STUDIES ON INSULIN ACTION. I. THE STEADY LEVEL OF GLUCOSE ACCUMULATION IN INSULIN-TREATED FROG MUSCLE AT 0°C.*

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SUMMARY

Glucose transport is maintained by frog muscles incubated at **0°C** while at the same time phosphorylation is reduced to an undetectable level. Based on these findings, a new method for studying the control of glucose transport by insulin and other hormones and drugs is introduced and used to study the effect of preincubation with insulin at **25°C** on subsequent uptake of glucose by frog muscle at **0°C.** In the absence of added insulin the steady level of glucose in the tissue water after overnight incubation at **0°C** is no higher, and usually lower than the external concentration. Following preincubation with insulin, at low external glucose concentrations **(<18 mM)** the steady level of glucose in the tissue water is higher than the external concentration. At high external glucose concentrations **(>25 mM)** the steady level in the tissue water is lower than the external concentration. This result is not changed when the data are corrected for the glucose in the extracellular space. The implications of these results are discussed in terms of the membrane theory and the association-induction hypothesis.

Höber[1] and Lundsgaard[2] suggested that insulin controls glucose metabolism by regulating the permeability of the cell membrane to sugar. At the time, however, the prevailing view was that insulin controlled the activity of the enzyme hexokinase which catalyzes the formation of glucose-6-phosphate (see Levine and Goldstein[3] for review). Increased glucose transport was held to be a secondary consequence of the increased utilization. In 1949 and 1950, Levine, Goldstein and their co-workers[4,5] showed that insulin promotes the uptake of D-galactose by the tissues of eviscerated-nephrectomized dogs. Since D-galactose is not metabolized in dog tissues, insulin must act directly on its transport into the cells. The implication was that insulin promotes the transport of glucose in a similar manner. Direct evidence in support of this concept was first provided by Park and his co-workers using rat diaphragm.[6-8] They saturated the hexokinase system with a high glucose concentration and found that free glucose was accumulated at a higher concentration in the presence than in the absence of insulin. The same effect could be seen at normal glucose concentrations if the enzymatic activity of hexokinase was decreased by lowering

* **A preliminary report of this work was given at the Federation Meetings (see Ling[71]).**

the temperature to 10°C. These results have been confirmed and extended to other tissues in a number of laboratories (see refs. 27,38,60-70). Narahara, Özand and Cori' and Özand et al.[10] were the first to show an increased accumulation of free glucose in frog sartorius muscles in response to treatment with bovine insulin.

The rapid advances in the understanding of insulin action in the last two decades attest to the power of the basic methods introduced by Levine, Park and others. Nevertheless, it has not been possible to study the transport of glucose uncomplicated by its removal due to phosphorylation. In the present communication we describe a method which allows a quantitative study of glucose transport under conditions where phosphorylation is reduced to an undetectable level. This method has been used to study the steady level of free glucose attained in freshly isolated as well as insulin-treated frog muscle at varying external glucose concentrations.

MATERIALS AND METHODS

Materials

Each unit of tissue consisted of one each of sartorius, iliofibularis, tibialis anticus longus, and semitendinosus muscles isolated with care from different leopard frogs (*Rana pipiens*, Schreber). After isolation, the tissues were used directly or kept overnight at 4°C in Ringer-phosphate solution (see reference 11, Appendix F).

Chemicals were C.P. grade. Labeled glucose (U-C-14) was obtained from Calbiochem (Los Angeles, Calif.); from International Chemical and Nuclear Corp. (City of Industry, Calif.), glucose-1-C-14, Lot #C1203 and glucose-1-H-3, Lot #10211; and from Schwartz (Orangeburg, N.Y.), U-C-14, Lot #6301.

In some early experiments, commercial insulin was used (Iletin, Eli Lilly). In most other experiments we employed a crystalline insulin preparation containing 24 units/mg from Sigma Chemical Co. (St. Louis, Mo., Lots #26B-0700 and 55B-1820). A preparation of crystalline beef zinc insulin containing 24 units/mg of insulin which was practically free of glucagon (<0.0003%)* (Eli Lilly, Lot #PJ4609) was used to check occasionally for possible glucagon interference.

Care of Frogs in the Laboratory

Frogs were kept in a large stainless steel tank with running water at room temperature. Since July 1967, they were force-fed about 3 grams of lean beef hamburger immediately upon arrival from the dealer and then once every 3-7 days. Such frogs are in a far more uniform nutritional condition than unfed frogs and give more consistent results, particularly in glucose experiments.

Method of Insulin Treatment

To study the effect of exogenous insulin, the tissue was subjected to a preliminary

*We are indebted to Dr. O. K. Behrens of Eli Lilly for this sample.

incubation (henceforth referred to as preincubation) in Ringer-phosphate solution containing insulin (400 mg of tissue to 500 ml of solution) and maintained at 25°C with gentle shaking at a rate similar to that used by Narahara, Özand and Cori.[9] In some experiments the preincubation medium was changed one or more times.

Uptake Studies

A. Indirect Method: Estimating loss from the bathing medium.

One to 8 g of muscle tissue were shaken in 3-20 ml of solution in a small Erlenmeyer flask or L-shaped tube placed in an Aminco constant temperature bath (0° ± 0.05°C). Aliquots of the bathing solution were taken at measured intervals after the introduction of the muscles. Uptake of labeled glucose by the tissue was calculated on the basis of loss of radioactivity from the solution and expressed in terms of moles/g of fresh tissue.

B. Direct Method: Direct estimation of tissue uptake.

One unit of tissue was placed on the side of a small glass specimen vial containing 1.5 to 3 ml of incubation medium. The vial was closed with poly-seal screw cap and submerged vertically in a 0°C bath for 10 minutes to bring the temperature of all components to 0°C. The muscles were then immersed in the medium by gently shaking and the entire tube submerged in the 0°C bath with its long axis in the direction of the shaking motion (shaking rate as above). The bathing solution was a Ringer-phosphate solution modified by substituting a specified concentration of glucose for its osmotic equivalent of NaCl. Approximately 0.25 μc/ml of either H^3 or C^{14}-labeled glucose were added. Glucose uptake was not changed by increasing the amount of bicarbonate in the Ringer solution from 6.5 mM to 25.3 mM as used by other workers in the field.[12,13]

Following incubation (usually 18 hours) muscles were removed from the vials and blotted in a stack of moist prechilled filter paper. The muscles were then frozen in liquid air and weighed.

Method for the Extraction of Tissue Glucose

In earlier experiments we followed the procedure of Somogyi:[14] boiling water extraction was followed by precipitation of protein with $Ba(OH)_2$ and $ZnSO_4$. In later work we relied on a new simplified method of extraction with cold trichloracetic acid (TCA). Table 1 shows that soaking the frozen muscle in 5% TCA for 18 hours at 4°C without grinding or shaking is adequate to extract the labeled sugar.

Measurement of Radioactivity

C-14 or H-3-labeled compounds were assayed in a Packard Tricarb liquid scintillation counter (Model 314EX). 0.5 ml of sample containing 5% TCA was added to 5 or 10 ml of Bray's scintillation fluid[15] in a glass vial. The use of TCA allows this ratio of sample to scintillation fluid to be used with no precipitation. Although a consistent concentration of TCA was used throughout (counting efficiency about 48% for C^{14}- and 4% for H^3-labeled compounds in 5% TCA) it is necessary to have correction factors when there are

	18 hours extraction		44 hours extraction			
	A	B	C	D	E	F
Muscle Type	Extracted counts	% of total counts	Extracted counts	% of total counts	Excess or deficient counts in homogenized residue	Total counts
1	9.25×10^4	99	9.31×10^4	100	+388	9.35×10^4
2	9.17×10^4	100	9.18×10^4	100	+150	9.20×10^4
3	8.55×10^4	98	8.70×10^4	100	- 18	8.70×10^4
4	6.15×10^4	100	6.15×10^4	100	+ 65	6.16×10^4
Average		100%		100%		

Table 1. Recovery of labeled glucose by simplified extraction procedure.
Muscles were incubated at 0°C for 48 hours in Ringer-phosphate solution containing 10 mM of glucose and 0.25 μc/ml glucose-C^{14}. After freezing and weighing, muscles were introduced into 2.0 ml of 5% TCA and kept in a coldroom (2°-4°C). 0.5 ml aliquots of the TCA extract were taken at 18 hours (Column A) and 44 hours (Column C), added to 10 ml of Bray's fluid and counted. The residues were then weighed and homogenized in 2.0 ml more 5% TCA. After centrifugation 0.5 ml aliquots were counted. The total counts obtained from this extraction were subtracted from the counts anticipated on the basis of the water content of the residue and the counts assayed in the second extraction. The difference and total counts are shown in Columns E and F respectively. Muscle types are 1. sartorius, 2. semitendinosus, 3. tibialis anticus longus, and 4. iliofibularis.

significant differences in composition between the samples and standards (e.g., initial sample of bathing solution vs. final bathing solution, variation in glucose concentration, etc.). These factors were obtained from a "silent" experiment in which everything was closely similar to the actual experiment except that no radioactivity was added. Using a repeat-delivery micropipette with less than 1% error (Hamilton Co., Whittier, Calif.) about 30 microliters of labeled glucose were added to each of the vials containing scintillation fluid plus the final diluted standard, the initial or final bathing solution, or a tissue extract, all from the silent experiment. Average values from quadruplicate determinations gave the relative counting efficiency of each of the types of samples added. These values were used to make suitable corrections in the actual experimental data. Both tritium and C-14-labeled compounds were handled similarly and after suitable correction for differences in the quenching factors gave entirely similar experimental results.

Thin Layer Chromatography (TLC)

Ready-made plastic sheets precoated with cellulose powder (polygram Cel 300), were obtained from Brinkmann Inst. Inc., Westbury, N.Y. Traces of heavy metals were removed by washing with 1% oxalic acid, followed by rinsing and washing with 0.1% EDTA. After a thorough final washing with distilled water, the sheets were dried in a gentle stream of dust-free air.

Tissues were extracted with 5-10% TCA in the cold (see above). The extract was neutralized with NH_4OH in the cold and treated with ethyl alcohol to precipitate the acid soluble glycogen, which is then removed by centrifugation. The clear supernatant was then passed through a small column of sulfonate exchange resin in the NH_4^- form. The volume of the extract was reduced in a vacuum at room temperature and 2 to 10 λ

were spotted on the thin layer sheets.

Two dimensional chromatograms were obtained using the Phase I and Phase II solvents as well as developing solutions recommended by Waring and Ziporin.[16]

Radiochromatography

Radiochromatograms of the developed chromatograms were made on Kodak non-screen X-ray film (Blue Brand) exposed from 2 days to 3 weeks.

Enzymatic Assay of Glucose

D-glucose was assayed with the glucose oxidase-peroxidase system commercially available from Worthington Biochemical Corporation, Freehold, N.J. (Glucostat). Developed color was read on a Klett-Summerson colorimeter.

Assay of Water Content

The water content of muscle tissue was determined by weighing before and after drying at 100°C for 24 hours.

Assay of K^+ and Na^+ Ion Content

These ions were extracted by placing the muscles in 3 ml of 0.1 N HCl overnight in the cold. Samples of the extracts were diluted with NaCl (for K^+ assay) or KH_2PO_4 (for Na^+ assay) to give a final concentration of 100 mM. These "radiation buffers" eliminate mutual and self-interference among the various ions. K^+ and Na^+ ion contents were assayed on a Beckman DU spectrophorometer with flame attachment.[17]

Measurement of the Resting Potential

The resting potentials of individual muscle fibers were measured by the microelectrode technique described by Ling and Gerard.[17]

RESULTS

PART I: EFFECT OF 0°C INCUBATION ON PHOSPHORYLATION

Survival of Isolated Frog Muscles in Vitro

The standard procedure adopted in the present series of studies includes 6 to 8 hours of preincubation at 25°C followed by 16-18 hours of incubation at 0°C with shaking. Table 2 shows the K^+ ion content and the resting potential of the muscles following this treatment. Both values compare favorably with the normal values of 85.8 μmoles/g for the K^+ ion content (Ling,[11] page 217) and 84.5 mV for the resting potential.[17] Table 3 shows the percentage of water in frog muscles following the standard treatment in the absence and presence of insulin (0.1 unit/ml) and 24 mM glucose. There is no essential difference between the experimental values and the percentage of water in freshly isolated muscles.

Thus, judging from the ion and water contents as well as the resting potential, the procedures of preincubation and incubation produce no measurable harmful effect. This

is in general agreement with the finding of Narahara and Özand[18] that reducing the shaking rate of free-floating sartorius muscles from 110 to 60 oscillations per minute does not alter the rate of uptake of 3-methyl glucose by frog sartorius muscles.

	K^+ ion μmoles/g	Resting Potential mV
Sartorius	85.7 ± 0.62 (4)	85.3 ± 2.4 (28)
Iliofibularis	74.6 ± 1.79 (4)	88.2 ± 3.8 (24)
Semitendinosus	81.6 ± 2.40 (4)	84.9 ± 3.9 (24)
Tibialis Anticus Longus	73.9 ± 2.98 (4)	86.4 ± 3.2 (24)
Mixed	79.8 ± 1.4	85.7 ± 3.3

Table 2. K^+ ion content and resting potential of mixed frog muscles after preincubation and incubation procedures.
Mixed muscles were dissected and preincubated 6 hours at 25°C with shaking in 100 ml of Ringer phosphate containing 24 mM glucose. They were then transferred to small vials containing the same Ringer solution and incubated 19 hours with shaking at 0°C. For flame photometry muscles were removed, blotted, weighed, and placed in 3 ml 0.1 N HCl overnight for extraction of ions. The extracts were diluted with NaCl to a concentration of 0.1 M Na^+ ion and analyzed by flame photometry. Resting potential measurements were made on separate muscles from four frogs. Values are means ± standard error (number of measurements).

	Control	Experimental		
		A.	B	C
Sartorius	81.3 ± 0.4 (4)	80.4 ± 0.4 (4)	79.9 (2)	81.8 (2)
Semitendinosus	80.5 ± 0.5 (4)	81.8 ± 0.1 (4)	78.9 (2)	81.5 (2)
Tibialis Anticus Longus	80.6 ± 0.5 (4)	80.8 ± 0.4 (4)	79.6 (2)	80.0 (1)
Iliofibularis	80.6 ± 0.9 (4)	79.8 ± 0.9 (4)	79.6 (2)	81.7 (2)
Average	80.7 ± 0.3	80.4 ± 0.3	79.5 ± 0.4	81.3 ± 0.5

Table 3. Water content of frog muscles.
Control muscles freshly isolated, weighed, dried and dry weights obtained. Experimental muscles preincubated 6 hours at 25°C and incubated 17 hours at 0°C before obtaining wet weight. A. Preincubation and incubation solutions contained 0.1 u/ml insulin and 24 mM glucose. B. Solutions contained no insulin, 24 mM glucose. C. Solutions contained no glucose, no insulin. Value expressed as per cent water in fresh or incubated tissue ± standard error (number of muscles). The overall average is 80.7%.

Frog	Experiment No.	Insulin (u/ml)	$[glucose^*]_{tis}$ (μmoles/g)
A	1	0	9.47 ± 1.30
A	2	0.1	9.01 ± 0.71
B	3	0	9.68 ± 0.90
B	4	0.1	9.28 ± 0.65

Table 4. The lack of detectable effect of insulin at 0° on glucose uptake.
Paired muscles from the same frogs were incubated at 0°C for 20 hours in Ringer solution containing 24 mM glucose and labeled glucose with and without insulin. Following incubation radioactive glucose was extracted with TCA and counted as in the text. Each set of data represents the mean of four determinations ± standard error.

The Time Course of Glucose Uptake by Frog Muscles at O°C

When freshly isolated frog muscles are introduced into a Ringer solution containing labeled glucose (0°C) the radioactivity in the bathing medium falls, reaching a constant level in about 10 hours (Fig. 1). This new steady level of labeled glucose is maintained as long as we measured it (27 hours in the experiment shown in Fig. 1). In the absence of detectable glucose phosphorylation (see below) such a steady level indicates that the rates of entry and exit of glucose are equal.

When insulin (0.1 unit/ml) is introduced into the bathing solution at O°C, no discernible effect can be seen within 24 hours (Table 4). If, on the other hand, the muscles are preincubated at 25°C in a solution containing the same concentration of insulin and glucose for a few hours, followed by incubation at 0°C in the presence of labeled glucose, a marked uptake of labeled glucose from the bathing solution is observed. However, again after 10 hours a new steady level of labeled glucose is reached and maintained for as long as we observed (Fig. 2, 23 hours). Similar results are obtained at both low (5 mM) and high (Fig. 3, 70 mM) glucose concentrations.

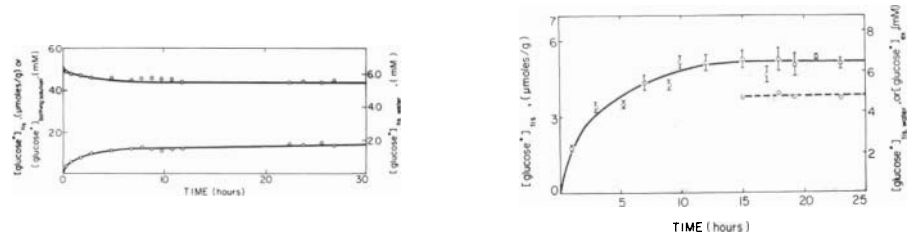


Fig. 1. Time Course of Glucose Uptake by Freshly Isolated Frog Muscles. Mixed muscles were isolated and stored overnight at 4°C in Ringer phosphate. In the morning they were placed in 10 ml of Ringer phosphate containing 5.0 mM glucose with 0.5 μc/ml glucose-H^3, but no insulin, in a 20 ml L-shaped tube at 0°C. This tube was placed in a water bath at 0°C ± 0.05°C with the long axis in the direction of the shaking motion. Samples were removed at appropriate intervals and their radioactivity determined as in the text. Labeled glucose remaining in the bathing solution is shown on the left-hand ordinate in mM. Uptake by muscles is calculated from the bathing solution loss in terms of μmoles/g tissue (left-hand ordinate) and μmoles/ml tissue water (right-hand ordinate).

Fig. 2. Time Course of Glucose Uptake by Frog Muscles Following Preincubation with Insulin. Mixed muscles were preincubated with 24 mM glucose and 0.1 u/ml insulin for 7% hours at 25°C. Groups of 20 muscles each were placed in 30 ml of Ringer solution containing 5 mM glucose and 0.2 μc glucose-H^3 at 0°C for incubation. At intervals 4 muscles were removed for analysis of glucose uptake. Results are shown as the mean; the line through the points represents twice the standard error. Samples of bathing solution were also removed from time to time for analysis to obtain the final external glucose concentration shown by the dotted line.

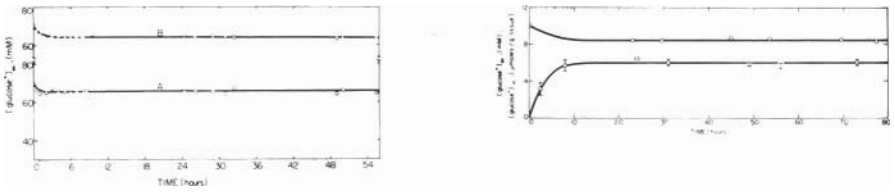


Fig. 3. Time Course of Loss of Labeled Glucose from the Bathing Solution at High (70mM) External Glucose Concentration. A. 2.77 g of mixed muscles were dissected and stored overnight in Ringer solution at 0°C. In the morning they were placed in 15 ml of Ringer phosphate containing 70 mM glucose and 1 μc/ml glucose-H^3 in a U-tube. The tube and its contents were maintained at 0°C and shaken in the horizontal direction. Samples of the bathing solution were removed at intervals and analyzed for their labeled glucose content. Points shown are the average of duplicate samples. B. 3.63 g of muscles treated as in A except that they were preincubated at 25°C for 7 hours with 24 mM glucose and 0.1 u/ml insulin.

Fig. 4. Time Course of Glucose Loss from Bathing Solution and Glucose Uptake by Frog Muscle. Upper curve shows data from 16 muscles preincubated with 0.1 u/ml insulin (Sigma) 5 hours at 25°C and 16 hours at 0°C. At zero time they were placed in 7 ml of Ringer phosphate containing 24 mM glucose and 50 μc/ml glucose-H^3 and maintained at 0°C in a water bath with shaking. Samples of the bathing solution were removed at appropriate times and counted to determine radioactivity. At 99 hours the labeled glucose concentration in the bathing medium was 7.70 mM. Lower curve shows data from muscles treated exactly as in upper curve except that 32 muscles were divided between two vials containing 10 ml solution each for incubation at 0°C. Four muscles were removed at each time and their glucose-H^3 activity determined from TCA extracts as described in the text. Values shown on graph are the mean tissue concentrations of glucose ± standard error.

Fig. 4 shows the time course of decrease of labeled glucose concentration in the bathing medium as well as the time course of the uptake of labeled glucose separately assayed on the tissue extracts. Once having reached a steady level, the radioactivity in the bathing solution as well as that in the muscles remains unchanged to 72 hours. This maintained radioactivity in the bathing solution between the 10th and 72nd hour suggests that no significant amount of labeled glucose could have been oxidized to CO_2 within this period of time

That the glucose accumulation observed here had truly reached a steady level is indicated by the experiment shown in Fig. 5. Following preincubation with 24 mM glucose and 0.1 u/ml insulin, muscles were incubated at 0°C with 70 mM glucose and 0.1 u/ml insulin. Glucose uptake was followed by analysis of muscles removed at intervals using boiling water extraction and the glucose oxidase assay test. As Fig. 5 shows, after 17 hours the tissue glucose reached an average level of 31 μmoles/g. The muscles were then removed from this solution, and placed in a second Ringer phosphate containing 20 mM glucose. Muscles were removed from this solution at intervals and again analyzed for their glucose content. By 65 hours of incubation the glucose concentration in the tissues was found to have fallen to an average level of 13.8 μM/g. The final external glucose concentration was 17.9 mM. This level is not significantly different from the final tissue concentration shown in Fig. 10, for this external concentration (13.6 μmoles/g). Thus, whether approached from above or below, a steady level of glucose accumulation is reached. This

experiment also shows that similar results are obtained by either radioactive or enzymatic determination of glucose accumulation.

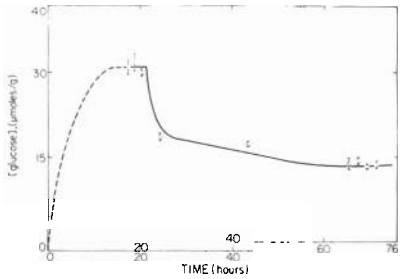


Fig. 5. Reversal of Glucose Uptake by Frog Muscles Incubated at 0°C. Mixed muscles were isolated and stored overnight in Ringer phosphate at 4°C. Following preincubation at 25°C with 24 mM glucose and 0.1 u/ml insulin far 6 hours, muscles were placed in a flask containing 300 ml Ringer phosphate with 70 mM glucose (0°). Groups of five muscles were removed at intervals and analyzed to determine their glucose uptake using boiling water extraction and glucostat. At exactly 21.5 hours the muscles were removed, lightly blotted with filter paper and placed in a second flask (0°C) containing Ringer phosphate with 20 mM glucose and shaken at 0°C. Groups of five muscles were again removed at intervals and analyzed as before. The final external glucose concentration was determined on samples of the bathing solution by the glucostat method. Muscle glucose concentration is shown as μmoles/g tissue. Bars above and below points indicate the standard error of the mean.

Two Dimensional Chromatography of Muscle Extract

Fig. 6B shows a radioautograph from a two-dimensional thin layer chromatogram of a TCA extract of muscle that had been preincubated at 25°C with insulin and glucose followed by incubation at 0°C with glucose and labeled glucose. TCA effectively extracts free glucose as well as glucose-6-phosphate and other intermediates of carbohydrate metabolism.[19] Nevertheless, the radioautograph shows a single spot with an R_f corresponding to that of free glucose (compare with Fig. 6A). The minimum discernible radioactivity was 5 x 10^{-5} μc, which is about 2% of the total amount placed on the chromatogram. This shows that no more than 2% of the glucose could have been transformed into glucose-6-phosphate during the course of the 0°C incubation; this amount is within our experimental error. This experiment has been repeated 6 times with similar results.

The Identification of the Source of Radioactivity in Muscle as Labeled "Free" Glucose by Enzymatic Determination

To be certain that the radioactively labeled glucose taken up during the 0°C incubation was completely mixed with any pre-existing nonlabeled glucose in the tissues (e.g., that carried over from the preincubation medium), we used glucose oxidase to determine the specific activity of the glucose both in the tissue and in the external solution. Table 5 shows that within experimental error, no difference exists.

Balance Sheet of Radioactivity Lost from the Solution Compared with that Gained by the Tissue

Table 6 shows that all the radioactivity lost from the bathing solution is quantitatively recovered from the tissue.

In summary, the two dimensional chromatograms and the balance sheet taken together indicate that all the labeled glucose added to the tissue is quantitatively recovered as glucose in the TCA extract. This proves that detectable glucose phosphorylation is absent in frog muscle incubated at 0°C. That the radioactively labeled glucose is representative of the nonlabeled glucose in the tissue is shown by the equality of the specific activities of the tissue and the external solution, respectively as well as by the experiment shown in Fig. 5. Thus, the steady levels of tissue glucose obtained here represent either steady state or equilibrium accumulation of glucose. This point will be amplified in the discussion.

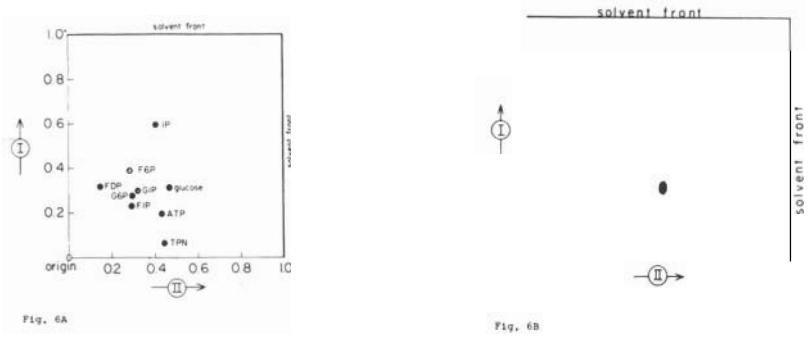


Fig. 6. Two-dimensional Thin Layer Chromatograms of Some Sugar Phosphates, Glucose and other Phosphate Compounds (A) and a Radiochromatogram of a Frog Muscle Extract (B). The diagram on the left (A) is reconstructed from average Rf values experimentally observed: (Fructose-6-phosphate from Waring and Ziporin[16]).

COMPOUND	PHASE I SOLVENT	PHASE II SOLVENT
Inorganic phosphate	0.60	0.40
Fructose-1, 6-diphosphate	0.32	0.14
Glucose-I-phosphate	0.30	0.32
Glucose-6-phosphate	0.28	0.29
Fructose-1-phosphate	0.25	0.29
Adenosine triphosphate	0.19	0.43
Triphosphopyridine nucleotide	0.06	0.49
Glucose	0.31	0.47

The radiochromatogram (B) was from a thin layer chromatogram spotted with a concentrated TCA extract of 344 mg of frog muscles. The muscles were preincubated for 6 hours at 25°C in a Ringer solution containing 24 mM glucose and 0.1 unit/ml of insulin (Sigma) followed by incubation in 1 ml of Ringer phosphate containing 24 mM glucose and 10 μc/ml of C-14-labeled glucose at 0°C for 27 hours. After freezing in liquid nitrogen, muscle extract was prepared as in the text. 11.2 h of extract were spotted on MN-polygram. After developing (but before spraying) the chromatogram was used to produce the radiochromatogram shown above. Total exposure time of the X-ray film was 11 days. The small zero indicates the origin.

Table 5

Supernatant			Muscle				
Glucose Concentration	Radioactivity	Specific Activity	Glucose Concentration Tissue	Radioactivity	Specific Activity	Average Specific Activity	Ratio
µmole/ml	$\frac{cpm}{ml} \times 10^{-5}$	$\frac{cpm}{\mu mole} \times 10^{-5}$	µmole/g	$\frac{cpm}{gm} \times 10^{-5}$	$\frac{cpm}{\mu mole} \times 10^{-5}$	$\frac{cpm}{\mu mole} \times 10^{-5}$	
Series A							
8.4	40.11	4.76	9.28	44.96	4.64	4.77	1.00
			9.60	45.24	4.71		
11.76	34.50	2.94	12.47	44.73	3.58	3.47	1.12
			12.72	42.90	3.37		
16.07	39.45	2.45	15.07	37.18	2.46	2.38	0.97
			16.61	38.26	2.30		
19.57	41.10	2.10	16.50	35.46	2.15	2.07	0.99
			17.69	35.49	2.01		
Series B							
3.78	41.56	10.99	3.27	36.67	11.21	11.18	1.02
			3.19	35.60	11.15		
7.98	44.73	5.60	6.20	34.10	5.50	5.65	1.01
			5.90	34.41	5.80		
11.97	44.37	3.71	7.66	27.96	3.65	3.69	0.99
			8.72	32.57	3.73		
16.59	41.74	2.52	10.19	27.99	2.74	2.75	1.09
			9.55	26.49	2.77		

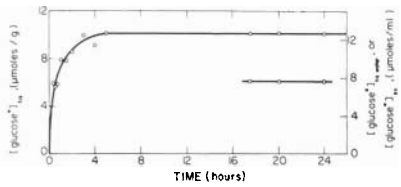
Average ratio $\left(\frac{\text{Specific Activity Muscle}}{\text{Specific Activity Bathing Solution}}\right) \pm$ Standard Error 1.01 • .02

Table 5. Comparison of Specific Activity of Muscle Extracts with Specific Activity of Bathing Solution. Mixed muscles were isolated and kept in glucose-free Ringer phosphate overnight at 4°C. Series A preincubated 5% hours in Ringer phosphate with 24 mM glucose at 25°C. Series B preincubated 5% hours in glucose-free Ringer phosphate at 25°C. Both preincubation solutions contained 0.1 u/ml insulin. Muscles incubated at 0°c overnight: 6 muscles were placed in a vial containing 1.5 ml of Ringer phosphate with varying glucose concentrations and 0.5 µc/ml glucose-H^3. Following incubation muscles were blotted, frozen and weighed and the glucose extracted with distilled water, $Ba(OH)_2$ and $Zn(SO_4)$. Nonlabeled glucose concentration of the muscle extracts and supernatants determined by the glucose-oxidase-peroxidase method. Radioactivity of bathing solution and TCA extracts of muscles determined as described in the text.

Sample No.	Total counts/min lost from bathing solution	Total counts/min gained by muscles	Difference	% Recovery
1	637,640	678,518	+40,878	106.4
2	1,811,491	1,877,650	+66,158	103.7
3	2,213,540	2,198,791	- 14,749	099.4
4	1,098,791	1,113,287	+14,495	101.3
5	1,384,332	1,356,599	+14,267	101.0

Average ± Standard Error 101.5 ± 1.36

Table 6. Balance sheet of labeled glucose recovery after 0°C incubation.
Mixed muscles from 10 frogs were isolated and stored overnight at 0°C in glucose-free Ringer phosphate. In the morning the muscles were divided among 3 flasks (250 ml) containing 100 ml each Ringer phosphate with 2 mM glucose and 0.1 u/ml insulin and preincubated 6 hours at 25°C. For the 0°C incubation about 1 gram of muscle was incubated overnight in each of 7 vials containing 1.5 ml of the above solution. Six of the vials also contained 50 μc/ml of C^{14}-glucose. The seventh vial provided a silent experiment for the calculation of correction factors for counting efficiency (see Methods). After 18 hours of incubation at 0°C muscles were removed, blotted, frozen in liquid nitrogen, weighed and their glucose extracted with 12% TCA. The radioactivity of the extracts and supernatants determined as in the text. In this experiment, all volumes (incubation solution, extraction solution, etc.) were determined to five significant figures by weighing.



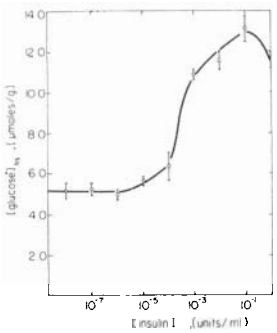


Fig. 7. The effect of the duration of preincubation in insulin at 25°C upon subsequent glucose uptake of muscle cells at 0°C.
Muscles preincubated in 50 ml of Ringer phosphate containing 0.1 u/ml insulin (Iletin) and 24 mM glucose for varying periods of time. Incubation with labeled glucose at 0°C lasted 24 hours. Lower curve indicates the final concentration of labeled glucose in the external bathing solution.

Fig. 8. Effect of the concentration of insulin in the preincubation medium (25°C) on the subsequent uptake (0°C) of glucose by frog muscle.
Reincubation solution contained 24 mM glucose and varying concentrations of insulin (Sigma). Preincubation lasted 6 hours at 24°C and was followed by incubation at 0°C with 24 mM glucose for 18 hours. Each point represents the average of determinations on eight different muscles. Distances between the horizontal bars represent twice the standard error.

PART II: PREINCUBATION CONDITIONS

Effect of the Duration of Preincubation with Insulin on Glucose Uptake

Fig. 7 shows the effect of the duration of preincubation with insulin on the equilibrium level of subsequent glucose uptake at 0°C. This experiment has been repeated 3 times with similar results. These data indicate that insulin requires about 6 hours of preincuba-

tion at 25°C to achieve its full effect. Further preincubation to as long as 24 hours did not produce any significant change in the ultimate steady level of glucose uptake. In these experiments the incubation solution (0°C) contained no insulin; no material difference was noticed when insulin was included in this medium.

The Effect of Insulin Concentration in the Preincubation Medium Upon the Steady Level of Labeled Glucose Reached During Subsequent Incubation (0°C).

Fig. 8 shows that the concentration of insulin in the preincubation medium determines the steady level of labeled glucose taken up during subsequent overnight incubation at 0°C. A discernible effect of insulin is seen at a concentration of 10^{-5} units/ml and reaches a maximum at 10^{-1} u/ml. The decrease in insulin action at 1 u/ml has been seen in several experiments. These data are in general agreement with the findings of Narahara, Özand and Cori[g] for insulin action on frog sartorius muscle at a higher temperature (19°C).

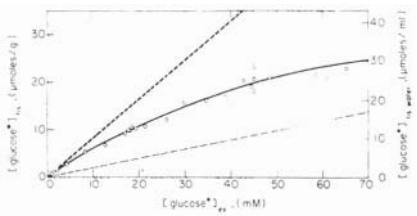


Fig. 9. Steady levels of glucose in freshly isolated frog muscles following overnight incubation at 0°C with varying labeled glucose concentrations.
Muscles isolated day before and kept overnight at 4°C in glucose-free Ringer phosphate before use. Uptake determined by analysis of loss of glucose from the bathing medium.

PART III: THE STEADY LEVEL OF GLUCOSE IN FROG MUSCLE AS A FUNCTION OF THE EXTERNAL GLUCOSE CONCENTRATION

Steady Glucose Levels in Freshly Isolated Muscles

Fig. 9 shows the steady levels of labeled glucose in frog muscles obtained after overnight incubation at 0°C in solutions containing varying labeled glucose concentrations. The data are expressed both in terms of pmoles per gram of fresh muscle tissue (left-hand ordinate) and in terms of pmoles per ml of tissue water (right-hand ordinate). The shape of the curve resembles those which have been published for nonmetabolizable sugars in other tissues.[20-23] The dashed line below shows the tissue space available to poly-L-glutamate (PLG), a recently introduced probe material for the extracellular space.[24] The dotted line shows equal distribution between the tissue water and the external solution. At no point is the steady level of glucose in the cell water significantly higher than the external labeled glucose concentration.

Steady Glucose Levels in Insulin-Treated Muscles

Mixed frog muscles were preincubated for 6 hours in the presence of insulin (0.1 unit/ml), and 24 mM glucose followed by overnight incubation with varying external labeled

glucose concentrations. The steady levels of glucose attained by the tissue are illustrated in Fig. 10, where again, as in Fig. 9, the data are expressed in terms of μmoles of labeled glucose per gram of whole muscle tissue and per milliliter of tissue water. The same data have been used in plotting the intracellular glucose concentrations in Figs. 11A and B. In calculating Fig. 11A, the value of 23.5% given by Narahara, Özand and Cori[9] for the extracellular space was used; in Fig. 11B an extracellular space of 8% was used.[24]

The data show that, in general, the overall steady level of glucose is higher following preincubation with insulin than in freshly isolated muscles (which contain some endogenous insulin; to be published). Nevertheless, at high external glucose concentrations the intracellular glucose concentration in μmoles per liter of cell water remains lower than the concentration in the external medium. This is the case whether one uses an extracellular space value of 8% or 23.5%

To determine whether the high cell glucose seen in the presence of insulin at low external glucose concentrations is really significant, we preincubated 16 muscles with 0.1 u/ml insulin and 24 mM glucose and then incubated them overnight with 10 mM labeled glucose at 0°C. The final glucose concentration of the incubation medium was determined by the glucose-oxidase-peroxidase method to be 9.56 mM. The final glucose concentration of the tissues is shown in the 3rd column of Table 7A. When this data is expressed in terms of concentration in the tissue water (column 4) all the tissue concentrations are found to exceed the external concentration. This is true even after a correction for extracellular space is made. Column 5 shows the concentration of glucose in the cell water calculated on the basis of an extracellular space of 8%;[24] column 6 shows the same value calculated on the basis of an extracellular space of 23.5%.[9] Columns 7 and 8 show the ratio of intracellular to extracellular concentration calculated from values given in columns 5 and 6 respectively. The average ratio is 1.26 for an extracellular space of 8%, 1.32 for an extracellular space of 23.5%; in both cases this ratio is significantly different from 1.0 ($p<0.001$) indicating that the concentration of glucose in the cell is indeed higher than the external concentration at low external glucose concentrations. That this high ratio of intracellular to extracellular glucose is not the result of a systematic error is indicated by similar data obtained from muscles treated in exactly the same way except that insulin was omitted from the preincubation medium. Here the average ratio is 0.325 (Table 7C).

That the ratio of less than 1.0 for the intracellular to extracellular glucose concentrations at high external glucose concentration is significant is shown by Tables 7B and D. In this case, although the total uptake by insulin-treated muscles is significantly higher than that of the control muscles preincubated without insulin, both ratios are significantly less than 1.0 ($p<0.001$).

In Table 8, data for the low concentration range from the majority of experiments performed on insulin-treated muscles over the last four years are tabulated. Although in occasional experiments the ratio of the internal glucose concentration to the external at low external glucose concentrations is less than one, the great majority of these experi-

ments show ratios greater than one. Preliminary experiments indicate that extreme starvation and low pH (about 7.0) in the Ringer solution tend to depress this ratio. Experiments from 1966 are not included; at that time we routinely treated our frogs with tetracycline (see Gibbs et al.[33]) which we later found to depress the insulin response of the muscles. Table 9 shows data from the higher concentration range obtained during 1964 and 1965. At external concentrations above about 20 mM, the ratio of the glucose in the cell water to that in the external solution is uniformly less than one.

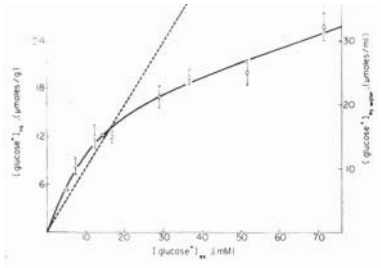


Fig. 10. The steady level of labeled glucose uptake in frog muscle at 0°C following preincubation with insulin (0.1 u/ml) at 25°C.
Muscles (mixed) preincubated 6 hours at 25°C in Ringer phosphate containing 0.1 u/ml insulin and 24 mM glucose followed by incubation overnight in Ringer phosphate containing varying concentrations of glucose and glucose-H^3. Labeled glucose content of muscles determined on TCA extracts. Final glucose concentration of bathing medium determined by glucose-oxidase-peroxidase method. Each point is average of four muscles ± standard error. Left-hand ordinate: Glucose content of whole tissue. Right-hand ordinate: Glucose in tissue water.

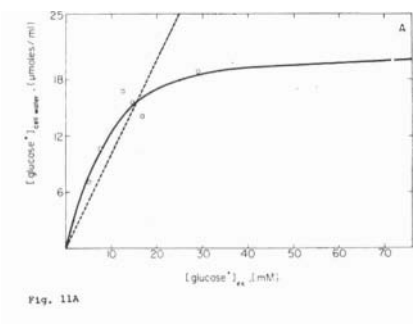


Fig. 11A

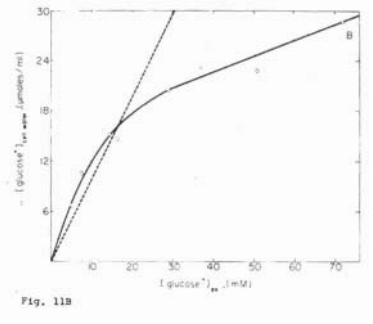


Fig. 11B

Fig. 11. The steady intracellular level of labeled glucose uptake in frog muscle at 0°C following preincubation with insulin (0.1 u/ml) at 25°C.
Calculated from the data of Fig. 9 on the basis of an extracellular space of 23.5% (A) and 8% (B).

A.

(1) Experiment No.	(2) Muscle Type	(3) [glucose*]tis (μmoles/g)	(4) [glucose*]tis water (mM)	(5) [glucose*]cell water (mM) I	(6) [glucose*]cell water (mM) II	(7) [glucose*]cell water / [glucose*]ex I	(8) [glucose*]cell water / [glucose*]ex II
A 1	1	9.45	11.7	12.0	12.7	1.26	1.33
A 2	2	10.1	12.5	11.7	13.8	1.22	1.44
A 3	3	9.20	11.4	11.6	12.2	1.21	1.28
A 4	4	8.45	10.5	10.6	10.8	1.11	1.13
A 5	1	10.8	12.4	12.7	13.6	1.33	1.39
A 6	2	10.4	12.9	13.3	14.3	1.39	1.50
A 7	3	9.60	11.9	12.2	12.9	1.35	1.35
A 8	4	11.1	13.7	14.3	15.5	1.62	1.69
B 1	1	10.0	12.4	12.7	13.6	1.42	1.49
B 2	2	9.16	11.4	11.6	12.1	1.27	1.33
B 3	3	10.3	12.7	13.1	14.1	1.48	1.55
B 4	4	12.1	15.0	15.6	17.3	1.81	1.90
B 5	1	14.6	18.1	19.1	21.6	2.26	2.36
B 6	2	9.94	12.3	12.6	13.5	1.41	1.47
B 7	3	9.50	11.8	12.1	12.8	1.26	1.34
B 8	4	9.40	11.7	11.9	12.5	1.31	1.37
Mean ± S.E.				12.9 ± 0.5	12.4 ± 0.6	1.26 ± 0.07	1.32 ± 0.07
p						<0.001	<0.001

B.

1	39.2	48.8	48.2	42.1	0.740	0.646
2	28.4	35.4	32.4	23.1	0.498	0.354
3	23.0	28.8	24.8	13.8	0.381	0.212
4	30.4	38.0	35.0	26.7	0.538	0.410
5	21.3	26.7	22.4	10.7	0.344	0.164
6	28.1	35.1	31.8	22.6	0.488	0.337
7	29.9	37.4	34.4	25.9	0.528	0.398
8	29.4	36.7	33.5	24.9	0.514	0.382
9	27.6	34.4	31.0	21.6	0.476	0.332
10	27.6	34.4	31.0	21.6	0.476	0.332
11	25.7	32.2	28.5	18.5	0.437	0.284
12	31.7	39.6	36.8	29.0	0.565	0.446
13	28.3	35.4	32.1	23.1	0.493	0.355
14	28.7	35.8	32.5	23.6	0.492	0.363
15	28.4	35.5	32.2	23.2	0.494	0.356
16	28.9	36.1	32.9	24.0	0.505	0.368
Average ± Standard Error			32.4 ± 1.71	23.4 ± 2.03	0.498 ± 0.026	0.358 ± 0.031
p					<.001	<.001

C.

(1) Experiment No.	(2) [glucose*]tis (μmoles/g)	(3) [glucose*]tis water (mM)	(4) [glucose*]cell water (mM) I	(5) [glucose*]cell water (mM) II	(6) [glucose*]cell water / [glucose*]ex I	(7) [glucose*]cell water / [glucose*]ex II
1	2.41	3.02	2.35	0.54	0.261	0.060
2	3.05	3.82	3.24	1.67	0.360	0.186
3	2.85	3.56	2.95	1.30	0.328	0.144
4	2.39	2.99	2.32	0.50	0.258	0.055
5	2.67	3.34	2.71	1.00	0.301	0.110
6	3.34	4.18	3.64	2.18	0.404	0.242
7	2.22	2.78	2.08	0.21	0.231	0.023
8	3.01	3.76	3.17	1.53	0.352	0.170
9	2.82	3.53	2.92	1.26	0.325	0.140
10	3.60	4.50	4.00	1.72	0.444	0.191
11	3.48	4.35	3.83	2.42	0.425	0.269
12	2.14	2.77	2.07	0.19	0.230	0.021
13	2.55	3.19	2.54	0.78	0.282	0.087
14	2.75	3.44	2.82	1.14	0.313	0.127
15	3.27	4.08	3.53	2.04	0.392	0.226
16	2.70	3.38	2.75	1.05	0.305	0.115
Average ± Standard Error			I.. ± 0.19	1.22 ± 0.a	0.325 ± 0.021	0.135 ± 0.022
p						<.001

D.

1	22.29	27.9	24.2	14.08	0.397	0.232
2	18.25	22.8	18.6	6.90	0.305	0.113
3	16.31	20.4	15.9	3.52	0.261	0.058
4	18.34	22.9	18.7	7.04	0.307	0.115
5	17.58	22.0	17.7	5.77	0.290	0.094
6	16.7	20.9	16.4	4.22	0.269	0.069
7	16.9	21.2	16.8	4.64	0.276	0.074
8	17.8	22.2	17.9	6.05	0.294	0.099
9	21.9	27.4	23.7	13.38	0.389	0.220
10	16.9	21.1	16.7	4.50	0.274	0.074
11	19.0	23.8	19.7	8.30	0.323	0.136
12	16.6	20.8	16.3	4.08	0.267	0.067
13	15.2	19.0	14.3	1.54	0.234	0.025
14	14.3	17.9	13.1	0.00	0.215	0.000
15	17.3	21.6	17.2	5.21	0.282	0.086
16	14.3	17.9	13.1	0.00	0.215	0.000
Average ± Standard Error			17.5 ± 0.923	5.57 ± 1.40	0.287 ± 0.018	0.091 ± 0.023
p					<.001	<.001

Table 7. The Effect of Insulin Pretreatment on Glucose Uptake by Frog Muscles at Low (10 mM) and High (70 mM) External Glucose Concentrations. Two grpups (A and B) of 16 mixed frog muscles each were isolated and preincubated with 24 mM glucose and 0.1 u/ml insulin for 7 hours at 25°C. Control groups (C and D) were treated in exactly the same manner except that insulin was omitted from the preincubation medium. Following preincubation, Groups A and C were placed in 6.0 ml Ringer solution containing 10 mM glucose and 0.25 μc/ml glucose-H^3. Groups B and D were placed in 6.0 ml Ringer solution containing 70 mM glucose and 0.25 μc/ml glucose-H^3. After overnight incubation at 0°C muscles were blotted, weighed and their radioactive glucose content analyzed on 5% TCA extracts as in the text. [Glucose*]tis water was calculated on the basis of 80% tissue water. Glucose in the cell water calculated on the basis of 8% extracellular space (I) and 23.5% extracellular space (II). For part A muscle types are: 1. sartorius, 2. semitendinosus, 3. tibialis anticus longus, and 4. iliofibularis. The final external glucose concentrations are: Group A, 9.56 mM; Group B, 65.2 mM; Group C, 9.00 mM; Group D, 61.0 mM.

Date	Preincubation (25 C)		Incubation (0 C)	Method of Analysis	Glucose, ex		Glucose* Conc. in Cell		Glucose cell water / Glucose ex
	Insulin Conc. (u/ml)	Hours	Hours		Initial (mM)	Final (mM)	mmoles/kg. cells	mmoles/L. cell water	
12- 4-64	0.15	3	23	1	2.0	1.41	1.79	2.26	1.62
12-11-64	0.15	3	21	1	2.0	1.67	1.50	1.92	1.15
12-11-64	0.15	3	21	1	10.0	8.16	6.43	8.34	1.02
12-15-64	0.15	3	20	1	1.0	0.81	0.88	1.12	1.38
12-15-64	0.15	3	20	1	2.0	1.62	1.79	2.30	1.42
12-15-64	0.15	3	20	1	3.3	2.77	2.75	3.51	1.27
1-21-65	0.1	6	17.0	2	10.0	7.79	6.95	8.90	1.14
2- 2-65	0.1	6	16.75	2	10.0	7.30	8.12	10.43	1.43
2- 8-65	0.1	6	17	2	10.0	8.30	7.49	9.56	1.15
2-22-65	0.1	6	17.5	2	1.0	0.74	1.05	1.34	1.82
3-22-65	0.1	6	17	1	10.0	8.0	10.2	13.1	1.64
3-30-65	0.1	6	17	1	10.0	7.85	10.2	13.1	1.67
4- 8-65	0.1	4.5	18	2	2.5	1.48	1.64	2.09	1.28
4- 8-65	0.1	4.5	18	2	5.0	3.46	3.16	4.04	1.17
5- 5-65	0.1	6.25	16	2	2.5	1.91	2.14	2.74	1.43
5- 5-65	0.1	6.25	16	2	5.0	3.90	3.63	4.63	1.19
9-14-65	0.1	6	18	2	5.0	4.56	4.41	5.65	1.22
12- 9-65	0.1*	6	18	2	2.5	1.66	2.54	3.26	1.96
12- 9-65	0.1*	6	18	2	5.0	3.63	5.13	6.57	1.85
12-21-65	0.1*	6	19	2	2.5	1.98	1.92	2.46	1.24
12-21-65	0.1*	6	18	2	5.0	4.24	3.58	4.58	1.08
2-8 -67	0.1	6	20.5	2	10.0	10.01	5.92	7.58	.76
2-16-67	0.1	6	17	2	10.0	9.07	8.42	10.79	1.21
2-22-67	0.1	6	18	2	10.0	8.58	8.42	10.78	1.27
3- 9-67	0.1	4	17	2	10.0	8.22	6.13	7.90	0.96
3-16-67	0.1	5.5	16	2	2.5	2.20	1.43	1.83	0.83
3-16-67	0.1	5.5	16	2	3.33	2.83	2.30	2.95	1.04
3-16-67	0.1	5.5	16	2	5.0	4.23	4.03	5.19	1.22
3-16-67	0.1	5.5	16	2	10.0	8.12	8.87	1.30	1.40
3-22-67	0.1	5.5	24	2	2.5	2.06	1.92	2.46	1.19
3-22-67	0.1	5.5	24	2	3.33	2.75	2.51	3.22	1.17
3-22-67	0.1	5.5	24	2	5.0	3.52	3.30	4.23	1.20
3-22-67	0.1	5.5	24	2	10.0	6.92	6.32	8.10	1.17
10- 9-67	0.1	6	25	2	2.0	1.97	2.01	2.58	1.31
10-18-67	0.1	6	18	2	5.0	4.13	3.43	4.40	1.07
10-18-67	0.1	6	18	2	5.0	3.84	3.88	4.98	1.27
10-18-67	0.1	6	18	2	5.0	4.12	2.24	2.87	0.70
10-18-67	0.1	6	18	2	5.0	3.82	3.19	4.08	1.07
3- 7-67	0.1	6	18	2	10.0	8.00	9.16	11.71	1.46
3- 7-67	0.1	6	18	2	10.0	7.20	10.25	13.15	1.82
3- 8-67	0.1	4.5	18	2	10.0	6.93	10.24	13.1	1.89
3-17-67	0.1	5.5	18	2	5.0	8.40	11.10	14.2	1.69
3-17-67	0.1	5.5	18	2	10.0	11.76	15.50	19.8	1.67
10-10-67	0.1	6	22	2	5.0	5.04	5.31	6.93	1.34
10-10-67	0.1	6	22	2	7.5	7.56	8.28	10.83	1.39
10-10-67	0.1	6	22	2	15.0	12.79	11.65	15.15	1.28

Average 1.33 ± .037 (S.E)

P <.001

Table 8. Tabulated Data on Glucose Uptake by Insulin-treated Frog Muscles at Low External Glucose Concentrations. Data from experiments carried out over 4 years. Data for 1966 not included, since at that time frogs were routinely treated with tetracycline which was later discovered to depress the insulin effect. All muscles preincubated at 25°C with the concentration of insulin indicated and 24 mM glucose. Methods of analysis: 1. Indirect, from assay of the loss of labeled glucose from the bathing medium; 2. Direct, from assay of the final glucose concentration in the muscles. All glucose concentrations calculated on the basis of an 8% extracellular space.

*Glucagon-free insulin from Eli Lilly.

Date	Preincubation (25°C) Insulin Conc. (u/ml)	Hours	Incubation (0°C) Hours	Method of Analysis	$[Glucose]_{ex}$ Initial (mM)	Final (mM)	Glucose Conc. in Cell mmoles / kg. cells	mmoles / l. cell water	$\frac{[glucose^-]_{cell\ water}}{[glucose^*]_{ex}}$
12- 4-64	0.15	3	23	1	20	16.8	7.37	9.41	0.56
12-11-64	0.15	3	21	1	20	17.8	8.93	10.5	0.59
12-11-64	0.15	3	21	1	60	54.5	26.3	33.7	0.56
12-11-64	0.15	3	21	1	70	66.5	26.4	33.8	0.50
12-15-64	0.15	3	20	1	22	20.4	7.12	8.35	0.41
12-15-64	0.15	3	20	1	65	61.2	16.2	21.0	0.34
12-18-64	0.15	3	21	1	60	55.0	22.1	28.4	0.51
1-21-65	0.1	6	17	2	30	25.4	6.80	8.70	0.34
2- 2-65	0.1	6	16.75	2	30	24.6	6.60	8.45	0.34
2- 8-65	0.1	6	17	2	30	26.4	5.81	7.43	0.28
2-22-65	0.1	6	17	2	30	24.8	10.2	13.0	0.52
4- 8-65	0.1	4.5	18	2	25	17.7	10.6	13.6	0.76
4- 8-65	0.1	4.5	18	2	45	34.4	17.0	20.8	0.63
5- 5-65	0.1	6.25	16	2	25	22.1	14.4	18.5	0.84
9-14-65	0.1	6	18	2	25	23.1	8.3	10.6	0.46
9-14-65	0.1	6	18	2	35	31.3	9.6	12.3	0.39
12- 9-65	0.1*	6	18	2	25	19.9	9.95	12.7	0.64
12- 9-65	0.1*	6	18	2	45	38.9	14.3	18.3	0.47
12-21-65	0.1*	6	18	2	25	21.5	10.6	13.6	0.63
12-21-65	0.1*	6	18	2	45	40.3	16.6	21.3	0.53
Mean ± S.E.									0.492 ± 0.033
P									<0.001

Table 9. Tabulated Data on Glucose Uptake by Insulin-treated Frog Muscles at High External Glucose Concentrations. Data from experiments carried out during 1964 and 1965; there is no material difference in later experiments. All muscles preincubated at 25°C with the indicated concentration of insulin for the time indicated. Methods of analysis as in Table 8. All glucose concentrations calculated on the basis of an 8% extracellular space.
*Glucagon-free insulin from Eli Lilly.

DISCUSSION

Under any conditions a steady level of intracellular glucose results from a balance between: 1. flux into the cell; 2. flux out of the cell; and 3. phosphorylation and thus removal of glucose by the action of hexokinase.[25] When phosphorylation is effectively

removed, as in the present experiments, a steady level of intracellular (or tissue) glucose represents equality of the inward and outward flux rates. If under this condition there is a difference between the external concentration of glucose and the intracellular glucose concentration (and the intracellular glucose concentration is not zero), it can be the result of either a steady state or an equilibrium state. The former demands a continuous input of energy directly linked to the movement of glucose (i.e., a pump) into or out of the cell or both.* In the latter case, the concentration difference results from a difference between the physical-chemical properties of the cytoplasm and those of the external milieu. No direct energy input is needed to maintain the concentration difference.**

In terms of the membrane theory, the cytoplasm is an aqueous solution of salts, protein, etc. whose physical-chemical properties are not much different from those of the external solution; any differences between the internal and external steady level concentrations of solutes are determined by the properties or activities of the cell membrane or its constituent parts.

In general, accumulation of sugars against a concentration gradient has not been found (see Randle and Morgan,[26] and Park et al.;[25] but see Haft et al.,[23] Wick and Drury[27] and Troschin[28] for exceptions) so that most workers in this field have not felt the need to propose an energy-consuming pump for glucose transport as has been proposed for Na^+ ion.[29-31] A nonenergy-consuming carrier mechanism for the entry and possibly the exit of sugars is considered necessary to explain selectivity and competition between sugars and the fact that the rate of entry of many sugars in the presence of insulin has been found to follow adsorption kinetics.[26-32]

The fact that glucose is found in the cell water at a level lower than that of the external medium under certain experimental conditions (insulin, high glucose concentration, etc.[8]) is considered to be the result of a balance between the rate of entry and the rate of conversion of glucose into glucose-6-phosphate. It has been something of a puzzle that non-metabolizable sugars such as D- and L-xylose, D-galactose, 3-0-methyl glucose, and D- and L-arabinose, have been found to reach steady levels within the cells lower than that in the external medium (see refs. 8,13,21,25,28,38,72-74). Some workers have explained this on the basis of accessible and nonaccessible compartments within the cell.[26,34]

Our present data show that when phosphorylation is effectively brought to a stop the

* A concentration of glucose in the cell water lower than that of the cytoplasm could be explained on the basis of impermeable compartments within the cell. This possibility is discussed in detail below.

** From a macroscopic point of view, if two phases are in equilibrium, the total number of concerned species in each phase is constant; this does not mean, however, that microscopically there is no interchange between the two phases. Quite the contrary; at the temperature of most investigations, there is a constant exchange. Thus in a Donnan equilibrium, while the total number of water molecules in both the sac and the surrounding solution remains constant; there is, nevertheless, continuous exchange of water between the two phases. A metastable equilibrium is a true equilibrium state although it may be less stable than another equilibrium state. Thus, a coin standing on its edge represents a metastable equilibrium state.

steady level of glucose in insulin-treated muscle is, in general, not the same as the concentration of glucose in the external medium. At high external glucose concentrations it is lower than the external concentration (Table 7B and D). Within the framework of the membrane theory, this suggests the operation of an outward energy-consuming glucose pump or the presence of a compartment in the cell inaccessible to glucose. At low external glucose concentrations, where glucose concentration in the cell water is higher than that of the bathing medium, it is necessary in terms of the membrane theory to postulate an inward energy-consuming pump.

Let us first consider the possibility of a compartment or compartments in the cell completely inaccessible to glucose. Such a compartment is generally considered to have an anatomical basis either in a well-defined structure such as the mitochondria, nucleus or sarcoplasmic reticulum, or in some less well-defined anatomical "barrier" to the entry of glucose into some portion of the cell. Whatever the nature of the compartment, however, the "barrier" must be an absolute barrier, completely impermeable to glucose. From a physical standpoint there is some difficulty in providing such a barrier within the cell. Thus, probably the least permeable substances within the cell are lipid in nature. Nevertheless, the exchange of water is very rapid throughout the cell.[35] In general, therefore, these lipids do not form a barrier to its passage. Now, water has an oil-water partition coefficient of about 10^{-5}; that of glycerol is about 10^{-6}; that of the 6-carbon sugars cannot be very much smaller.* It is difficult to imagine that such a relatively small difference in lipid solubility should make the difference between complete permeability (i.e., to water) and absolute impermeability (i.e., to sugar) into any region of the cell. In fact, it bears pointing out that whenever the actual passage of sugar through cells can be measured as in the aqueous humor of the eye,[36] or plant cell sap,[37] no sugar has been found to be completely impermeable.

Further, it is probably necessary to postulate a rather complex series of compartments since different nonmetabolizable sugars have been found to have different volumes of distribution in the cell water.[38-39] Moreover, the volume of distribution of D-xylose, L-arabinose and D-galactose is increased to different degrees in the presence of insulin;[40,38] insulin must therefore be postulated to change the size of the compartments according to the nature of the sugar present.

Experiments on Na^+ ion uptake by actomyosin, which cannot realistically be considered to have anatomical subdivisions, show that the water in this gel accommodates only 60% of the ion concentration in the external solution (see below; Ling and Ochsenfeld, in preparation). This indicates that partial exclusion of solutes from the water of protein-water systems is possible even in the absence of anatomically defined compartments.

A second possible explanation for the low intracellular glucose concentration at high

* Ross[36] (1951) found the ether-water partition coefficient for glucose to be about 10^{-4}, that of glycerol is 10^{-3}.

external glucose concentrations is the operation of an outward energy-consuming glucose pump. This, combined with an inward energy-consuming pump operating at least in the low external glucose concentration ranges, could explain the entire glucose distribution curve in the presence of insulin.

Examination of Fig. 10 shows a smooth transition from the low external concentration range in which the inward pump must operate to the high external concentration range where the outward pump must be operative. If one postulates that only one of these pumps can be operating at any one time, one must also postualte a very elaborate switching mechanism for no readily understandable reason other than to account for this smooth transition. One may also recall that in terms of the membrane theory it is necessary to postulate pumps to maintain the intracellular concentrations of ions such as Na^+, Ca^{++}, and Mg^{++} ions.[11,32,41] These pumps alone have been shown to demand an excessive amount of energy at 0°C (as well as at 25°C).

A third alternative explanation for the results seen in the present experiment is given by the association-induction hypothesis (see refs. 11,32,41-44). According to this model the steady level intracellular concentration of any solute is the result of an equilibrium determined by two processes: 1. partial exclusion from the water within the cell which is considered to be in a different state from the water in the bathing solution; 2. adsorption onto specific sites on the protein within the cell. Let us discuss these two processes separately.

1. The Physical State **of** Water in Resting Cells: The senior author has suggested that the water in living cells is organized by the protein (largely actomyosin in muscle cells) into polarized multilayers whose detailed arrangement is determined by the structural organization of the proteins involved. There is increasing evidence in support of this concept. Thus, freezing experiments carried out by Chambers and Hale[45] and Rapatz and Luyet[46] indicated that the water in muscle cells does not freeze in the hexagonal patterns seen in distilled water.[44] Instead it freezes in the form of long spikes following the direction of orientation of the myofilaments. Chapman and McLauchlen[47] have concluded from studies of the nuclear magnetic resonance of rabbit sciatic nerve that the bulk of the cell water must be oriented by the cell proteins (see also Bratton et al.[48] and Fritz and Swift[49]). Thus a number of lines of evidence lead to the conclusion that the bulk of the water within the cell is in a different state from the water in the external solution.

On theoretical grounds one would expect a protein-polarized water system to partially exclude solutes like sugars and Na^+ ion (see refs. 41,42,50). Cope[51] concluded from nuclear magnetic resonance studies of Na^+ ion in actomyosin solutions, that the solubility of Na^+ ion is much reduced in the water of actomyosin gel. Recent experiments in our laboratory (Ling and Ochsenfeld, in preparation) have shown that at low pH's the water in actomyosin gel accommodates Na^+, Rb^+, and Cs^+ ions at equilibrium to a concentration only 60% of that of the external solution. This gel contains no more than 5% protein, whereas most cells contain about 20% protein leading one to expect a much higher degree

of solute exclusion from the resting cells.

2. Specific Adsorption Sites for Sugars: The phenomena of selectivity and competition in the transport of sugars,[25,26] often taken as evidence for the involvement of a carrier in glucose transport really are evidence for selectivity and competition in the adsorption of sugars onto specific sites. It is well known from studies in enzymology that proteins may carry highly specific adsorption sites for sugars. Thus enzymes such as glucose oxidase and glucokinase must bear stereospecific sites for glucose because its adsorption is a prerequisite step to the enzyme-catalyzed reaction. Dastoli and price[S2] have recently isolated proteins from bovine taste buds which carry nonenzymatic stereospecific adsorption sites for various sugars.

Assuming the sites involved in the selectivity of sugar transport to be on carriers in the cell membrane, Stein and Danielli[53] and Bowyer and Widdas[54] concluded that only proteins offer sufficient stereospecificity for distinguishing between similar sugars. There is, however, no a ***priori*** reason to suppose that proteins bearing such sites are limited to a component of the cell membrane and are not found within the cytoplasm itself.

The question really becomes: Could a cell possess enough stereospecific adsorption sites to account for the observed accumulation? Thus enzymes usually possess only one or two specific adsorption sites per protein molecule. Assuming that cell proteins were similarly limited to a low molar ratio of nonenzymatic sites, Cohen and Monod[SS] argued that there would not be enough sites in E. ***coli*** to adsorb the large amount of galactosides known to accumulate in these organisms.

There is, however, no a ***priori*** reason to set a low limit on the capacity of all protein molecules to adsorb sugars on the basis of the known molar ratios of enzyme sites. The basic requirement of a stereospecific sugar adsorption site can theoretically be met by any set of H-bonding groups on the backbone of the protein molecule which form the proper stereospecific relation to one another. The following evidence strengthens this view:

1. Debourge[S6] has shown that each mole of bovine serum albumin adsorbs 12 moles of D-glucose. The molecular weight of this protein is 65,000.[57] Thus each gram of this protein adsorbs $\frac{12}{6.5 \times 10^4}$ or 1.8×10^{-4} moles of D-glucose. Muscle cells contain from 20 to 25% (average 22.5%) of protein. If all this protein has sugar binding properties similar to bovine serum albumin, there would be a total of $2.25 \times 10^2 \times 1.8 \times 10^{-4}$ or 42 mM of D-glucose adsorption sites per kilogram of fresh muscle. This is of the same order of magnitude as we have interpreted to occur in insulin-treated muscle (see below).

2. Another example will further strengthen this point. Phenol is an H-bonding solute resembling the sugars. In fact, phenol and sugars form H-bonded complexes.[75] Küntzel and Schwank[S8] have shown that as much as 7 moles of phenol can adsorb on one kilogram of dry collagen. This in fact corresponds roughly to one phenol molecule per NHCO group on the backbone. (For evidence that the phenolic compounds are indeed adsorbed on the backbone, see Ellis and Pankhurst.[59]) If all muscle proteins behaved toward sugar

as collagen does toward phenol and if each sugar adsorption site consisted of two NHCO groups the **potential** concentration of sugar-binding sites in one kilogram of fresh muscle would amount to 900 mM!

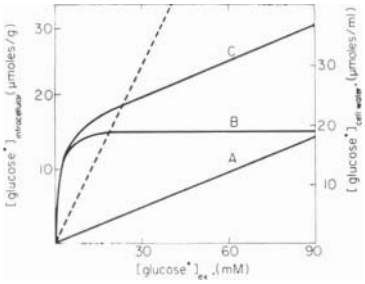


Fig. 12. Theoretical curve for the equilibrium distribution of glucose between the cell and the external medium.
Curve A represents the distribution of glucose between the cell water and the external medium assuming that the cell water accommodates at equilibrium, 20% of the external glucose concentration. Curve B represents adsorption of glucose onto specific adsorption sites assuming 15 mM of binding sites per kg of cell with dissociation constant of 10^{-3} M. The adsorption is assumed to be noncooperative or Langmuir type. Curve C is the sum of surves A and B representing the total glucose uptake by the cell. The dotted line shows the results to be expected if the glucose were equally distributed between the cell water and the external solution. Left-hand ordinate: intracellular glucose in μmoles/g cells; right-hand ordinate: glucose in the cell water, calculated on the basis of a water content of 80%.

Let us now, for purposes of illustration, consider a model cell in which the bulk of the water exists in polarized multilayers. As such, glucose is accommodated to a fraction, say 20%, of the concentration in the external medium. This is shown by line A in Fig. 12. In this figure the dotted line shows the intracellular glucose concentration to be expected on the basis of equal distribution between the cell water and the external solution. Let us further consider that this model cell contains 15 mM glucose binding sites per gram of cell with a dissociation constant of 10^{-3} to 10^{-4} M (similar to the dissociation constant for the adsorption of sugars onto bovine taste bud proteins). As the external glucose concentration increases, the amount of glucose adsorbed will increase reaching a maximum at about 30 mM external glucose as shown by line B in Fig. 12, which is simply a Langmuir adsorption isotherm.* The total glucose in the cell will be the sum of fractions A and B as shown by line C. At external concentrations above 23 mM, the internal concentration will be below the external concentration. The similarity of Fig. 12 to the experimental data presented in Figs. 10 and 11 is obvious. It is important to reiterate that according to this interpretation, the steady level of glucose in the resting cell is an expression of a metastable equilibrium; as in all equilibrium states its maintenance does not directly require the expenditure of free energy. Because of its organization and polarization by the cytoplasmic proteins, the intracellular water is a poorer solvent for glucose than the extracellu-

* It is likely that under many conditions glucose uptake follows not the simple Langmuir adsorption isotherm shown here for purposes of illustration, but a more complex cooperative adsorption isotherm. A detailed analysis of this possibility will be presented in a subsequent paper of this series.

lar water. Thus, one would expect a linear relationship between the intracellular and extracellular glucose with a distribution coefficient less than one (Ling[11] Chap. 12). It will be shown in a subsequent publication that this is precisely what one finds. Insulin is considered to act by making available specific cytoplasmic sites for the adsorption of glucose. A specific molecular mechanism for this action has been given previously (see refs. 11, p. 458, and 42).

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