TABLE I. Analyses of Eluate 2, in Per Cent of Lyophilized Dry Weight.

	Total eluate 2	Ppt. sat/2-$(NH_4)_2SO_4$	Ppt. sat-$(NH_4)_2SO_4$	Supernate
Mg recovered	197	57	99	26
Hexose*	13.3	8.2	14.9	24.1
N	14.41	15.92	13.35	8.04
Hexosamine†	7.4	2.7	5.1	6.7

* Anthrone method; standard was equal weights galactose and mannose.

† As glucosamine base.

migrating like albumin was seen. The supernate contained material which moved in the α_2 to γ region at pH 8.6, being a broad peak centered in the β region. Evidently, on separation as described, some further portion of RS-1 had become soluble in saturated $(NH_4)_2SO_4$.

The analyses (Table I) as compared with serum globulins(12,13), indicate some contamination with mucosubstances of the "serum globulin" fraction precipitated by half saturation with $(NH_4)_2SO_4$. However, all detectable material behaving like serum globulins at pH 4.5 was present in this fraction, and in 70-fold concentration as compared with the total nondialyzable solids(8). Five components, including some albumin, were seen by disc electrophoresis. Incomplete solubility of the fraction in water and solubility in dilute salt solutions confirms the presence of globulins. The results indicate that normal excretion of recognized serum globulins (including γ globulins) does not exceed about 6 mg/day.

Summary. A procedure for concentration of albumin and globulins from normal urine is described. The albumin appears to be identical with serum albumin, and excretory rate is about 16 mg/day, under conditions of normal activity. Excretion of globulins amounts to less than 6 mg/day.

We thank Dr. Karl Schmid, Harvard Med. School, for the ultracentrifugation and Dr. G. Grant, Shrewsbury, England, for immunoelectrophoretic examination of fraction UF-3.

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Received September 28, 1961 P.S.E.B.M., 1961, v108

Alternate Pathways of Glucose Metabolism in *Azotobacter agilis.** (27048)

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Johnson, Sobek, and Clifton(1) found that more C_2-carbon from glucose-2-C^{14} than C_1-carbon from glucose-1-C^{14} was assimilated by *Azotobacter agilis*, suggesting that a pentose pathway, involving an immediate decarboxylation, might be of quantitative importance in the metabolism of this organism. The presence of an active glucose-6-phosphate dehydrogenase had been demonstrated by Mortenson and Wilson(2,3). Extracts used by these workers also oxidized 6-phosphogluconate (6-PG) rap-

* This investigation was supported in part by a grant from Nat. Inst. of Allergy and Infectious Diseases, P.H.S.

idly. However, they were unable to demonstrate reduction of triphosphopyridine nucleotide (TPN) or diphosphopyridine nucleotide (DPN) in presence of 6-PG and found no stimulation by TPN of oxygen uptake on 6-PG. Mortenson, Hamilton, and Wilson(4) found that 6-PG was split quantitatively to pyruvate and glyceraldehyde-3-phosphate (G-3-P), presumably following a dehydration reaction. The work to be presented here represents a reinvestigation of the possibility of 6-PG dehydrogenase activity in *A. agilis* and a study of other reactions connected with 6-PG dissimilation.

Materials and methods. For spectrophotometric experiments, cells of *A. agilis* were grown on a shaker in 2-liter flasks containing 1 liter of Burk's nitrogen-free medium with 2% glucose or sucrose. Sucrose-grown cells were harvested at 24 hours. A shorter growth period of 16 hours was used with glucose-grown cells to avoid gum formation. The cells were collected by centrifugation, washed twice with distilled water, suspended in 0.05 M tris (hydroxymethyl)-aminomethane (Tris) buffer at pH 8.0 at a concentration of about 2 g in 8 ml, and then disrupted by sonication for 15 minutes in a 9-kc Raytheon Sonic Oscillator. Whole cells and debris were removed by 2 centrifugations at 2,000 g for 10 minutes. The supernatant fluid from the final centrifugation is designated as the crude extract. Fractionation of the crude extract was accomplished by centrifugation for 120 minutes at 144,000 g in the Spinco Model L Ultracentrifuge. The supernatant fluid from this centrifugation, designated the 144s120 fraction, was the source of enzyme for spectrophotometric assays, unless otherwise indicated.

Assays were performed with the Beckman Model DU Spectrophotometer by measurement of increase in optical density at 340 mμ. Unless otherwise indicated, cuvettes contained 1.5 ml glycyl-glycine buffer (0.4 M, pH 7.4), 0.2 ml TPN or DPN (2 mg/ml), 0.1 ml of 0.1 M $MgCl_2$, 0.1 ml enzyme preparation (about 200 μg protein), and 0.6 ml distilled water. TPN (98%-100% pure) and DPN (95%-100% pure) were obtained from Sigma Chemical Co. After taking a zero reading, 0.1 ml of 0.02 M substrate was added, and readings taken at 15- or 30-second intervals. No change in optical density occurred in absence of substrate.

For manometric experiments, cells were grown as previously described, and the crude extract was prepared in the same manner except that concentration of cells was reduced to 1 g of cells per 10 ml of suspending medium. Fractionation procedures included recovery of the particulate fraction (144p120) after ultracentrifugation, and homogenation of the particle in 0.05 M Tris, pH 7.2. The volume of Tris used for homogenation was calculated so that 0.2 ml of suspension would be equivalent to 1 ml of crude extract.

Oxygen uptake was measured in the Warburg constant volume respirometer at 30°C.

Results. Crude extracts of *A. agilis* showed no reduction of TPN in presence of 6-PG, even when cyanide was added. The 144s120 fraction of the crude extract, however, catalyzed a rapid reduction of TPN (Fig. 1). Rate of reduction of DPN was much slower than that observed with TPN.

TABLE I. Effect of TPN on Oxidation of 6-Phosphogluconate by Crude Extracts of *Azotobacter agilis*.*

System	Total Oxygen Uptake 15 min	30 min
	μl	μl
Endogenous	4	12
" with TPN	1	10
6-Phosphogluconate	22	53
" with TPN	45	64

* Flask contents: Main compartment—1.0 ml crude extract, 0.3 ml phosphate buffer (0.66 M, pH 7.0), 0.1 ml $MgSO_4$ (0.1 M), Tris buffer (0.05 M, pH 7.2) to a total vol of 2.2 ml; Sidearm—where indicated 0.2 ml TPN (2 mg/ml), 0.3 ml 6-phosphogluconate (0.02 M); Center well—0.1 ml 10% KOH.

Table I shows the stimulation of TPN of the oxidation of 6-PG by a crude extract. This effect could be demonstrated only under carefully controlled conditions. Inconsistent results were obtained when the cells used were more than 24 hours old or when the cell suspensions used for preparation of extract contained much more than 1 g (wet weight) of cells per 10 ml of suspending medium. It was necessary also to take readings of oxygen uptake at intervals shortly after mixing of extract and substrate, as differences between flasks with and without TPN were obscured in about 30 minutes. TPN was found to stimulate crude extracts of both glucose- and sucrose-grown cells.

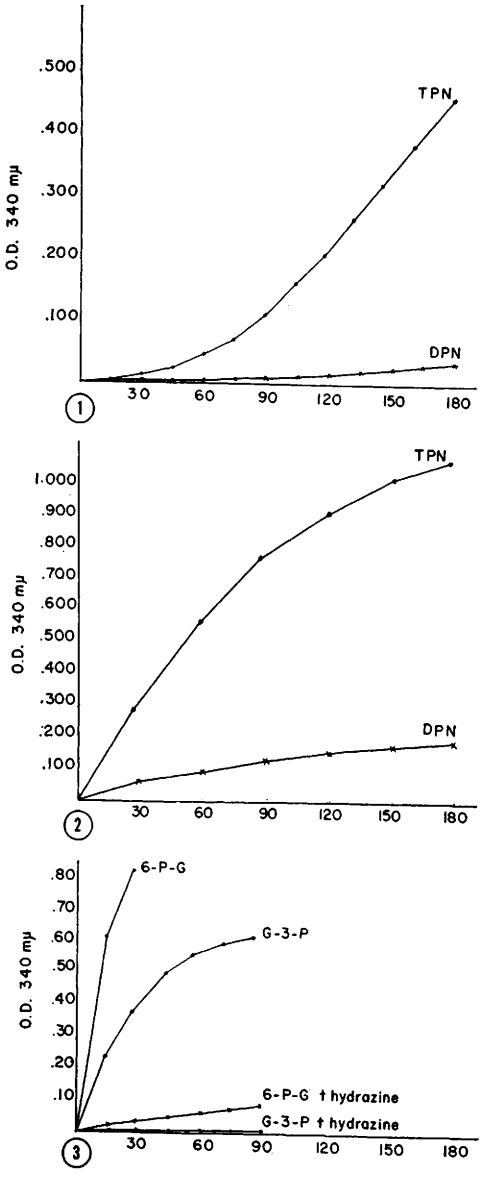

FIG. 1. Reduction of TPN and DPN in presence of 6-phosphogluconate by 144s120 fraction from *Azotobacter agilis*.

FIG. 2. Reduction of TPN and DPN in presence of glyceraldehyde-3-phosphate by the 144s120 fraction from *Azotobacter agilis*.

FIG. 3. The effect of hydrazine on TPN reduction in presence of gyceraldehyde-3-phosphate and 6-phosphogluconate. Substrate, hydrazine, and enzyme were combined first, and reaction started by addition of TPN.

No reduction of TPN or DPN by the 144s120 fraction occurred with pyruvate as substrate. The results with G-3-P are shown in Fig. 2. With G-3-P a rapid reduction occurred with TPN, but DPN served only poorly as hydrogen acceptor. Good activity with G-3-P occurred when extracts were prepared from cells that had been frozen for several months. Once prepared, however, the extract lost its activity rather rapidly and no TPN reduction could be demonstrated with extracts more than 2 days old.

Attempts to fractionate the 144s120 preparation further with ammonium sulfate to separate the 6-PG and G-3-P activities were unsuccessful. Almost all of the activity on 6-PG was lost on fractionation.

Fig. 3 shows the effect of 0.12 M hydrazine on TPN reduction when 6-PG and G-3-P served as substrate. The reduction of TPN was completely inhibited with G-3-P, whereas an extensive, but not complete, inhibition was noted with 6-PG.

The sodium salt of *p*-chloromercuribenzoate was also tested. At a concentration of 1×10^{-3} M complete inhibition of activity on both G-3-P and 6-PG was observed.

The 144s120 fraction was tested for aldolase by addition of fructose-1,6-diphosphate. Aldolase would yield G-3-P which the extract would oxidize with the reduction of TPN. However, no TPN reduction occurred. To test the possibility that reoxidation of TPN was obscuring the result, crystalline aldolase and fructose-1,6-diphosphate were added to the system. TPN reduction then occurred at the same rate as with G-3-P, showing that no such reoxidation was occurring. The particulate fraction was tested for aldolase activity by incubation with fructose-1,6-diphosphate, heating to destroy enzymes which reoxidize reduced TPN, and then measurement of TPN reduction upon the addition of 144s120 fraction. No TPN reduction occurred.

Triose phosphate dehydrogenase activity at various hydrogen ion concentrations showed an optimum at pH 8.5, with 80% of maximum activity at pH 7.5 and 50% at pH 9.5. Activity was not increased by pre-incubation with cysteine.

In an investigation of the mode of oxidation of reduced TPN, incubation of reduced TPN with crude extract resulted in a very low rate of reoxidation (Table II). Addition of DPN greatly increased this rate, demonstrating the

TABLE II. Oxidation of Reduced Pyridine Nucleotides by Crude Extract of *Azotobacter agilis*.

Substrate	Decrease in OD at 340 mμ between 60 and 90 sec
TPNH	.030
TPNH + DPN	.135
DPNH	.200

Each cuvette contained: 1.0 ml Tris buffer (0.05 M, pH 7.2), 0.1 ml $MgCl_2$ (0.1 M), 0.3 mg DPN (where indicated), 0.3 mg TPNH or DPNH, sufficient distilled water to bring final volume to 3.0 ml. The reaction was started by addition of 0.2 ml crude extract.

presence of TPN-DPN transhydrogenase. This table also shows the presence of DPNH oxidase in the extract.

Discussion. Our results agree with those of Mortenson and Wilson(3) with regard to the lack of demonstrable TPN reduction with 6-PG as substrate, even in the presence of cyanide, when crude extract was used as a source of enzyme. The fact that we were able to show a rapid TPN reduction, using the fractionated extract, indicates that the crude extract catalyzed a rapid reoxidation of the reduced TPN. The shape of the curve in Fig. 1, representing an increase in the rate with time, suggested the possibility of some other reaction(s) preceding the dehydrogenation. Since Mortenson, Hamilton, and Wilson(4) reported that their extracts split 6-PG to pyruvate and G-3-P, we tested the possibility that one of the latter products was actually the compound which was being dehydrogenated. The results shown in Fig. 2, the fact that TPN reduction proceded more rapidly with G-3-P than with 6-PG, and the additional finding (Fig. 3) that pre-incubation of enzyme and substrate eliminated the lag we had observed in rate of TPN reduction with 6-PG, suggest that the reduction we were observing with 6-PG actually resulted from dehydrogenation of G-3-P formed from 6-PG by the splitting system. The fact that activity on both G-3-P and 6-PG is inhibited by *p*-chloromercuribenzoate also indicates that there may be a common enzymatic reaction involved. The extensive inhibition by hydrazine of TPN reduction with 6-PG strengthens this interpretation, since hydrazine completely inhibited TPN reduction with G-3-P. The low level of activity observed with 6-PG in the presence of hydrazine may, however, reflect another route of dissimilation of this substrate. Pyruvate is apparently not involved, since no TPN reduction occurs with this substrate.

The finding of a TPN-dependent triose phosphate dehydrogenase in this organism led us to a reexamination of the possibility of the operation of the Embden-Meyerhof pathway. Mortenson and Wilson(2) suggested that this pathway might be quantitatively unimportant because their extracts showed no phosphohexokinase activity. They did find indirect evidence for aldolase. We were unable to show any aldolase activity in our preparations.

The Azotobacter triose phosphate dehydrogenase is similar to that isolated from green leaves(5) in its pH optimum. It differs from the plant enzyme, however, in that activity is not increased by pre-incubation with cysteine. A similar enzyme from *Alcaligenes faecalis* has been described by Brenneman and Volk (6). The purified enzyme in this case required arsenate or phosphate.

In view of the major role played by TPN in the metabolism of *Azotobacter agilis*, we investigated the possibility of a mode of oxidation of reduced TPN other than the TPN-DPN transhydrogenase system. We found no evidence for a direct TPNH-oxidase. However, transhydrogenase and DPNH-oxidase were present in the crude extract.

Summary. Fractions from *Azotobacter agilis* reduce TPN in the presence of 6-phosphogluconate. This reduction is due largely although perhaps not entirely, to oxidation of glyceraldehyde-3-phosphate formed by the splitting system described by Mortenson, Hamilton, and Wilson. This organism possesses a TPN-linked triose-phosphate dehydrogenase, similar to that found in green leaves. The reduced TPN is reoxidized via the TPN-DPN transhydrogenase system.

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