{14}C]glucose 6-phosphate was used as substrate, the pattern of labelling of metabolites was found to be quantitatively and qualitatively similar to the pattern found with 10mM-[U-^{14}C]glucose, except that incorporation into [^{14}C]lactate was decreased, and significant amounts of [^{14}C]glucose and [^{14}C]mannose phosphate and [^{14}C]fructose phosphate were formed. 3. Unlabelled glucose (10mM) caused a tenfold decrease in the incorporation of 25mM-[U-^{14}C]glucose 6-phosphate into all metabolites except [^{14}C]glucose and [^{14}C]mannose phosphate and [^{14}C]fructose phosphate. In contrast, unlabelled glucose 6-phosphate (25mM) had no effect on the metabolism of 10mM-[U-^{14}C]glucose other than to increase markedly the incorporation into, and amount of, [^{14}C]lactate, the specific radioactivity of this compound remaining approximately the same. 4. The effect of glucose 6-phosphate in increasing lactate formation from glucose was found to occur also with a number of other phosphate esters and with inorganic phosphate. Further investigation indicated that the effect was probably due to binding of medium calcium by the phosphate moiety, thereby de-inhibiting glucose uptake. 5. Incubations carried out in a high-phosphate high-potassium medium gave a pattern of metabolism similar to that found when slices were subjected to depolarizing conditions. Tris-buffered medium gave similar results to bicarbonate-buffered saline, except that it allowed much less lactate formation from glucose. 6. Part of the glucose formed from glucose 6-phosphate was extracellular and was produced at a rate of 12μmol/h per g of tissue in Krebs tris medium when glycolysis was blocked. The amount formed was much less when 25mM-P_i or 26mM-HCO_3^- was present, the latter being in the absence of tris. 7. Glucose 6-phosphate also gave rise to an intracellular glucose pool, whereas no intracellular glucose was detectable when glucose was the substrate.

The specificity of the mammalian brain towards glucose as a primary substrate both *in vivo* and *in vitro* is well documented (for reviews see Sacks, 1969; Bachelard, 1969; Quastel, 1969; Bradford, 1968). The efficiency of most other substrates in maintaining respiration and energy-rich phosphate concentration is poor even *in vitro* where the blood–brain barrier is no longer restricting the access of these substrates to the tissue. Glucose 6-phosphate, which is accepted as the first product of glucose utilization, is present in both plasma and brain tissue in only small amounts, and is not normally available extracellularly either as an energy source or for formation of metabolites. This report describes the extent to which cerebral cortex maintained *in vitro* in a range of incubation media is able to metabolize this compound, and employ it for maintenance of energy-rich phosphates. Its performance in these respects is compared with that of glucose.

MATERIALS AND METHODS

Preparation of cortex slices. Wistar or Sprague–Dawley rats (200–250g body weight) of either sex were used. Cortex slices were cut from the outer surface of whole cerebral cortex by using a strip of razor blade and a McIlwain guide recessed to give slices 350–400μm thick

(McIlwain & Rodnight, 1962). For experiments with radioactive substrates two slices per hemisphere were taken in each vessel; otherwise only the top slice from each cortex was used, one per vessel.

Incubation with radioactive substrates. Top and second-cut slices from one cortex were incubated in Warburg flasks in 2ml of either Krebs tris medium [composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 0.75; $MgSO_4$, 1.3; tris–HCl, 26; pH7.4], Elliott–Henderson medium [composition (mM): NaCl, 98; KCl, 27; KH_2PO_4, 4; $MgSO_4$, 1.2; NaH_2PO_4, 17.5; pH7.4] (Elliott & Henderson, 1948), or Krebs bicarbonate medium [composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 0.75; $MgSO_4$, 1.3; $NaHCO_3$, 26; pH7.4, equilibrated with O_2+CO_2 (95:5)]. Small filter-paper wicks soaked with Hyamine (Packard Instrument Company, Ill., U.S.A.) were placed in the centre wells of the vessels where appropriate. Total incubation at 38°C was 60–70min which included a brief period of gassing and temperature equilibration. Approx. 10μCi of [U-^{14}C]glucose or 20μCi of [U-^{14}C]glucose 6-phosphate (The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the vessels before incubation. After incubation the slices were separated from the medium by brief centrifugation at room temperature and extracted with ethanol as described below.

Purification of radiochemicals. All radioactive substrates were purified by one-dimensional descending paper chromatography in butan-2-ol–formic acid–water (70:13.2:16.8, by vol.). After drying, a radioautogram was made overnight and the pure radioactive band was eluted with water. The solution was evaporated to dryness at 40°C on an Evapomix (Buchler Instruments, New York) and redissolved in a small volume of water. The radiochemical was used within a week of purification.

Extraction for paper chromatography. Sedimented slices were extracted three times with ice-cold 60% (v/v) ethanol and twice with water. After brief centrifugation in the cold the supernatants were evaporated to dryness on the Evapomix at 40°C. The extract was redissolved in 0.5ml of water and duplicate 50μl samples were taken for chromatography. Duplicate 50μl samples of medium were also taken for chromatography. $^{14}CO_2$ was measured by transferring the Hyamine-soaked wick directly to a scintillation vial containing 1ml of water. This was left for 20min before addition of scintillant.

Paper chromatography. Two-dimensional paper chromatography was carried out as described by Bradford, Chain, Cory & Rose (1969). The presence of fructose 6-phosphate in tissue extracts was confirmed by chromatography on columns of Dowex 50 eluted with ammonium acetate–boric acid buffers (we are indebted to Mr K. Blanshard for these analyses). Mannose 6-phosphate was identified by R_F value.

Warburg experiments to determine ATP or lactate concentrations. Top slices were incubated for 60–70min in 2ml of medium with the appropriate substrate. Hyamine-soaked wicks were in the centre well. For lactate determination the slice was removed by brief centrifugation at room temperature and the assay performed directly on samples of the medium. For ATP assay, the flask contents were tipped into a beaker, the slice picked up with forceps, drained for 2–3s and transferred into 1ml of ice-cold 1M-$HClO_4$ for extraction as described below.

Incubation in quick-transfer holders. Top slices held in McIlwain quick-transfer holder electrodes (McIlwain & Rodnight, 1962) were incubated in 5ml of Krebs tris medium containing approx. 1μCi of [*carboxy*-^{14}C]inulin (The Radiochemical Centre) and 0.25% (w/v) of inulin per vessel. The medium was gassed throughout the 1h incubation with 100% O_2. At the end of the incubation the slice was rapidly rinsed twice in substrate-free Krebs tris medium containing 0.25% non-radioactive inulin, then released into 5ml of ice-cold 1M-$HClO_4$ for extraction as described below. The incubation medium was made 1M with respect to $HClO_4$ and cooled in an ice bath for extraction.

Perchloric acid extraction. Slices were homogenized by using all-glass homogenizers (Jencons Ltd., Hemel Hempstead, Herts., U.K.) in 1M-$HClO_4$ which was 0.2M with respect to tris. After being left for 20min the homogenate was centrifuged in the cold at 5000*g* for 15min in a PR2 centrifuge (I.E.C., Mass., U.S.A.) and the pellet extracted with a further 0.5ml of 0.4M-$HClO_4$. The combined extracts were neutralized with 10M-KOH, the precipitated $KClO_4$ was spun off in the cold and the supernatant either assayed directly or quick-frozen in a solid CO_2–acetone mixture and stored frozen at −20°C.

Lactate assay. Duplicate 50 or 200μl samples of medium were assayed enzymically by the method of Hohorst (1965). Internal standards of lactate were used in every determination.

ATP assay. Method 1. The glucose 6-phosphate dehydrogenase–hexokinase method (Lamprecht & Trautschold, 1965) was used, slightly modified in that the reagent buffer had the composition (mM): $MgCl_2$, 100; NADP, 0.3; glucose, 5; tris–HCl, 200; pH7.6; 2.5ml of buffer plus 0.5ml of sample per cuvette. A 10μl sample of glucose 6-phosphate dehydrogenase (0.5mg/ml) and 10μl of hexokinase (1mg/ml) [Boehringer Corporation (London) Ltd., London W.5, U.K.] were added successively and the reaction monitored at 340nm. Internal standards of ATP (5–10nmol) were added as a routine. Standards were taken through the extraction procedure to allow for adsorption on the $KClO_4$ precipitate. This was found to be very low.

Method 2. The phosphoglycerate kinase–glyceraldehyde 3-phosphate dehydrogenase method (Adam, 1965) was used, modified in that 2.5ml of reagent buffer plus 0.5ml of sample per cuvette were used. Phosphoglycerate kinase (Boehringer) was diluted to 1mg/ml with water; the treatment of glyceraldehyde 3-phosphate dehydrogenase suggested by Adam (1965) was followed except that the final enzyme concentration in sodium pyrophosphate buffer was 1mg/ml, and mercaptoethanol was used instead of glutathione.

Glucose assay. Method 1. The glucose 6-phosphate dehydrogenase–hexokinase method (Slein, 1965), was modified in that the buffer had the composition (mM): $MgCl_2$, 10; ATP, 0.5; NADP, 0.3; tris–HCl, 200; pH8.0; 2.5ml of buffer plus 0.5ml of sample per cuvette. Enzymes were used as in the ATP assay, method 1.

Method 2. The glucose oxidase–peroxidase method of Bergmeyer & Bernt (1965) was used. Composition of reagent buffer: NaH_2PO_4, 500mM; tris, 100mM; *o*-dianisidine, 0.005%; glucose oxidase, 12.5mg/100ml; peroxidase, 4mg/100ml. A 0.5ml sample plus 2.5ml of

reagent was incubated at 37°C for 30min, and the extinction read at 440nm. A standard curve was run for every batch of reagent buffer used.

Amino acids. Medium amino acids were determined by using the automated ion-exchange column method described by Bradford & Thomas (1969) with added norleucine as standard.

Lactate relative specific radioactivity determination. Duplicate 50μl samples of incubation medium from Warburg experiments were applied to Whatman no. 1 paper and descending one-dimensional chromatography was performed in butan-2-ol–formic acid–water (70:13.2:16.8, by vol.). A marker of [U-^{14}C]lactate was run in parallel as a routine. After drying in an atmosphere of NH_3, autoradiograms were prepared over a period of 1–5 days. The spots were cut out and transferred directly into scintillation vials with 1ml of water. An interval of 20min was allowed to elapse before scintillant was added. Lactate mass was determined on duplicate 0.2ml samples of the original medium as described above. The lactate relative specific radioactivity was calculated as the lactate/added substrate specific radioactivity ratio.

Phosphate. This was assayed by the method of Martin & Doty (1949).

Titration of phosphates. An automatic titration system was used in conjunction with a scale expander (Radiometer, Model TTT 1c/SBR2c/SBU1/TTA31). Temperature was controlled at 38°C by using a water jacket. A 4.0ml sample of NaH_2PO_4 (1mM) was titrated against 0.5ml of KOH (8mM), and 4.0ml of disodium glucose 6-phosphate (1mM) was titrated against 0.5ml of HCl (8mM). Both solutions were titrated in the presence or absence of Ca^{2+} (as $CaCl_2$; 25 or 50mM). The ionic strength of all solutions was adjusted at 0.2 by using tetramethylammonium bromide before titration. Glucose 6-phosphate–calcium titrations were also carried out at I0.4 to show that tetramethylammonium bromide, unlike KCl, had no interaction with the phosphate group. The presence of calcium caused a displacement of the titration curve to the acid side, through formation of $CaHPO_4$ or calcium glucose 6-phosphate and this displacement (ΔpH) was used to calculate the stability constants for complex formation:

$$K = \frac{[CaHPO_4]}{[Ca^{2+}][HPO_4^{2-}]}$$

or

$$K = \frac{[\text{calcium glucose 6-phosphate}]}{[Ca^{2+}][\text{glucose 6-phosphate}^{2-}]}$$

by using the relationship of Trevelyan, Mann & Harrison (1952):

$$\Delta pH = \log(1+[Ca^{2+}]K)$$

RESULTS

Respiration, ATP and lactate. The respiratory response of cortex slices in Krebs tris medium to increasing concentrations of both glucose 6-phosphate and equimolar glucose is shown in Fig. 1. The maximal respiratory response was stimulated by only 2mM-glucose, but required glucose 6-phosphate at 25mM. Three times as much lactate accumulated in the medium in a 1h incubation

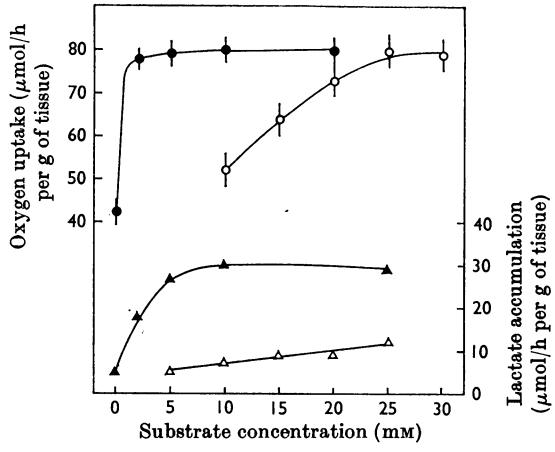


Fig. 1. Respiration and lactate production of cortex slices with different substrates. Cortex top slices were incubated for 1h at 37°C in 2ml of Krebs tris medium containing either glucose (●, ▲) or glucose 6-phosphate (○, △). Lactate was determined as described in the Materials and Methods section. Respirations (●, ○), means±S.D. of eight experiments. Lactate production (▲, △), means of six experiments.

period with 10mM-glucose than accumulated with 25mM-glucose 6-phosphate (Fig. 1), confirming the observation that respiratory saturation is not coincident with maximal lactate formation (Chain, Rose, Masi & Pocchiari, 1969). At 25mM-glucose 6-phosphate, tissue concentrations of ATP were similar to those obtained with 10mM-glucose, but with 10mM-glucose 6-phosphate, when respiration was not maximal, ATP concentrations were less than half the values with glucose (Table 1). On the basis of these results, incubations were carried out with a minimal glucose 6-phosphate concentration of 25mM throughout the rest of this work where optimum metabolic performance was required.

Metabolic patterns. After 1h incubation of cortex slices with 25mM-[U-^{14}C]glucose 6-phosphate, the metabolic pattern obtained was strikingly different from that seen after incubation with [U-^{14}C]-glucose (Plate 1). This difference was of both a qualitative and a quantitative nature. Thus, incorporation of ^{14}C into both fructose phosphate and mannose phosphate occurred with [U-^{14}C]glucose 6-phosphate, together with incorporation into glucose, but incorporation into lactate was less than with [U-^{14}C]glucose in agreement with the lower total lactate accumulation (Table 2 and Fig. 1). To determine whether the mannose phosphate and fructose phosphate were formed intracellularly or extracellularly from enzymes that had leaked from the tissue, cortex slices were incubated for 30min in Krebs tris medium containing either no substrate,

Table 1. *Adenosine triphosphate concentrations in cortex slices*

Cortex slices (approx. 50 mg weight) were incubated at 38°C in 5 ml (method 1) or 2 ml (method 2) of Krebs tris medium for 1 h before extraction with $HClO_4$. The ATP analysis methods are described in the Materials and Methods section. Values are mean±S.D. with numbers of experiments in parentheses.

Substrate	Concn. of ATP (μmol/g wet wt.) Method 1	Method 2
None	0.20±0.03 (6)*	
Glucose, 1 mM	0.79±0.23 (6)	
Glucose, 10 mM	0.90±0.19 (31)	1.00±0.15 (6)
Glucose 6-phosphate, 10 mM	0.40±0.10 (8)*	
Glucose 6-phosphate, 25 mM	0.72±0.36 (6)	0.93±0.09 (6)
Glucose 6-phosphate, 25 mM plus glucose, 10 mM	1.13±0.21 (6)	1.10±0.09 (6)

* Difference from 10 mM-glucose value significant, $P<0.01$

Table 2. *Comparison of* [U-^{14}C]*glucose 6-phosphate and* [U-^{14}C]*glucose metabolism in cortex slices*

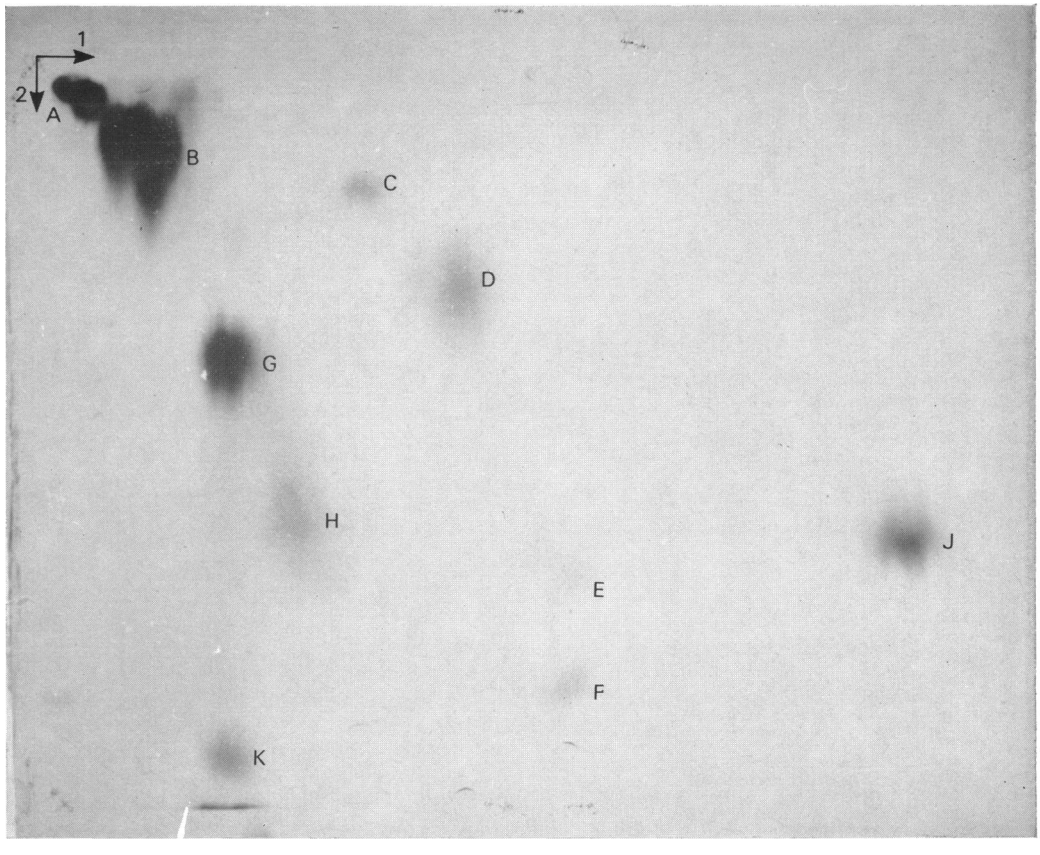
Top and second-cut cortex slices (approx. 50 mg weight each) from 1 hemisphere were incubated together in 2 ml of Krebs tris medium at 38°C for 1 h with 20 μCi of [U-^{14}C]glucose 6-phosphate or 10 μCi of [U-^{14}C]-glucose. The tissue was extracted with ethanol and water. Extracted metabolites and incubation medium were chromatographed separately and radioactivity was measured in the chromatographic spots as described in the Materials and Methods section. Values are those from tissue and incubation medium combined and are mean±S.E.M. adjusted for specific radioactivity of substrate at 500 d.p.m./nmol.

Metabolite	Radioactivity incorporated (d.p.m./mg wet wt.) [U-^{14}C]Glucose 6-phosphate (25 mM)	[U-^{14}C]Glucose (10 mM)	[U-^{14}C]Glucose 6-phosphate (25 mM) plus glucose (10 mM)
Aspartate	720±62	452±71*	trace
Glutamate	1296±112	1441±260	84±9*
Mannose phosphate and fructose phosphate	3288±291	not detected	4339±421
Glucose	1411±121	—	4504±326*
Glucose in tissue only	1036±81	—	2478±165*
Glutamine	not detected	403±82	—
Alanine	275±53	374±51	40±6*
γ-Aminobutyric acid	354±54	460±66	91±8
Lactate	3614±371	6381±397*	866±88*
CO_2	2909±256	2232±202	336±48*
Unknown	398±49	not detected	28±5*
Total	14265	11743	10288
Respiration (μmol of O_2/h per g of tissue)	85±5	80±9	89±14
Number of experiments	12	12	12

* Difference from value with 25 mM-[U-^{14}C]glucose 6-phosphate is significant, $P<0.01$.

or 10 mM-glucose, or 25 mM-glucose 6-phosphate. The slice was then sedimented at room temperature and discarded, the supernatant was collected and made 25 mM with respect to [U-^{14}C]glucose 6-phosphate. After a further hour of incubation at 38°C, no evidence of formation of radioactive mannose phosphate and fructose phosphate was found.

Effects of non-radioactive glucose on metabolic patterns. Addition of 10 mM-glucose to cortex slices incubated with 25 mM-[U-^{14}C]glucose 6-phosphate caused a tenfold decrease in the incorporation of



EXPLANATION OF PLATE 1

Radioautogram showing pattern of metabolites produced from [U-^{14}C]glucose 6-phosphate by cortex slices incubated for 1 h in Krebs tris medium. Solvent 1, butan-2-ol–formic acid–water (70:13.2:16.8, by vol.); solvent 2, phenol–water–ammonia (80:20:3, w/v/v). A, origin; B, glucose 6-phosphate; C, aspartate; D, glutamate; E, alanine; F, γ-aminobutyric acid; G, glucose; H, mannose phosphate and fructose phosphate; J, lactate; K, unknown.

radioactivity into glutamate, aspartate, alanine and γ-aminobutyrate and the d.p.m. recovered in [^{14}C]-lactate decreased by 80% (Table 2). Actual lactate accumulation, however, increased threefold to values substantially higher than those characteristic of glucose alone at 10mM (Table 4). Respiration did not significantly change though incorporation of ^{14}C into carbon dioxide was decreased eight- or nine-fold (Table 2). Radioactivity recovered in glucose, however, increased threefold, most of this increase being in medium glucose, and mannose phosphate and fructose phosphate did not show a decreased incorporation.

Metabolic patterns with [U-^{14}C]*glucose and non-radioactive glucose 6-phosphate.* Except for one metabolite, the overall metabolic pattern of 10mM-[U-^{14}C]glucose was not significantly changed either quantitatively or qualitatively by addition of unlabelled glucose 6-phosphate at 25mM or 50mM (Table 3). Lactate, however, showed a massively

Table 3. *Effect of non-radioactive glucose 6-phosphate on* [U-^{14}C]*glucose metabolism*

Top and second-cut cortex slices (approx. 50mg weight each) from 1 hemisphere were incubated together for 1h in Krebs tris medium with the substrates indicated; 10μCi of [U-^{14}C]glucose was added to each flask. Tissue extraction, chromatography and measurement of radioactivity was as described in the Materials and Methods section. Values are means±S.E.M. adjusted to a specific radioactivity of [U-^{14}C]glucose of 500d.p.m./nmol.

	Radioactivity incorporated (d.p.m./mg wet wt.)		
Metabolite	[U-^{14}C]Glucose (10mM)	[U-^{14}C]Glucose (10mM) plus glucose 6-phosphate (25mM)	[U-^{14}C]Glucose (10mM) plus glucose 6-phosphate (50mM)
Aspartate	452±71	350±15	385±98
Glutamate	1441±260	1331±105	1984±458
Glutamine	403±82	408±30	272±100
Alanine	374±51	232±15	345±125
α-Aminobutyric acid	460±66	331±25	411±160
Lactate	6281±397	8810±800	10980±1200
CO_2	2232±202	1941±195	1861±225
Total	11643	13403	16238
Lactate relative specific radioactivity calculated from values in the medium	0.29±0.04	0.30±0.07	0.32±0.07
Respiration (μmol of O_2/h per g of tissue)	80±9	87±3	91±6
Number of experiments	12	3	3

Table 4. *Effect of various compounds on lactate formation from glucose and pyruvate*

Cortex slices (50mg) were incubated for 1h at 38°C in 2ml of Krebs tris medium, pH7.4, containing 10mM-glucose, 10mM-pyruvate or 20mM-fructose, and the additional compounds indicated. Lactate in the medium was measured as described in the Materials and Methods section. Values are means±S.E.M. of number of experiments in parentheses.

	Lactate in medium (μmol/g of tissue)		
Added compound (25mM)	Glucose (10mM)	Fructose (20mM)	Pyruvate (10mM)
None	35.6±2.2 (23)	30.7±2.6 (6)	24±2 (3)
Glucose 6-phosphate	56.5±3.5 (30)		41±2 (3)
Glucose 1-phosphate	64.5±7.6 (6)		
Sodium glycerophosphate	59.7±4 (6)		
Sodium phosphate	87.7±2.5 (5)	39.2±1.8 (6)	
Sodium sulphate	39.9±2.2 (6)		
Glucose 6-phosphate, with Ca^{2+} in the medium increased to 4.1mM	39.9±1.7 (6)		
Glucose 6-phosphate, with Mg^{2+} in the medium increased to 7.3mM	58.6±2.4 (6)		

increased incorporation of radioactivity in parallel with an equivalent increase in total lactate formation (Table 4), the specific radioactivity showing no significant change. This effect of glucose 6-phosphate of elevating the lactate formation from glucose increased with the glucose 6-phosphate concentration (Fig. 2). However, increasing total lactate formation was not an effect that was specific to glucose 6-phosphate nor was it specific to glucose, since glucose 1-phosphate, glycerophosphate, and even phosphate itself were also able to elevate lactate accumulation from glucose as well as from pyruvate. Sulphate, however, was without effect (Table 4). Further investigation of the capacity of added glucose 6-phosphate to increase lactate accumulation by cortex slices showed that it could be decreased or prevented by increasing the calcium concentration in the medium (Table 4). This suggests that an interaction occurs between glucose 6-phosphate and calcium that restricts the availability of calcium in the free ionic form. Mg^{2+} could not replace Ca^{2+} in these experiments indicating the specificity of the lactate response to the presence of Ca^{2+}.

Incubation in different media. The experiments described above were carried out with Krebs tris buffer as incubation medium. When the high-phosphate, high-potassium and calcium-free Elliott–Henderson medium was employed certain quantitative differences in the metabolism of glucose 6-phosphate were observed (Table 5). Thus respiration showed a small significant increase (13%), as did total lactate and [^{14}C]lactate formation (Table 5). Incorporation into glutamate in the tissue was closer to aspartate than was observed with Krebs tris medium but analysis showed that glutamate and aspartate were lost to the medium (Table 7). When the total incorporation into glutamate and aspartate was examined, their ratio was similar to that measured after metabolism of [U-^{14}C]-glucose. This shows the importance of measuring metabolites in both tissue and incubation medium, and helps to explain the differences between these results and those of other workers (Balliano, Masi & Pocchiari, 1969). Formation of [^{14}C]glucose and [^{14}C]hexose phosphates was, however, decreased to approximately one-half. Bicarbonate medium showed fewer differences from the tris medium, most of the metabolites from glucose 6-phosphate showing a similar extent of radioactive incorporation. Mannose phosphate and fructose phosphate did, however, show decreased incorporation (Table 5). Lactate formation from glucose in the bicarbonate medium was also sensitive to calcium concentration, its accumulation in the presence of 0.75mM-Ca^{2+} was 40% greater than in the presence of 1.3 to 3.75mM (Table 6). In the absence of Ca^{2+} and presence of the specific calcium-complexing agent EGTA [ethanedioxybis(ethylamine)tetra-acetate] the accumulation of lactate increased by more than 100% compared with its production in the presence of 1.3mM-Ca^{2+}. Addition of phosphate to 25mM had little further effect, whereas when tris was the buffering ion, lactate accumulation in the presence of 25mM-phosphate was substantially greater than when Ca^{2+} was omitted (Table 6).

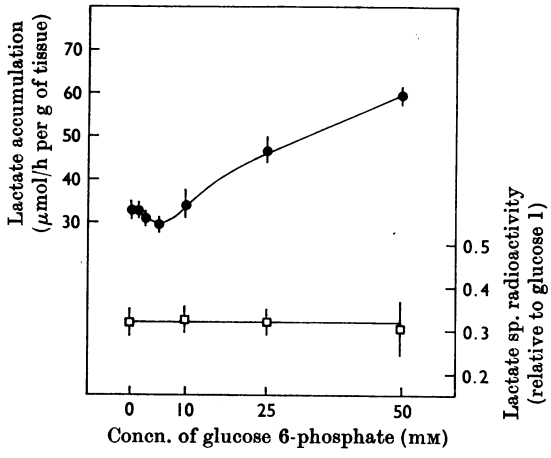


Fig. 2. Effect of glucose 6-phosphate on lactate production and lactate relative specific radioactivity from glucose substrate. Cortex slices were incubated for 1h at 37°C in 2ml of Krebs tris medium containing 10mM-[U-^{14}C]glucose, and glucose 6-phosphate at the indicated concentration. Lactate, and lactate relative specific radioactivity, were determined as described in the Materials and Methods section. ●, Lactate accumulated, means± S.E.M.; □, lactate relative specific radioactivity, means± S.E.M.

Formation of glucose from glucose 6-phosphate. Part of the radioactive glucose formed from added [U-^{14}C]glucose 6-phosphate was recovered from the medium, and some was found in the tissue extracts (Table 5). To examine whether this latter glucose was intracellular or was present within the extracellular spaces and in medium adhering to the tissue, [*carboxy*-^{14}C]inulin was included in the medium as a marker of extracellular space. Table 8 shows that the total glucose/[^{14}C]inulin ratio in the tissue was tenfold higher than this same ratio in the medium. When these ratios were measured with 1mM-glucose as substrate the opposite ratio was found, suggesting that a large proportion of the glucose generated from glucose 6-phosphate is intracellular.

The measurement of the amount of glucose produced in the medium from glucose 6-phosphate is difficult, since actively metabolizing tissue will rapidly take it up. Therefore, glycolysis and oxidative metabolism were inhibited with iodo-

Table 5. *Comparison of* [U-^{14}C]*glucose 6-phosphate metabolism in Elliott–Henderson, Krebs tris and Krebs bicarbonate media*

Top and second-cut cortex slices (approx. 50 mg each) from 1 hemisphere were incubated together for 1 h in 2 ml of the media indicated with 25 mM-glucose 6-phosphate and 20 μCi of [U-^{14}C]glucose 6-phosphate. Tissue extraction, chromatography and measurement of radioactivity was as described in the Materials and Methods section. Values are mean of the number of experiments indicated. Standard deviations are less than 10% of the mean.

	Radioactivity incorporated (d.p.m./mg wet wt.)								
Medium ...	Elliott–Henderson			Krebs tris			Krebs bicarbonate		
Metabolite	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total
Aspartate	1035	trace	1035	560	not detected	560	500	not detected	500
Glutamate	1770	325	2095	1370	not detected	1370	1058	not detected	1058
Glucose	720	—*	(720)	1305	—*	(1305)	1490	—*	(1490)
Mannose phosphate and fructose phosphate	1140	370	1510	907	1554	2461	407	not detected	407
Alanine	275	trace	275	275	not detected	275	20	not detected	20
γ-Aminobutyric acid	315	trace	315	305	not detected	305	140	not detected	140
Lactate	2009	4726	6735	1100	2450	3550	976	2962	3938
CO_2			3670			2080			not determined
Total			16355			11906			(7553)
Respiration (μmol of O_2/h per g of tissue)			97±11			85±5			
Number of experiments			8			8			3

* Medium glucose incorporation was not determined because of the poor separation from [^{14}C]glucose 6-phosphate.

acetate and cyanide. The effectiveness of the inhibitors is shown by the low O_2 uptake and lactate formation in their presence (Table 9). It is likely that the glucose produced under these conditions is coming directly from glucose 6-phosphate due to the action of a (not necessarily specific) phosphatase since an equimolar quantity of P_i is produced (Table 9). If this is the case, the phospholytic activity is probably unaffected by cyanide and iodoacetate, since the amount of P_i produced is similar whether these poisons are present or absent (Table 9). A substantial decrease in the amount of glucose formed when both cyanide and iodoacetate were present was found when the P_i concentration was increased in Krebs tris saline buffer, and also when Krebs bicarbonate saline buffer was used instead of Krebs tris saline. It is notable in this context that P_i and HCO_3^- show a marked inhibitory effect on isolated liver glucose 6-phosphatase (Vianna & Nordlie, 1969; Dyson, Anderson & Nordlie, 1969).

Another possibility was that the glucose 6-phosphatase activity was present only in the regions at the slice periphery that were degenerating due to the damage they received during tissue slicing. This was investigated by comparing glucose formation from slices cut from the outer convexity of the cerebral cortex and having only one cut surface (top slices), with that from slices cut subsequently from the cortex and with two cut surfaces (2nd slices). Analysis showed no increased glucose production/h per g of tissue from 2nd slices indicating no correlation with tissue damage. In the presence of iodoacetate and cyanide, the value for top slices was 12.6±1.1 μmol of glucose/h per g of tissue; for the second slice it was 11.5±1.4 μmol of glucose/h per g of tissue (means±S.D. from five experiments each).

Glucose 6-phosphate and hexose monophosphate shunt activity. The effect of glucose 6-phosphate in increasing the conversion of glucose into lactate (Fig. 2; Table 3) could be the result of increased hexose monophosphate shunt activity in the presence of high concentrations of glucose 6-phosphate. Thus increased NADPH concentrations could lead to a similar change in NADH through the activity of the transhydrogenase (Stein, Kaplan & Ciotti, 1959), and lactate formation could be facilitated.

Table 6. *Effect of calcium on lactate formation from glucose in different media*

Cortex slices (50mg) were incubated for 1h at 38°C in 2ml of indicated medium with pH adjusted to 7.2–7.4. Lactate in the medium was measured as described in the Materials and Methods section. Values are means±S.E.M. of the number of experiments in parentheses.

Substrate (mM)	$CaCl_2$ (mM)	NaH_2PO_4 added (mM)	Lactate in medium (μmol/g of tissue)
(*a*) Krebs-tris medium			
None	0	0	4.5±0.2 (6)
None	0.75	0	2.8±0.5 (5)
Glucose (10)	0	0	51.6±3.2 (6)
Glucose (10) with EGTA (0.5)	0	0	51.8 (3)
Glucose (10)	0.75	0	35.6±2.2 (23)
Glucose (10)	2.6	0	33.5±2.7 (6)
Glucose (10)	0.75	24	87.7±2.5 (5)
Glucose 6-phosphate (25)	0.75	0	16.5±2.2 (8)
Glucose 6-phosphate (25)	5	0	11.4±0.7 (6)
Glucose 6-phosphate (25)	0.75	24	13.0±0.9 (6)
(*b*) Krebs bicarbonate medium			
Glucose (10) with EGAT (0.5)	0	0	160 (3)
Glucose (10)	0.75	0	105.2±2.6 (25)
Glucose (10)	1.3	0	69.0±2.4 (6)
Glucose (10)	2.6	0	67.1±1.0 (20)
Glucose (10)	3.75	0	64.8±1.3 (4)
Glucose (10) with EGTA (0.5)	0	24	170 (3)
Glucose 6-phosphate (25)	0.75	0	13.8±0.4 (6)

Table 7. *Loss of amino acids from cortex slices to the incubation medium*

Cortex slices (50mg approx.) were incubated in 2ml of the medium indicated for 1h at 38°C with 25mM-glucose 6-phosphate as substrate. Amino acids were measured in the medium, and in tissue $HClO_4$ extracts, as described in the Materials and Methods section.

	Elliott–Henderson medium		Krebs tris medium	
Compound	nmol of amino acid/g of tissue in medium (means±S.E.M.)	Medium amino acid (% of total amino acid)	nmol of amino acid/g of tissue in medium (means±S.E.M.)	Medium amino acid (% of total amino acid)
Aspartate	725±180	26	90±45	4
Glutamate	1540±230	30	505±95	11
Glycine	750±90	49	330±35	29
Alanine	820±110	58	285±50	43
γ-Aminobutyric acid	200±40	17	90±30	8
No. of experiments	14		6	

However, no increase in hexose monophosphate shunt activity could be detected when glucose 6-phosphate was present at 25mM (Table 10).

Titration of phosphate and glucose 6-phosphate. Interaction between calcium and phosphate and calcium and glucose 6-phosphate could be demonstrated by titration. Table 11 shows the pH values due to these interactions. Stability constants for the calcium phosphate ($CaHPO_4$) and calcium glucose 6-phosphate ($CaPO_4$–glucose) complexes were 1.87 and 1.46 (log values) respectively, the constant for $CaHPO_4$ being close to published values (Sillén & Martell, 1964). This allowed some estimation of the proportion of calcium bound in these complexes in incubation media. Thus, in Krebs medium containing Ca^{2+} at 0.75mM and phosphate at 10mM, 45% of the calcium is bound. With phosphate at 25mM, 65% of the calcium is bound in this form. In addition, 25mM-P_i contains a concentration of PO_4^{3-} ions at pH7.4 which is significant in relation to the solubility product of $Ca_3(PO_4)_2$, and a further amount of calcium would be bound in this form. Krebs tris medium containing 0.75mM-calcium and 25mM-glucose 6-phosphate would contain 40% of the calcium in this un-ionized form.

Table 8. [*carboxy*-^{14}C]*Inulin and glucose content of cortex slices and incubation medium*

Cortex slices (50mg approx.) held in McIlwain quick-transfer holders were incubated for 1h in 5ml of Krebs tris medium containing the substrate indicated and 0.25% (w/v) inulin together with 1μCi of [*carboxy*-^{14}C]inulin. Slices were rinsed in medium containing 0.25% non-radioactive inulin before extraction and analysis of glucose and inulin as described in the Materials and Methods section. Tissue and medium extract volumes were identical. Values are means±S.D. of the number of experiments indicated.

	Glucose 6-phosphate (25mM)			Glucose (1mM)		
	Glucose (μmol/g of tissue) (*a*)	[^{14}C]Inulin (d.p.m. per extract) (*b*)	$10^5 \times$ Ratio *a*/*b*	Glucose (μmol/g of tissue) (*a*)	[^{14}C]Inulin d.p.m. per extract) (*b*)	$10^5 \times$ Ratio *a*/*b*
Tissue	0.29	5640	5.14	0.050	518	9.66
Medium	2.23	468270	0.476	61.42	8963	684
Tissue *a*/*b* / Medium *a*/*b*		10.75			0.143	
Number of experiments		6			6	

Table 9. *Formation of glucose and phosphate from glucose 6-phosphate*

Cortex slices (50mg approx.) were incubated for 1h at 38°C in 2 ml of the medium indicated (pH7.4) containing 25mM-glucose 6-phosphate with or without 1mM-KCN and 10mM-sodium iodoacetate. Glucose and phosphate were measured as described in the Materials and Methods section. Values are means±S.E.M. of the number of experiments indicated. Phosphate, lactate and glucose values are for medium only. Values are expressed as μmol/h per g of tissue.

	Glucose		Phosphate		Oxygen uptake		Lactate	
Medium	Poisons added	No poisons	Poisons added	No poisons	Poisons added	No poisons	Poisons added	No poisons
Krebs tris	12.2±1.2	2.1±0.2	16.1±1.1	15.0±1.0	14±1	83±3	4.7±0.3	16.5±2.2
Krebs tris containing 21mM-sodium phosphate and no Ca^{2+}	2.5	0.9	—	—	18±2	96±5	not determined	
Krebs bicarbonate	3.3±0.2	not detectable	not determined		—	—	7.0±0.2	18.7±0.3
Number of experiments	6	6	6	6	8	6	8	6

In tris medium, interaction between cationic tris and anionic phosphate is likely to occur, and titration allowed estimation of the stability constant for the resulting tris–phosphate complex (log value approx. 1.0). These experiments were more difficult to interpret because of the buffering capacity of the tris ion. However, the possibility arises that tris, like calcium, could also interact with membrane phosphate moieties, and this could help to explain the relatively low lactate formation from glucose found in Krebs tris medium as compared with Krebs bicarbonate medium (Table 5).

DISCUSSION

Several observations of particular note arise from this study of glucose 6-phosphate metabolism by cortex slices. Outstanding among these are the accumulation of fructose phosphate and mannose phosphate and the formation of an apparently intracellular pool of glucose. In addition, glucose 6-phosphate appears to have little influence on the quantitative or qualitative pattern of glucose metabolism over a period of 1h in spite of its demonstrable entry to intracellular regions of the tissue. This is an important observation in view of the inhibition of isolated brain hexokinase by low concentrations of glucose 6-phosphate (Crane & Sols, 1954).

Glucose and glucose 6-phosphate as substrates. Except for glucose and hexose phosphate production, the overall metabolic patterns of glucose and glucose 6-phosphate were basically similar. The considerable incorporation of radioactivity into glucose and into fructose phosphate and mannose phosphate was previously shown by Balliano *et al.* (1969).

Both substrates were able to stimulate respiration to a maximal rate of 80–90μmol of O_2/h per g fresh wt. though these rates were achieved with

Table 10. *Effect of glucose 6-phosphate on hexose monophosphate-shunt activity*

Cortex slices (50 mg approx.) were incubated for 1 h at 38°C in Krebs tris medium containing either $^{14}C_1$- or U-^{14}C-labelled substrate. Relative specific radioactivity in lactate was determined as described in the Materials and Methods section. Theoretically, shunt activity = 0 when lactate relative specific radioactivity from [$^{14}C_1$]glucose (or [$^{14}C_1$]glucose 6-phosphate) = lactate relative specific radioactivity from [U-^{14}C]glucose (or [U-^{14}C]glucose 6-phosphate). Shunt activity > 0 is indicated by a ratio < 1. Values are means ± S.E.M. of the number of experiments in parentheses.

(*a*) Shunt activity from glucose (10 mM)

Glucose 6-phosphate added (mM)	Lactate relative sp. radioactivity from [$^{14}C_1$]glucose / Lactate relative sp. radioactivity from [U-^{14}C]glucose ratio
0	1.2±0.2 (12)
10	0.9±0.2 (6)
25	1.2±0.1 (7)

(*b*) Shunt activity from glucose 6-phosphate

Substrate	Lactate relative sp. radioactivity from [$^{14}C_1$]glucose 6-phosphate / Lactate relative sp. radioactivity from [U-^{14}C]glucose 6-phosphate ratio
Glucose 6-phosphate (10 mM)	1.1±0.2 (4)
Glucose 6-phosphate (10 mM) plus glucose (10 mM)	1.3±0.3 (4)
Glucose 6-phosphate (25 mM)	1.3±0.2 (4)
Glucose 6-phosphate (25 mM) plus glucose (10 mM)	1.0±0.2 (5)

Table 11. *Titration of P_i and glucose 6-phosphate in the presence and absence of calcium*

NaH_2PO_4 was titrated against alkali, and disodium glucose phosphate against acid, as described in the Materials and Methods section. The calcium concentration was increased, at constant ionic strength, and the titration repeated. The difference in pH between the curves, in the presence and absence of calcium, (ΔpH) was measured in the region of pK. The ΔpH was then substituted in the equation (Materials and Methods section) to calculate the stability constant, K. All titrations were carried out at 38°C. For calcium glucose phosphate, mean log K = 1.46.

Solution	I	Concn. of calcium (M)	ΔpH	log K
$H_2PO_4^-$	0.2	0.022	0.43	1.87
Glucose phosphate^{2-}	0.2	0.0248	0.23	1.45
Glucose phosphate^{2-}	0.2	0.0496	0.40	1.48
Glucose phosphate^{2-}	0.4	0.0248	0.23	1.45
Glucose phosphate^{2-}	0.4	0.0496	0.39	1.47

glucose 6-phosphate at 25 mM and glucose at only 1–2 mM, as shown by Balliano *et al.* (1969). At these concentrations lactate accumulation from the two substrates and tissue ATP concentrations were similar. This concentration of glucose 6-phosphate generates insufficient glucose through the action of the phosphatase to account stoicheiometrically for both the observed respiration and lactate formation by the slice. It cannot therefore be argued that glucose 6-phosphate causes local production of glucose on the membrane surface which would be equivalent to a medium glucose concentration of 1–2 mM. It appears, therefore, that glucose 6-phosphate enters the cell as the intact molecule as well as being first converted into glucose extracellularly. Moreover, it seems likely that mannose phosphate and fructose phosphate are formed intracellularly from glucose 6-phosphate since the enzymes responsible for their production, namely glucose phosphate isomerase (EC 5.3.1.9) and mannose phosphate isomerase (EC 5.3.1.8) are soluble enzymes in brain (Johnson, 1960; Bruns, Noltmann & Williamsen, 1958) and no evidence was found of leakage of these enzymes into the incubation medium.

Glucose formation from glucose 6-phosphate. Brain is usually thought to be free of glucose 6-phosphatase activity (Scrutton & Utter, 1968).

Nevertheless, experiments reported here show appreciable generation of glucose from glucose 6-phosphate. The generation of glucose has also been observed during the passage of glucose 6-phosphate through human brain *in vivo* (Sacks & Sacks, 1968).

Some of the glucose formed in the absence of metabolic poisons is associated with the non-inulin space and is likely to be intracellular. Since incubation with 1mM-glucose did not give rise to a similar pool, the tissue glucose formed from glucose 6-phosphate is unlikely to have come from externally formed glucose. If glucose 6-phosphate were converted into glucose in the same cellular compartment as the main glycolytic sequence, it should presumably suffer the same fate as glucose entering the compartment from outside, since brain hexokinase is very active (Bachelard, 1969). However, 1mM-glucose externally did not give rise to intracellular glucose. It is possible, therefore, that tissue glucose formation from glucose 6-phosphate takes place in a compartment other than containing the main glycolytic sequence. This is supported by the observation that incorporation into tissue [^{14}C]-glucose from [^{14}C]glucose 6-phosphate is not diminished by added non-radioactive glucose; in fact there is a substantial increase in the amount of ^{14}C present as glucose.

The rate of glucose formation from 25mM-glucose 6-phosphate in the presence of metabolic poisons was 12μmol/h per g fresh wt., which is only half that required to account for the observed respiration and lactate formation in Krebs tris medium in the absence of poisons. As argued above, only part of the observed glucose 6-phosphate utilization is therefore likely to occur through initial formation of glucose. The proportion of glucose 6-phosphate utilized via this pathway would be even less in Krebs bicarbonate or Elliott–Henderson medium since glucose formation was decreased to one-quarter or less in the presence of HCO_3^- or P_i, with poisons present, yet respiration and lactate formation in the absence of poisons were similar in all three media.

Influence of glucose on glucose 6-phosphate metabolism. As shown by other workers, when glucose was present utilization of glucose 6-phosphate was greatly decreased. Non-radioactive glucose 6-phosphate was without effect on the metabolism of [U-^{14}C]glucose except for its influence in elevating lactate production without changing its specific radioactivity, i.e. without contributing to this lactate itself. Since isolated brain hexokinase is inhibited by low concentrations of glucose 6-phosphate (Crane & Sols, 1954), continued entry of glucose 6-phosphate could be expected to decrease the extent to which glucose is metabolized. Glucose 6-phosphate does in fact continue to enter the tissue in the presence of glucose as judged by its incorporation into fructose phosphate and mannose phosphate, and the continuing high rate of formation of glucose (Table 2). Thus, either glucose 6-phosphate does not enter the compartment in which hexokinase is phosphorylating glucose or its inhibition of hexokinase is not so effective with the enzyme *in situ*. Alternatively, there may exist a glucose phosphorylating mechanism that does not involve hexokinase.

Incubation in different media. The different metabolic pattern observed when Elliott–Henderson medium rather than Krebs tris medium is used can be explained by the high K^+ and phosphate concentration of the former. Thus, 31mM-K^+ is known to depolarize cortex tissue (Hillman, Campbell & McIlwain, 1963; Gibson & McIlwain, 1965), and raised potassium causes release to the medium of aspartate, glutamate and γ-aminobutyric acid (Machiyama, Balazs, Hammond, Julian & Richter, 1970; Arnfred & Hertz, 1971). P_i inhibits glucose 6-phosphatase activity and the observed lower incorporation of radioactivity into glucose is likely to have been the result of such an inhibition.

Effect of phosphates on lactate formation. The formation of lactate from glucose appears to increase with the concentration of phosphate in the incubation medium, and this effect is also caused by organic phosphates including glucose 6-phosphate. Moreover, this stimulatory effect of glucose 6-phosphate on glucose uptake and conversion into lactate can be prevented by a parallel increase in the calcium concentration of the medium, suggesting that interaction with calcium is involved. The specific involvement of calcium in this mechanism was shown by the ineffectiveness of changing the magnesium concentration to an equivalent extent, magnesium also forming soluble complexes with phosphate. Decreasing the calcium concentration to low values is known to stimulate glucose utilization and lactate output by brain slices, and considerable binding of calcium by both P_i and glucose 6-phosphate could be demonstrated by titration. It should be noted that in Krebs phosphate media buffered with 10 or 20mM-phosphate a very large proportion of the calcium is present as the un-ionized soluble monohydrogen phosphate.

Lactate formation from glucose occurs at a high rate in calcium-free bicarbonate medium (160μmol/g fresh wt.) but proceeds at only one-third of this rate when HCO_3^- is replaced by tris, though both rates are decreased by increasing calcium. Since evidence of interaction between tris and P_i was obtained by titration it is possible that both the calcium and tris cations could be decreasing glucose uptake by interacting with phosphate groups in the membrane that are involved in glucose transport.

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