Biochimica et Biophysica Acta, 307 (1973) 181–196

BBA 76265

EFFECT OF ANOXIA ON SUGAR TRANSPORT IN AVIAN ERYTHROCYTES

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(Received November 20th, 1972)

SUMMARY

1. Simple anoxia, without addition of metabolic poisons, was found to stimulate carrier-mediated sugar transport.

2. Development of an anoxic stimulation of transport immediately followed changes in intracellular levels of low and high energy intermediates, suggesting a role for these compounds as regulators of transport.

3. Adenine and ATP were the only metabolites found to alter transport rates, when they were added exogenously. Adenine partially inhibited the anoxic stimulation of transport while ATP stimulated sugar entry in both aerobic and anaerobic cells.

4. Neither monovalent nor divalent cations were absolutely required for the development of the faster rate of transport in anoxic cells.

INTRODUCTION

Glucose transport in avian erythrocytes resembled that in heart and skeletal muscle in being carrier-mediated and stimulated by inhibition of oxidative metabolism[1]. The mechanism by which energy depletion, resulting from anoxia or metabolic poisons, is transduced into an accelerated rate of sugar movement through the cell membrane is unknown. One proposed mechanism[2] suggested that transport in muscle could be regulated by the degree of phosphorylation of a membrane protein by a high energy intermediate such as ATP. If this mechanism were to operate, the time required for development of an anoxic stimulation of transport must be consistent with changes in intracellular metabolites.

In the present experiments, sugar transport in goose erythrocytes was studied during induction of simple anoxia without the addition of metabolic poisons. The time course of the anoxic effect was correlated with intracellular levels of nucleotides and inorganic phosphate. Requirements for cations for obtaining an anoxic stimulation of transport were investigated. The abilities of various metabolic intermediates to alter transport rates when added to the external medium also were determined.

EXPERIMENTAL PROCEDURE

Blood was collected from the neck veins of white domestic geese, into polycarbonate bottles containing heparin (2 mg/100 ml blood). The cells were washed three times by centrifugation and resuspension in cold buffer. Leukocytes were removed during the washing procedure. Cells were either used fresh or stored overnight at 4 °C. If stored, the cells were washed again immediately before use. The buffer contained 137 mM NaCl, 5.9 mM KCl, 1.3 mM $CaCl_2$, 2.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.2 mM imidazole, 7.6 mM glycylglycine, and 0.2% bovine serum albumin. The same buffer was used for incubation of cells and for measurement of glucose uptake and 3-*O*-methylglucose entry and exit.

Entry of 3-O-methylglucose, L-*glucose, and* D-*sorbitol.*

Cells were suspended in buffer at 10% hematocrit, placed in 2-l roller bottles that were equipped with perfusion caps, and incubated at 37 °C in a New Brunswick Rollertherm incubator. Bottles were gassed continuously with either water-saturated O_2 or N_2. After various periods of incubation, sufficient 3-*O*-[^{14}C]methylglucose was added through the perfusion cap to give an extracellular concentration of 12.5 μM. At various intervals after the addition of 3-*O*-methylglucose, duplicate samples (5 ml) of suspension were withdrawn through the prefusion caps with 10-ml syringes and transferred to weighed tubes sitting in an ice bath. The suspension was allowed to cool for 5–10 min and reweighed and the cells collected by centrifugation. The cells were washed three times in cold buffer (2 °C). The cells were hemolyzed with distilled water and the hemolysate was deproteinized with perchloric acid. Additional aliquots of the whole cell suspension were mixed with water and precipitated with perchloric acid. Aliquots of the samples were counted in a scintillation spectrometer.

Equilibration of intracellular and extracellular sugar concentration was calculated using the following formula:

$$\% \text{ equilibrium} = \frac{\text{dpm/ml of intracellular } H_2O \text{ at time } t}{\text{dpm/ml of intracellular } H_2O \text{ at equilibrium}} \cdot 100$$

$$\text{where dpm/ml at time } t = \frac{\text{dpm/ml of cell extract} \cdot \text{ml of extract,}}{\text{ml of intracellular } H_2O}$$

$$\text{and dpm/ml at equilibrium} = \frac{\text{dpm/ml of extract of cell suspension} \cdot \text{ml of extract,}}{\text{ml of } H_2O \text{ in whole suspension}}$$

Intracellular water of the suspension was calculated from measurements of dry weight of the cell suspension and buffer, and the hematocrit. Equilibrium distribution of 3-*O*-[^{14}C]methylglucose could be determined by this method since the sugar was not metabolized by avian erythrocytes[1].

3-O-Methylglucose exit

Washed cells were suspended in buffer at 50% hematocrit, gassed with either O_2 or N_2 and preloaded with 3-*O*-[^{14}C]methylglucose (15 mM) by incubation at 37 °C for 20 min. The suspensions were diluted with 20 ml of cold buffer (2 °C) and washed three times. Washed cells containing 3-*O*-[^{14}C]methylglucose were added to sugar-free buffer (37 °C) to give a 5% hematocrit. Samples (5 ml) were removed at intervals into

tubes sitting in an ice bath. After centrifugation, the supernatant was collected for determination of 3-*O*-[^{14}C]methylglucose.

Glucose uptake and utilization

Glucose uptake was determined in 40–80% cell suspensions by measuring disappearance of glucose from the medium[1]. In addition, glucose utilization was measured by determining production of tritiated water from [5-^{3}H]glucose[3]. This method provides an index of glucose utilization since the tritium on the fifth carbon of the sugar exchanged freely with water at the triose phosphate isomerase and enolase steps of glycolysis. Tritiated water formation was determined following incubation of the cell suspension (40% hematocrit) with O_2 or N_2 in a roller bottle.

L-*Glucose and sorbitol spaces*

Cell suspensions (40–50% hematocrit) were incubated with L-[^{14}C]glucose (40 μM) or [^{14}C]sorbitol (2.8 μM). Aliquots of the whole suspension and of the supernatant, obtained by centrifugation at $5000 \times g$ for 5 min, were collected, extracted with perchloric acid and counted. Spaces were defined as ml of water required to dissolve the sugar of the whole suspension at the concentration found in the extracellular phase, and were calculated as described earlier[1].

Nucleotide triphosphate analysis

Aliquots of suspension were weighed and centrifuged. The cells were homogenized with ice cold 0.6 M perchloric acid (2 ml per g cells) and the precipitate spun down. The extract was neutralized with KOH to pH 6.7–7.0, and the $KClO_4$ removed by centrifugation. Nucleotide triphosphates were determined by the hexokinase method[4]. Levels of ATP, GTP, ADP, AMP, and IMP were determined by high pressure liquid chromatography (Dupont Instruments, 830 Liquid Chromatograph) using a Permaphase column. Samples of cell extract (5–10 μl) were injected into the column, and the nucleotides were eluted with phosphate buffer, pH 3.3. A linear gradient of 1 mM to 0.5 M KH_2PO_4 was used to elute nucleotide mono- and diphosphates. During this portion of the chromatogram, the phosphate concentration increased at the rate of 3% per min. After ADP was eluted, the gradient was increased to 10% per min to elute ATP and GTP. ATP accounted for 90% of the total nucleotide triphosphate value obtained by the hexokinase assay.

Intracellular free glucose

Cell suspensions (70–80% hematocrit) were incubated with 15 mM glucose. At 5–30-min intervals, aliquots of suspension (2–3 ml) were removed into tubes sitting in an ice bath. After the tubes were weighed, ice-cold buffer (20 ml) was added to dilute the extracellular glucose, and the cells were spun down. The cell pellet was mixed with 2 ml of 1.5 M $Ba(OH)_2$ and 1.3 ml of 5% $ZnSO_4$ (final pH 7.0). The mixture was frozen, thawed and centrifuged to remove the protein. The supernatants were shaken with a small amount of Dowex-50W and Duolite A-4 to remove phosphorylated or charged intermediates[5]. Recovery of free glucose by this method was 90%. Aliquots of the clear extract and of the extracellular buffer were analyzed for glucose by the hexokinase assay[4]. Glucose concentrations within the cells were calculated from measurements of the glucose content of the cell pellet and extracellular buffer.

Ion analysis

Aliquots of cell suspension to be analyzed for Na^+, K^+, Ca^{2+} and Mg^{2+} were mixed with [^{14}C]sorbitol to measure the extracellular space. The cells were pelleted by centrifugation, the supernatant was aspirated and the cell pellet was mixed. Aliquots were taken for wet ashing, and for determination of extracellular fluid volume of the cell pellet. The residue from wet ashing was diluted with 0.1 M HCl and analyzed for Na^+, K^+, Mg^{2+} and Ca^{2+} using a Zeiss spectrophotometer with an Fa2 flame attachment. Ion concentrations were calculated according to the following formula:

$$\text{mequiv./l } IC_{H_2O} = \frac{\dfrac{\text{ion content of ashed cell suspension, mequiv.}}{\text{g of cell suspension}} - \dfrac{\text{(1-hematocrit, ml/ml)}}{\text{specific gravity of suspension, g/ml}} \cdot \text{(ion content of buffer, mequiv./ml)}}{\text{Fraction } H_2O \text{ in cells, ml} \cdot g^{-1} \cdot 0.001}$$

Cell water

Cell water was determined by drying an aliquot of cells to a constant weight, and calculating ml of intracellular water per g of dry cells using the specific gravity, extracellular fluid volume and hematocrit measurements that were made on aliquots of the same cell pellet[6]. The percent dry weight of the cells, w/w, was found using the following formula:

$$\text{w/w} = \frac{\text{dry wt of suspension, g} - \dfrac{\text{wet wt of suspension, g}}{\text{specific gravity of suspension, g} \cdot \text{ml}^{-1}} \cdot \text{(1-Hematocrit, ml/ml)} \cdot \text{Fraction dry in buffer, g} \cdot \text{ml}^{-1}}{\text{wet wt of blood, g} - \dfrac{\text{wet wt of suspension, g}}{\text{specific gravity of suspension, g} \cdot \text{ml}^{-1}} \cdot \text{(1-Hematocrit, ml/ml)} \cdot \text{Specific gravity of buffer, g} \cdot \text{ml}^{-1}}$$

Inorganic phosphate analysis

Intracellular inorganic phosphate was analyzed by a modification of the method of Wahler and Wollenberger[7]. This modification involved addition of 1 ml of perchloric acid extract to 3 ml of 12.5 mM Na_2MoO_4 rather than the 5 mM that was described. Extracellular inorganic phosphate was analyzed by the method of Harris and Popat[8].

Reagents

All inorganic chemicals used were reagent grade. Organic reagents were obtained from Sigma or Calbiochem. Heparin was purchased from Nutritional Biochemicals. 3-*O*-[^{14}C]Methylglucose was obtained from New England Nuclear or Amersham-Searle and [5-^{3}H]glucose and [^{14}C]sorbitol from New England Nuclear. Enzymes were obtained from Sigma. β,γ-Methylene-adenosine triphosphate was obtained from Miles Laboratories, Kankakee, Ill.

RESULTS

Specificity and reversibility of anoxic stimulation

Anoxia stimulated the rate of entry of 3-*O*-methylglucose into goose erythrocytes (Fig. 1). After a lag period of 45 min, rate of sugar entry into anaerobic cells

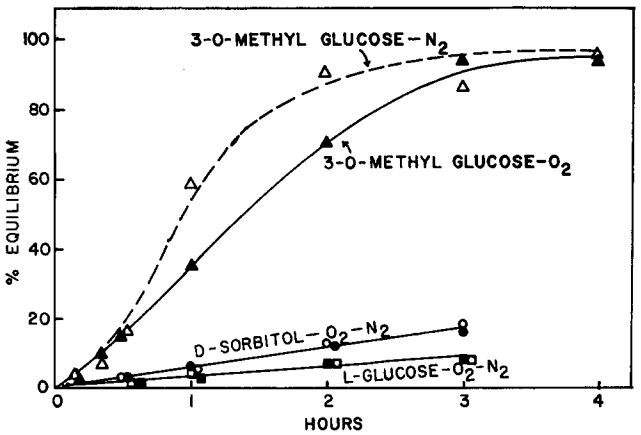


Fig. 1. Entry of 3-*O*-methylglucose, L-glucose, and D-sorbitol into goose erythrocytes. Suspensions (40%) were incubated in bottles gassed with O_2 or N_2 as described in Experimental procedures and sampled for determination of intracellular 3-*O*-[^{14}C]methylglucose, L-[^{14}C]glucose and D-[^{14}C]sorbitol. This complete experiment was done on two other days with similar results.

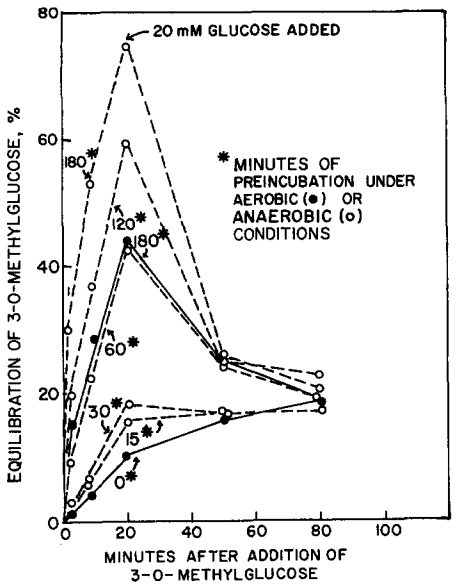
was 2–3 times that of aerobic cells. The lag period included the time required to wash O_2 out of the incubation bottle (10 min), and the time required to exhaust O_2 from the hemoglobin. The effect of anoxia was shown to involve stimulation of carrier-mediated transport rather than a non-specific increase in permeability by measurements of L-glucose and D-sorbitol entry. Both substances entered aerobic or anaerobic cells at a slow rate (Fig. 1). In other experiments, L-glucose and D-sorbitol spaces were calculated from analyses of whole blood and supernatants. These measurements confirmed the conclusion that only small amounts of these substances had entered the cells since the spaces did not exceed the extracellular volume as calculated from hematocrit measurements.

Stimulation of carrier-mediated sugar transport by anoxia also was demonstrated by observing counterflow of 3-*O*-methylglucose by glucose (Fig. 2). In these experiments, cells were incubated for various periods of time under O_2 or N_2, and allowed to accumulate 3-*O*-methylglucose during the last 20 min. At this point, glucose was added to a final concentration of 20 mM. In all cases in which the period of anoxia was sufficient to allow significant accumulation of sugar, 3-*O*-methylglucose left the cells against its concentration gradient in response to the addition of glucose. It can also be seen that the rate of entry of 3-*O*-methylglucose continued to increase with longer periods of anoxia.

The effect of anoxia on transport was reversible (Fig. 3). Half-time ($t_{\frac{1}{2}}$) for entry of 3-*O*-methylglucose into cells incubated 60 min with O_2 or N_2 were 60 and 25 min, respectively. In cells switched to O_2 after 1 h of incubation with N_2, the $t_{\frac{1}{2}}$ was 67 min, a value near that observed in aerobic cells. These results indicated that the accelerated rate of transport in anoxic cells was reduced when the cells were returned to aerobic conditions.

Entry and exit rates and cell water content

Progression of the anoxic effect on transport was monitored by determining the $t_{\frac{1}{2}}$ for entry of sugar into cells preincubated with O_2 or N_2 for various times



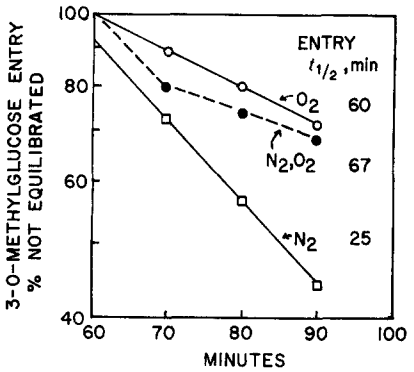


Fig. 2. Counterflow of 3-*O*-methylglucose from goose erythrocytes incubated for varying times under anaerobic conditions. After a period of preincubation in a N_2 or O_2 atmosphere, 3-*O*-methylglucose was added to the suspension and the cells allowed to equilibrate with the non-metabolized sugar for 20 min. After the equilibration period, a 1-M solution of glucose was added to each flask to give a final concentration of 20 mM. Incubation with both 3-*O*-methylglucose and glucose was continued for 1 h. This experiment was done on two other days with similar results.

Fig. 3. Reversibility of the anoxic stimulation of 3-*O*-methylglucose transport into goose erythrocytes. Cell suspensions were preincubated for 60 min in an O_2 or N_2 atmosphere. After 60 min, one bottle that had been preincubated with N_2 was switched to O_2. Sugar was added and its entry followed over the next 30 min. ○—○, experiments in which incubation was for 90 min with O_2; ●---●, 60 min with N_2 followed by 30 min with O_2; and □—□, 90 min with N_2. This experiment was repeated on two other days with similar results.

(Fig. 4). Half-times of entry in suspensions preincubated for 0–15 and for 60 min with O_2 were 81 and 47 min, respectively. After 20–35 min of anoxia, $t_{\frac{1}{2}}$ decreased to 35 min. Further preincubation for 60 and 120–180 min decreased the $t_{\frac{1}{2}}$ to 15 and 10 min, respectively. The anoxic effect on exit of 3-*O*-methylglucose also was investigated in order to determine if the stimulation of transport involved both influx and efflux. Cells that had been allowed to accumulate 3-*O*-methylglucose were washed and resuspended in buffer. Appearance of 3-*O*-methylglucose in the buffer was measured. Half-time for exit in both aerobic and anaerobic cells agreed well with $t_{\frac{1}{2}}$ for entry, being 47 and 26 min for cells incubated 60 min with O_2 and N_2, respectively.

Incubation under anaerobic conditions has been reported to cause cells to swell[9]. After 60 min of incubation, anoxic cells were found to have slightly more water than aerobic cells (0.628 ± 0.004 compared to 0.613 ± 0.008 ml water/g wet wt). When entry rates were calculated from the 3-*O*-methylglucose concentration, half-time, cell volume and dry cell mass[10], the effect of anoxia on the rate of entry was 3% less than indicated by comparison of only the half-times. When exit rates were calculated, the magnitude of the anoxic effect on exit was underestimated by the same fraction.

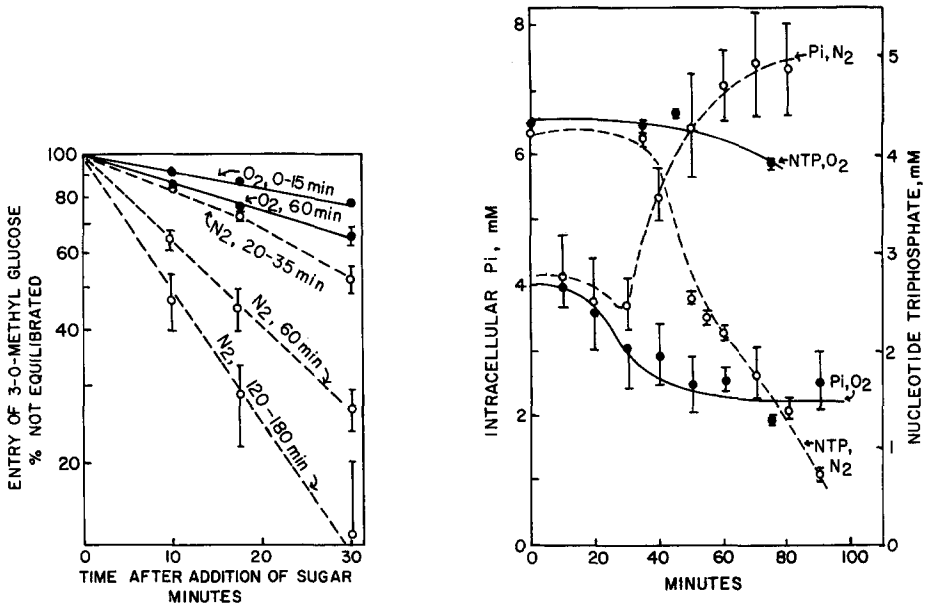


Fig. 4. Effect of anoxia on half-time for entry of 3-*O*-methylglucose into goose erythrocytes. Cell suspensions were sampled for determination of intracellular 3-*O*-[^{14}C]methylglucose at the times indicated. The period of preincubation under aerobic (●) or anaerobic conditions (○) is indicated on each line. Incubation was continued in the same gas phase during the period of 3-*O*-methylglucose accumulation. The values that are plotted are the mean ± S.E. of 3–7 determinations.

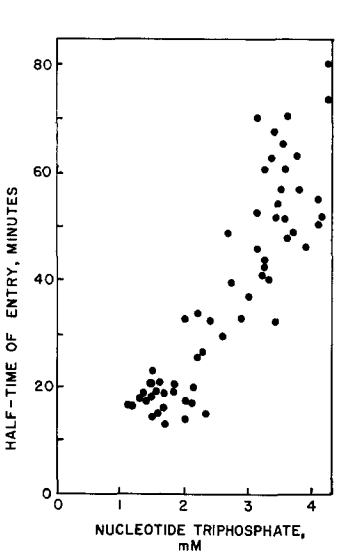
Fig. 5. Effect of anoxia on intracellular inorganic phosphate and nucleotide triphosphates. All cell suspensions (10%) were incubated in an atmosphere of O_2 (●) or N_2 (○), and samples were taken at various times for analysis of phosphate and nucleotide triphosphates as described in Experimental procedures. Values are the mean ± S.E. of 3–10 determinations.

Low and high energy intermediates during anoxia

Total nucleotide triphosphate, and inorganic phosphate were measured during anoxia in order to relate the time course of changes in these metabolites to the onset of the anoxic stimulation of transport. Nucleotide triphosphate was maintained at 4 mM during 90 min of incubation without substrate in aerobic cells (Fig. 5). Under anaerobic conditions, the levels of nucleotide triphosphate were maintained for 40 min. By 90 min the level of nucleotide triphosphate had decreased to less than 1 mM. Inorganic phosphate began to increase after 30 min of anoxia and was significantly higher by 50 min. After 90 min, P_1 was 7.5 mM. The time of onset of transport stimulation correlated with the beginning of the fall in nucleotide trisphosphate.

A better definition of the relationship between transport rate and nucleotide triphosphate levels was obtained by plotting $t_{\frac{1}{2}}$ of entry of 3-*O*-methylglucose as a function of nucleotide triphosphate levels in cells that were incubated for various times with O_2 or N_2 (Fig. 6). A decrease in nucleotide triphosphate from 4 to 2 mM was accompanied by a decrease in $t_{\frac{1}{2}}$ from approx. 60 to 20 min.

Values for ATP, GTP, ADP, AMP+GMP, and IMP during incubation in a N_2 atmosphere were determined to define the relationship between changes in levels



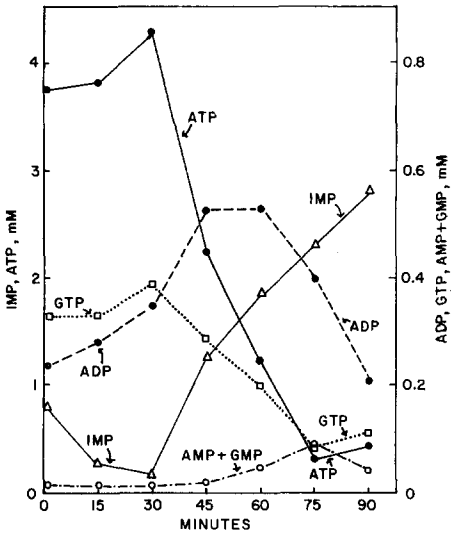


Fig. 6. Relationship between half-time of entry and nucleotide triphosphate levels. Cell suspensions were incubated in an atmosphere of O_2 or N_2 and samples taken after various time periods for determination of 3-*O*-methylglucose entry and content of nucleotide triphosphate. Levels of nucleotide triphosphate are plotted as the average values found during the period of time over which entry of 3-*O*-methyl glucose was measured.

Fig. 7. Nucleotide concentrations in anaerobic erythrocytes. A 10% suspension of cells was incubated with N_2 for 90 min. Aliquots of the cell suspension were analyzed for ATP, GTP, ADP, IMP, and AMP+GMP by high pressure liquid chromatography as described in Experimental procedures. This experiment was repeated on 3 other days with similar results both for concentrations of nucleotides and the time course of the changes.

of these metabolites and the rate of transport (Fig. 7). GTP levels declined in a manner parallel to that of ATP. ADP and IMP began to increase when ATP levels fell. Changes in these metabolites were apparent after 30 min of incubation, and before a change in transport rate was detected. AMP and GMP did not increase until cells had been incubated for 75 min in a N_2 atmosphere. The largest change was in the concentration of IMP, which increased about 5- to 10-fold. This finding indicated that, in the avian erythrocyte, AMP formed from ATP hydrolysis was rapidly converted to IMP rather than to adenosine. In aerobic cells, the relative amounts of ATP, GTP, ADP and IMP did not change significantly over 75 min of incubation. There was some loss of total nucleotide from the cells during incubation, but the loss was equivalent in aerobic and anaerobic cells. These studies demonstrated that loss of ATP and accumulation of ADP and IMP began immediately prior to the time that transport of 3-*O*-methylglucose started to rise.

Further investigation of the relationship between loss of nucleotide triphosphate and onset of transport stimulation was undertaken in cells by measuring glucose uptake. Anoxia increased the rate of glucose uptake, from 0.8 to 4.9 μmoles $\cdot g^{-1} \cdot h^{-1}$ (Fig. 8). Glucose disappearance from the medium was not stimulated significantly before 90 min, the time at which the nucleotide triphosphate levels began to fall. The time course of the anoxic stimulation of glucose uptake was confirmed by measuring

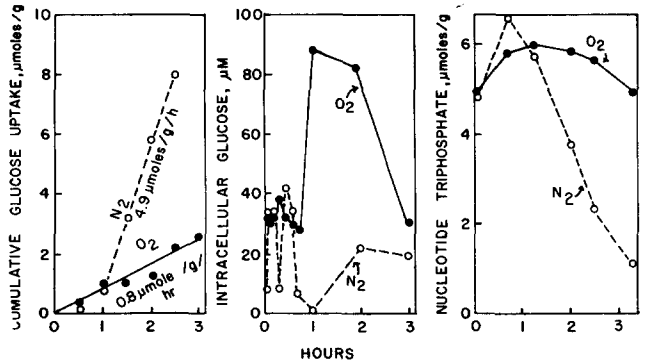


Fig. 8. Effect of anoxia on glucose uptake, intracellular glucose concentration, and nucleotide triphosphate levels. Glucose uptake (left panel) by a 50% cell suspension was calculated from measurements of glucose disappearance from the incubation medium. The initial glucose concentration was 3.5 mM. The uptake rate was calculated between 60 and 180 min of incubation. A similar time course of uptake by aerobic and anaerobic cells was found in 11 additional experiments. Intracellular glucose (middle panel) was estimated in 70% cell suspensions incubated with 15 mM glucose. At the time indicated, cell samples were taken for determination of intracellular free glucose. The higher intracellular free glucose in aerobic cells was found in two additional experiments. Nucleotide triphosphate levels (right panel) were measured at the times indicated, as described in Experimental procedures. Similar values were found for aerobic and anaerobic cells on two other days.

production of tritiated water from [5-H^3]glucose as described in Experimental procedures. In this case, an accelerated rate of tritiated water production also was found after 90 min of incubation in a N_2 atmosphere.

Measurement of intracellular free glucose showed that a large gradient of glucose existed across the cell membrane in both aerobic and anaerobic cells (Fig. 8). Although the extracellular glucose concentration was 15 mM, the intracellular concentration in aerobic or anaerobic cells initially was only about 30 μM. This finding indicated that glucose phosphorylation was able to keep pace with the entry of glucose into the cell, and that transport was the major restriction to glucose uptake. Intracellular glucose levels remained low in anaerobic cells as incubation was continued. An accelerated rate of glucose phosphorylation could account for the ability of anaerobic cells to maintain low intracellular glucose levels despite faster rates of glucose entry.

Effect of extracellular nucleotides, adenosine, adenine and inorganic phosphate on 3-O-methylglucose transport.

The possibility that metabolic intermediates whose levels changed in anaerobic cells would modify the rate of transport was explored by addition of these intermediates. Adenine (3.5 mM) was found to inhibit the rate of entry of 3-*O*-methylglucose in anaerobic cells (Fig. 9). Half-time for entry was increased from 20 to 32 min. The intracellular nucleotide triphosphate levels in cells incubated with adenine in the buffer were not different from those in the control cells.

ATP (as the sodium or magnesium salt) was found to significantly increase the rate of entry of 3-*O*-methylglucose into aerobic and anaerobic cells (Fig. 9, Table I). Half-times for entry of sugar were 70 min in the control and 41 min in cells incubated

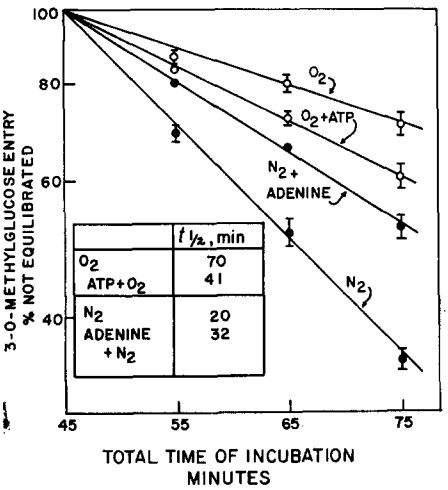


Fig. 9. Effect of exogenous ATP and adenine on entry of 3-*O*-methylglucose in aerobic or anaerobic cells. Cell suspensions (10%) were preincubated for 45 min and entry of 3-*O*-methylglucose was determined over the following 30 min. ○, indicate suspensions that were incubated aerobically with or without ATP (5 mM); ●, suspensions that were incubated anaerobically with or without adenine (3.5 mM). Values represent mean ± S.E. of 3–8 experiments.

TABLE I

EFFECT OF EXTRACELLULAR NUCLEOTIDES, BASES AND INORGANIC PHOSPHATE ON 3-*O*-METHYL GLUCOSE ENTRY INTO GOOSE ERYTHROCYTES

Entry of 3-*O*-methylglucose is given as the percent to which intracellular water had equilibrated with extracellular sugar. Values are those reached 30 min after addition of 3-*O*-methylglucose to cells that had been preincubated 45 min with the respective buffers. All compounds were present at 5 mM except as noted. Glucose was present in the buffer only during the period of 3-*O*-methylglucose accumulation. n.t., not tested.

	Buffer	*Aerobic*	*Anaerobic*
1	Control	19.7 ± 0.8 (8)	65.2 ± 2.9 (4)
2	Adenine, 3.5 mM	23.8 ± 1.5 (3)	49.9 ± 0.2 (4)*
3	ATP	29.2 ± 1.3 (8)*	73.0 ± 2.4 (4)*
4	Mg ATP	28.7 ± 1.9 (4)*	70.7 ± 3.4 (3)
5	ATP washed away	26.8 ± 2.1 (4)*	n.t.
6	GTP	22.1 ± 1.6 (3)	n.t.
7	UTP	21.3 ± 1.8 (3)	n.t.
8	ADPCP	22.9 ± 2.7 (5)	n.t.
9	ATP + glucose (20 mM)	12.6 ± 2.5 (3)*	n.t.
10	Adenosine	22.5 ± 1.5 (3)	60.7 ± 6.1 (4)
11	AMP	24.8 ± 0.4 (3)	65.4 ± 7.6 (4)
12	IMP	24.4	n.t.
13	ADP	21.5 ± 3.1 (3)	n.t.
14	AMP + P_i (10 mM)	14.3, 19.6	n.t.
15	ADP + P_i	22.4 ± 2.6 (3)	n.t.
15	P_i, 20 mM	21.8 ± 1.9 (6)	61.5 ± 6.8 (3)
17	IMP ± P_i (10 mM)	24.4, 19.0	n.t.

* $P < 0.05$ *vs* respective control by paired analysis.

with 5 mM ATP. In other experiments, the lowest effective concentration of ATP was 2 mM. Increasing the concentration to 10 mM did not increase the transport rate further. There was no lag period for the stimulatory effect of ATP. Faster entry of 3-*O*-methylglucose was seen without preincubation of the cell suspension or when ATP was added at 45 min along with the 3-*O*-methylglucose.

When ATP was added to the buffer, it was rapidly hydrolyzed to AMP and phosphate (Fig. 10). Even when samples of the suspension were removed as rapidly as possible (zero time), about half of the ATP had been lost and substantial amounts of AMP and ADP were present. ATP completely disappeared within 10 min. At this time, the concentration of inorganic phosphate had increased to 10 mM. When cells were incubated for 45 min with ATP, and then washed and resuspended in fresh buffer before transport was measured, the accelerated rate of entry of 3-*O*-methylglucose persisted (Table I). Other nucleotide triphosphates (GTP and UTP) were also hydrolyzed to the respective monophosphate but did not change transport rate. The methylene diphosphonate derivative of ATP (β,γ-methylene-adenosine triphosphate) was also without effect.

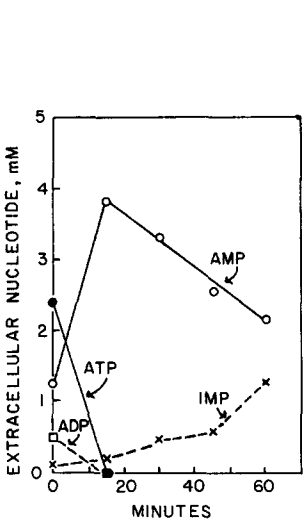


Fig. 10. Fate of ATP added to buffer in which goose erythrocytes were incubated. Cells (10% suspension) were incubated aerobically in buffer containing 5 mM ATP, and samples of the suspension were taken at various times. The cells were spun down and the buffer analyzed for ATP, ADP, AMP, and IMP. The experiment was repeated on three other days with similar results.

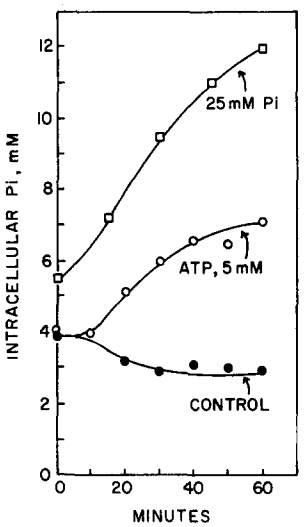


Fig. 11. Intracellular inorganic phosphate in cells incubated in the presence of ATP or increased extracellular phosphate. Cells were incubated in control buffer (1.2 mM phosphate) or in buffer containing 5 mM ATP or 25 mM phosphate. Samples of cell suspension (20%) were spun down and the content of inorganic phosphate in the cell pellet and extracellular fluid was determined. The experiment was repeated on three other days with similar results.

To verify that the stimulatory effect of ATP was on carrier-mediated transport and not on diffusion of 3-*O*-methylglucose, glucose (20 mM) was used as a competitor for the transport of 3-*O*-methylglucose. Glucose prevented the ATP stimulation of 3-*O*-methylglucose transport (Table I).

In view of the rapid hydrolysis of ATP, various combinations of the hydrolytic products were tested for their ability to alter transport rates. None of the combinations tested were found to reproduce the effect of ATP (Table I, Lines 10–17). Intracellular nucleotide triphosphate levels were not changed by addition of any of the compounds or combinations of compounds to the extracellular phase.

Cells incubated in the presence of 5 mM ATP had increased intracellular inorganic phosphate (Fig. 11). The P_i level was equivalent to that found in cells incubated in a N_2 atmosphere for 60 min. However, the increase in P_i alone was unlikely to be the factor responsible for stimulation of 3-*O*-methylglucose transport, since intracellular P_i could be increased to 10–12 mM by incubation of cells in buffer containing 25 mM P_i, with no effect on transport rate.

Effect of ionic composition of the buffer

Cells were incubated in buffers of varying ionic compositions in order to determine if there was a requirement for an ion in the carrier-mediated transport of sugar in nucleated erythrocytes (Table II). There was no difference in rate of sugar entry in

TABLE II

EFFECT OF BUFFER IONIC COMPOSITION ON 3-*O*-METHYLGLUCOSE ENTRY IN ANOXIC CELLS

Cells were washed 3–5 times in the buffer and then incubated in an atmosphere of O_2 or N_2. Entry of 3-*O*-methylglucose was calculated as the percent to which intracellular water had equilibrated with extracellular sugar. Values are those reached 30 min after addition of 3-*O*-methylglucose to cells that had been preincubated 45 min with the respective buffers. All ion substitutions were made isosmotically with choline, K^+ or Li^+ in place of Na^+, and Na^+ in place of K^+, Ca^{2+} or Mg^{2+}. Ion-free buffers were checked by flame photometry to show that they were initially free of the ion to be omitted. Osmolarity of buffer was measured on an Advanced Model 3R osmometer. Values are given as the percent of the value for anaerobic cells incubated in control buffer.

Buffer	*Equilibration of cell water (% of anaerobic control)*	*Nucleotide triphosphate (% of anaerobic control)*
Anaerobic cells		
Control	100 ±10 (11)	100 ±10 (11)
Na^+-free	99.0± 8.0 (7)	170.6± 8.5 (7)*
Ouabain, $5 \cdot 10^{-5}$ M	76.3± 6.0 (7)*	220.7±54.3 (7)*
K^+-free	87.4± 7.2 (9)	164.4±26.4 (9)*
Ca^{2+}-free	94.8±12.0 (4)	152.3±23.2 (4)*
Mg^{2+}-free	115.2±14.0 (4)	127.5±35.5 (4)
Ca^{2+}- and Mg^{2+}-free	98.3± 5.3 (7)	113.0±16.5 (7)
$5 \times Ca^{2+}$ (6.5 mM)	99.8±11.6 (5)	138.8±30.7 (5)
$5 \times Mg^{2+}$ (12 mM)	125.4± 8.7 (4)*	95.8± 9.5 (4)
$5 \times Ca^{2+}$ (6.5 mM) + Mg^{2+} (12 mM)	101.4± 6.6 (7)	124.1±26.1 (7)
Aerobic cells		
Control	43.9± 1.0 (4)	424.5±33.8 (4)
Ca^{2+}- and Mg^{2+}-free	44.3, 39.9	427.3, 508.4
$5 \times Ca^{2+}$ (6.5 mM) + Mg^{2+} (12 mM)	48.1± 2.2 (4)	413.2±28.1 (4)

* $P < 0.05$ *vs* anaerobic or aerobic control.

anaerobic cells when incubated in Na^+-free buffer or when Na^+ was replaced by K^+, Li^+ or choline. The anaerobic rate of 3-*O*-methylglucose entry was somewhat lower in cells incubated (a) with ouabain, (b) without K^+, or (c) without Ca^{2+}. In each of these instances, nucleotide triphosphate levels were higher than they were in the anoxic control cells. Transport rate was not modified by addition of higher levels of Ca^{2+} or Mg^{2+} to the incubation medium of anaerobic or aerobic cells.

Intracellular Na^+ and K^+ concentrations were measured during incubation of cells under aerobic and anaerobic conditions. During the first hour of incubation, the concentration of these ions did not change, but within the following 2 h, cells began to gain Na^+ and lose K^+ (Fig. 12). In addition, intracellular Ca^{2+} and Mg^{2+} were measured. No change could be detected in Ca^{2+} or Mg^{2+} concentration during the period over which entry of 3-*O*-methylglucose was measured.

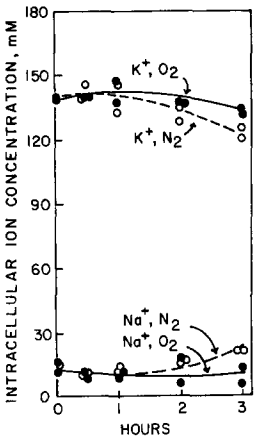


Fig. 12. Cell Na^+ and K^+ concentrations during aerobic and anaerobic incubations. Cell suspensions were incubated with O_2 (●) or N_2 (○) and samples taken at various times for water and ion analysis.

DISCUSSION

The effect of anoxia was shown to involve a stimulation of carrier-mediated transport of 3-*O*-methylglucose. This conclusion was supported by demonstrations of substrate stereospecificity and countertransport. Movement of sugar both into and out of the cells was enhanced, as shown by the agreement between rates of entry and exit of sugars, in cells incubated in a N_2 atmosphere. The magnitude of the effect involved a 2–3-fold decrease in half-times for entry or exit. Reversibility of the anoxic effect could be demonstrated in cells preincubated 60 min anaerobically and subsequently incubated in an O_2 atmoshpere. These findings indicated that this period of anoxia did not lead to irreversible damage of the membrane.

There was a time lag of 40–45 min before an anoxic stimulation of transport was apparent. An estimated 20 min of this lag was accounted for by the time needed to wash O_2 out of the incubation bottle and to desaturate the hemoglobin. The remaining 20 min appeared to represent the time required to generate the signal for stimulation of transport. The lag period suggested that the signal was generated either by depletion of intracellular stores of an inhibitory substance, or by a slow

buildup of a product which was stimulatory, or by combination of both of these possibilities.

Cells could be incubated aerobically, in substrate-free medium, for at least 3 h without appreciable loss of total nucleotide triphosphate or ATP. The substrate stores which maintained ATP during this time were not identified. Measurable quantities of creatine phosphate were not found. ATP was maintained for 30–40 min in cells incubated anaerobically. Following this period, ATP levels decreased rapidly, and were very low by 90 min. The decrease in ATP largely was accounted for by an increase in IMP in the cells. Neither AMP nor GMP accumulated to high levels within the cells. The decrease in ATP and increase in ADP, IMP and P_i occurred just prior to a measurable increase in transport rate, indicating that any or all of these compounds might be involved in transport regulation.

An increase in inorganic phosphate alone did not appear to be responsible for transport stimulation. When intracellular P_i was raised to the same level as that found in anaerobic cells by increasing the extracellular P_i concentration, no change in transport rate was noted. ATP concentrations in these experiments remained the same.

Glucose uptake in anaerobic cells did not increase until ATP levels began to fall. The onset of the anoxic effect on glucose uptake had a lag of 90 min. Glucose transport appeared to be the major rate-limiting step for glucose uptake in either aerobic or anaerobic cells with low rates of glucose utilization since only low levels of intracellular glucose were found. Once the transport rate had been accelerated, glucose phosphorylation could still keep pace with increased rates of entry, as indicated by lack of accumulation of free intracellular glucose.

The good correlation between ATP levels and transport rate does not indicate that ATP was the controlling factor in transport regulation. Equally good correlations can be made between transport rate and ATP/ADP ratios, P_i, IMP, or energy charge of the cell[11]. None of these intermediates changed in a manner which would be inconsistent with a model in which they regulated activity of the carrier.

Adenine, when added to the extracellular medium, partially inhibited the anoxic stimulation of transport. Since ATP concentrations in cells incubated with adenine were slightly lower than in control anoxic cells, this effect apparently was not due to maintenance of ATP levels. Moreover, with no other substrate or source of ribose available, adenine would not be expected to maintain ATP synthesis[12].

Likewise, the mechanism of the stimulation of sugar entry in cells incubated in the presence of ATP is not understood. Entry rate was stimulated both in aerobic and anaerobic cells. The magnitude of the effect was not dependent upon ATP concentration in the range from 2 to 10 mM, and was not reproduced by addition of any of the hydrolytic products of ATP which were found in the medium. Since the effect of ATP was (1) immediate, (2) was not removed by washing the ATP from cells before transport rate was measured, and (3) was seen even 50 min after ATP had disappeared from the incubation medium, several possibilities can be suggested for its effect. ATP may have (1) entered the cells, (2) bound tightly to a membrane component, (3) phosphorylated a membrane component which was not easily dephosphorylated, (4) changed the activity of some membrane contractile element, or (5) chelated divalent cations from the membrane and allowed more rapid carrier movement. Entry of ATP would have been expected to inhibit transport, unless it was hydrolyzed at the inner surface of the membrane, to products that facilitated sugar entry. Exogenous ATP

has been shown to stimulate Na^+ transfer[13], to increase short-circuit current[14] across small intestine, and to increase acid secretion by gastric mucosa[15]. In these studies, it was suggested that ATP may have acted after entering the cells.

In view of the rapid disappearance of ATP from the medium, a high affinity would have been required to maintain binding during rapid hydrolysis of the extracellular nucleotide. Other nucleotide triphosphates did not stimulate sugar entry. Lack of a stimulatory effect following addition of β,γ-methylene-adenosine triphosphate may indicate that the effect of ATP was related to provision of a high energy phosphate rather than to binding as an allosteric regulator.

ATP, acting from the outside of the plasma membrane, could have changed the activity of a contractile protein in the membrane and through this mechanism, altered carrier activity. In avian erythrocytes, a change in cell volume was not noted following addition of ATP. Volume changes have been reported to accompany addition of ATP to tumor and kidney tubule cells, but only in a Ca^{2+}-free medium[16,17]. This effect has been suggested to be due to a change in contractile elements of the membrane or to increased Ca^{2+} influx[16,17].

Acceleration of sugar transport resulting from removal of divalent cations by chelation with ATP did not appear to be likely. MgATP was as effective as Na_4ATP. Chelators such as EDTA, or incubation in divalent cation-free buffer did not increase sugar entry. Intracellular ATP levels were not altered by any of the above procedures.

The anoxic stimulation of sugar transport in goose erythrocytes was not absolutely dependent upon the presence of Na^+, K^+, Ca^{2+} or Mg^{2+} in the incubation medium. Although the cells were washed five times in ion-free buffer before incubation and estimation of entry of 3-*O*-methylglucose, efflux of ions from the cell could have partially restored the missing ion. The necessity for incubating the cells for 40–45 min in a N_2 atmosphere, in order to obtain the anaerobic effect, prevented short-term experiments in which efflux would have been minimized. Although the anaerobic rate of 3-*O*-methylglucose entry was somewhat lower in the presence of ouabain, or in the absence of K^+ or Ca^{2+}, the lower entry rate may have been due to a slower rate of energy depletion. Inhibition of ion transport by ouabain or lack of K^+ would have reduced ATP utilization. Lack of Ca^{2+} would restrain hydrolysis by ATPases dependent upon this ion. Lack of an effect of ions on 3-*O*-methylglucose entry and glucose uptake is in contrast to the results reported for muscle and adipose tissue[18,19]. However, comparison of effects of ions in those studies is difficult since ATP was not measured in these tissues. The role of ions in the passive sugar transport has recently been reviewed[20].

ACKNOWLEDGEMENT

The authors wish to thank Miss Linda Metzger for her technical assistance.

This research was supported by Grant No. HL-13029 from the National Institutes of Health.

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