hydrate metabolism (Huggett, Warren & Winterton, 1949) as well as by the high overall rate of oxygen consumption of the tissue (Wang & Hellman, 1943). The pattern of placental lipids, and in particular the small proportion of neutral fats, and the abundance of compounds which are high in phosphate, is in agreement with the conception of the placenta as an actively metabolizing tissue. Together with the complexity of the phospholipids, as indicated by the distribution of bases, these are the most striking features of the lipid components of placenta.

SUMMARY

1. The lipids of mature human placentae have been fractionated by countercurrent distribution in

a solvent system of carbon tetrachloride, methanol and water, which was further modified for use with polar and non-polar lipids.

2. The placental lipids have been compared with those of brain, distributed under the same conditions.

3. Ethanolamine, choline and serine each occur in several fractions of placental lipid.

4. Glutamic acid is distributed so widely as to indicate some general relation to the placental lipids.

5. The nitrogen and phosphorus contents are not consistent with the predominance of the usual phospholipids, but indicate that compounds of high phosphorus and low nitrogen content are present.

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The Oxidative Pathway of Carbohydrate Metabolism in *Escherichia coli*

1. THE ISOLATION AND PROPERTIES OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE

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It has been shown that some bacterial viruses change the metabolism of the host cell in such a way that virus deoxyribosenucleic acid is synthesized instead of host deoxyribosenucleic acid and ribosenucleic acid. The hypothesis has been advanced that the routes of carbohydrate metabolism leading to the production of pentose and deoxypentose were different and that the relative amounts of glucose used by the two pathways were changed during elaboration of virus by the host (Cohen, 1947, 1951a). The glycolytic, or Embden-Meyerhof,

breakdown of one molecule of glucose 6-phosphate gives rise to two molecules of triosephosphate. It has been shown by Racker (1951) that condensation of glyceraldehyde phosphate with acetaldehyde by enzymes from *Escherichia coli* and other cells yields deoxyribose 5-phosphate which may be used in the synthesis of deoxyribosenucleic acid.

By the oxidative, or Warburg-Lipmann-Dickens pathway (Warburg, Christian & Griese, 1935; Lipmann, 1936; Dickens, 1938), oxidation of glucose 6-phosphate yields 6-phosphogluconate. It

has been shown in this laboratory that ribose 5-phosphate is a product of the degradation of 6-phosphogluconate by enzymes isolated from yeast (Scott & Cohen, 1949; Cohen & Scott, 1950) or from *Escherichia coli* (Scott & Cohen, 1951; Cohen, Scott & Lanning, 1951). Evidence has been adduced by Horecker & Smyrniotis (1950) that this ribose 5-phosphate is derived from the isomerization of ribulose 5-phosphate, which is the more immediate product of the oxidative decarboxylation of phosphogluconate. It has also been shown (Horecker & Smyrniotis, 1952) that ribulose 5-phosphate can be broken down by enzymes to produce triose phosphate and an as yet unidentified two-carbon fragment. The reversibility of this reaction has not yet been demonstrated.

Studies in this laboratory on the utilization of [1^{14}C]glucose by *Esch. coli* gave values for recovery of the labelled carbon in carbon dioxide which indicated a use of approximately 37 % of the glucose by the oxidative pathway during growth, and a shift of some of this glucose to the glycolytic pathway on infection with bacteriophage (Cohen, 1951*b*). Thus it seemed that the use of the phosphogluconate pathway was reduced as ribonucleic acid synthesis was inhibited in the presence of the virus.

A study was undertaken to determine whether the dehydrogenases for glucose 6-phosphate and for 6-phosphogluconate were present in the bacterial cells in amounts sufficient to account for the apparent use of glucose by the oxidative pathway, as indicated by the isotopic data. In order to determine the amounts it was necessary to be able to assay these enzymes under optimal conditions. The determination of these conditions is the subject of this paper.

METHODS AND MATERIALS

The activities of the enzymes were determined in the Beckman spectrophotometer with a thermostatically controlled cell chamber. The increase in density at 340 $\text{m}\mu$. caused by the reduction of triphosphopyridine nucleotide (TPN) was measured at 0.5 or 1 min. intervals after the reaction was started by addition of the last component of the system. The reaction was carried out in 1 ml. vol. in a cuvette with a 1 cm. light path. Results are expressed as the increase in density (ΔE)/ml. of extract/min., or increase in density/mg. of protein/min., or as μmole TPN reduced/mg. protein/min. Unless it is otherwise stated, all estimations were carried out at 36°.

Protein was determined by a modification of the Nessler procedure (Miller & Miller, 1948), or by the precipitation of the protein in 2.5 % perchloric acid. With the latter method, turbidity was read at 420 $\text{m}\mu$. in a Klett photometer. The relation of turbidity to protein was linear and reproducible for 0.3–1.5 mg. protein in a total vol. of 5 ml. In addition, the ratios of the u.v. absorptions at 260 and 290 $\text{m}\mu$. were useful to indicate what proportion of nucleic acid was present in the protein solutions.

TPN was prepared by the method of LePage & Mueller (1949). Fractions eluted from the charcoal column were

37–55 % pure by enzymic assay and contained no diphosphopyridine nucleotide.

Glucose 6-phosphate was prepared by the method of Seegmiller & Horecker (1951). The synthetic Ba salt was 80 % pure by enzymic analysis. 6-Phosphogluconate, prepared by oxidation of either the natural or the synthetic glucose 6-phosphate, contained 81 % phosphogluconate and was free of glucose 6-phosphate.

The buffers used were glycylglycine or tris(hydroxymethyl)aminomethane (Tris) (Gomori, 1946).

The enzymes were prepared from *Esch. coli*, strain B, grown at 36° in a mineral medium (Cohen, 1949) of pH 7.8 to which glucose or gluconate was added as the sole source of carbon. For maximal aeration, the bacteria were grown in roller flasks revolving at 300–400 rev./min. Growth was followed by measurement of the turbidity of the culture with the 420 $\text{m}\mu$. filter in the Klett photometer. The number of cells was determined either by plating or from a standard curve for turbidity correlated with plate count.

Cells were harvested at the desired stage of growth, or immediately after growth had stopped because of exhaustion of the carbon source. The bacterial suspension was cooled in an ice bath, and all further procedures were carried out at 4°. The bacteria were centrifuged, washed with 0.85 % saline or saline containing 0.1 % glucose, and centrifuged again at 1800 g for 15 min. The wet pellet of bacteria was weighed and ground with 2.5 times its weight of alumina (Alcoa A 301) for 2 min. and then extracted with the desired volume of water or buffer (McIlwain, 1948). Phosphate buffer at pH 7.4 extracted more glucose 6-phosphate dehydrogenase and less phosphogluconate dehydrogenase. Extraction with glycylglycine or with water yielded less of the former and more of the latter enzyme. Of the solutions tried, Tris buffer was the best for extracting both enzymes (see Table 1). The extract was obtained by centrifugation in the cold at 10000 g for 30 min. Centrifugation for 2 hr. at 10000 g or for 1 hr. at 100000 g did not sediment any of the activity in the water extracts. The alumina pellet was re-extracted once or twice. The activities of these extracts in reducing TPN in the presence of glucose 6-phosphate (G 6-P) and phosphogluconate (PG) were assayed immediately. Typical preparations are shown in Table 1.

Separation and partial purification of the enzyme

The extracts (Table 2, O) containing about 5–10 mg. protein/ml. were treated with 0.1 vol. of 0.25 M - MnCl_2 added slowly with constant agitation. After standing 30 min., the precipitated nucleic acid was removed by centrifugation. Little or no enzyme activity was lost in this step.

Saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 7.6 was added to the supernatant fluid (S) to 45–50 % saturation. The slight precipitate (A) was removed by centrifugation and discarded.

Glucose 6-phosphate dehydrogenase. To the supernatant fluid, $(\text{NH}_4)_2\text{SO}_4$ was added to 60 % saturation. The precipitate (B) was dissolved in water and reprecipitated at 60 % saturation of $(\text{NH}_4)_2\text{SO}_4$. This precipitate (C) contained 35–80 % of the total glucose 6-phosphate dehydrogenase activity of the extract and 2 % or less of phosphogluconate dehydrogenase. This step accomplished a 3- to 7-fold increase in activity/mg. of protein. Dialysis of the enzyme against running cold distilled water caused precipitation and loss of 60–80 % of the activity. However, the enzyme could be dissolved in buffer or albumin solution and

Table 1. *Extraction of protein and enzymes*

(For details, see text.)

Wet weight of bacteria (g.)	Extractant	Vol. (ml./g.)	Extract	Protein (mg./ml.)	Glucose 6-phosphate dehydrogenase (ΔE /mg./min.)	Phosphogluconate dehydrogenase (ΔE /mg./min.)	% of total extracted			Ratio: Glucose 6-phosphate dehydrogenase / Phosphogluconate dehydrogenase	
							Protein	6-phosphate dehydrogenase			Phosphogluconate dehydrogenase
								Glucose 6-phosphate dehydrogenase	Phosphogluconate dehydrogenase		
Exp. 1											
1.00	Water	10	First	11.4	1.27	0.79	—	—	—	0.63	
1.00	Glycylglycine, 0.1M, pH 7.4	10	First	11.2	1.72	0.66	—	—	—	0.38	
1.00	Tris, 0.1M, pH 7.4	10	First	10.7	1.85	0.75	—	—	—	0.40	
1.00	Tris, 0.1M, pH 8.0	10	First	10.0	1.78	0.86	—	—	—	0.45	
1.00	Tris, 0.1M, pH 8.7	10	First	10.8	1.57	0.85	—	—	—	0.54	
Exp. 2											
2.28	Water	2	First	48.5	1.29	0.91	59	81.5	65	0.71	
		8	Second	6.53	0.47	0.71	35	17.5	30	1.5	
		10	Third	0.95	0.10	0.63	6	0.6	4.7	6.3	
Exp. 3											
1.64	Tris, 0.1M, pH 8.0	5	First	27.2	1.29	0.59	80	75	75	0.46	
		5	Second	5.1	1.85	0.84	17.3	20.2	20	0.45	
		5	Third	1.5	1.48	0.75	4.2	4.6	4.9	0.51	

Table 2. *Separation of the enzymes*

Fraction	Volume (ml.)	Protein		Glucose 6-phosphate dehydrogenase		Phosphogluconate dehydrogenase		Ratio: Phosphogluconate dehydrogenase
		(mg./ml.)	(Total mg.)	(Units/mg.*)	(Total units)	(Units/mg.)	(Total units)	
<i>Esch. coli</i> extract† (O)	30	16.4	490	0.274	138	0.109	54	0.40
Supernatant fluid (S) from Mn ²⁺ pptn.	60	4.18	250	0.460	116	0.188	46.3	0.41
50% (NH ₄) ₂ SO ₄ ppt. (A)	3.5	5.80	20.3	0.161	3.2	0.005	0.1	0.03
60% (NH ₄) ₂ SO ₄ ppt. (B)	3.3	20.3	67.0	1.00	62.4	0.021	1.4	0.021
60% (NH ₄) ₂ SO ₄ reprecipitate (C)	3.3	16.1	53.0	0.92	49.0	<0.014	<0.74	<0.015
62% (NH ₄) ₂ SO ₄ ppt. (D)	3.3	6.06	20.0	1.00	20.0	0.03	0.65	0.03
pH 5.0 ppt. (E)	5.5	7.84	43.0	0.267	11.4	0.48	20.8	1.8
pH 4.4 ppt. (F)	2.2	3.14	6.9	0.055	0.4	1.30	8.8	23.6
Sum of precipitates			157.2		97.4		31.6	

* A unit of enzyme reduces 1 μ mole TPN/min. at 36°.

† Cells extracted twice with 5 vol. of 0.1M-Tris, pH 7.4.

reactivated by incubation at 4° with glucose 6-phosphate. Freeze-dried dialysed enzyme could be similarly dissolved and reactivated (Table 3).

Phosphogluconate dehydrogenase. This was prepared from the supernatant fluid of the first 60% saturated $(\text{NH}_4)_2\text{SO}_4$ solution (B). The precipitate (D) formed at 62–64% saturation was discarded since it contained only a small amount of the desired enzyme and residual glucose 6-phosphate dehydrogenase (Table 2). At this point, several methods were tried for obtaining good yields of a purer phosphogluconate enzyme.

Precipitation by organic solvents at low temperature by the methods of Askonas (1951) resulted in 70% loss of activity. With acetone, enzyme activity was spread over the precipitates throughout the whole range of acetone concentration. Addition of ethanol to dialysed 60% $(\text{NH}_4)_2\text{SO}_4$ supernatant fluid at pH 6.35 gave a precipitate between 37 and 54% ethanol which contained 70% of the recoverable activity, that is, 27% of the original activity. This step produced a 10-fold increase in phosphogluconate dehydrogenase activity/mg. of protein. The precipitate was dissolved in 0.2M-glycylglycine buffer at pH 7.4 and dialysed immediately against cold running distilled water with no further loss of activity.

A second method, precipitation (F) of the enzyme between pH 5.1 and 4.4 from 63% saturated $(\text{NH}_4)_2\text{SO}_4$ supernatant fluid, produced an 8- to 12-fold increase of activity on phosphogluconate and greatly reduced the glucose 6-phosphate dehydrogenase. The precipitate at pH 5.0 (E) still contained appreciable amounts of the latter enzyme.

The third method was saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate (G) had 5 times the activity/mg. protein of the original extract and the yield was 56%.

Dialysis of these precipitates (F and G) dissolved in glycylglycine or water caused precipitation of the enzyme, which was then centrifuged and redissolved in buffer.

RESULTS

Variation of activity with concentration of enzyme

Glucose 6-phosphate dehydrogenase activity was proportional to the amount of enzyme over as wide a range as can be read in the instrument, if the proper precautions were taken in diluting the enzyme solutions. Dilution with water or buffer solution caused a decrease in activity but dilutions with buffer containing substrate maintained proportionality of activity (Table 4).

The rate of reduction of TPN by phosphogluconate dehydrogenase was also proportional to the amount of the enzyme (Fig. 1). However, dilution of the extracts was observed to increase the activity. This increase was most marked in glycylglycine buffer or albumin and was not affected by the presence or absence of substrate. This effect may be due to the dilution of an inhibitor. Therefore, phosphogluconate dehydrogenase activity of ex-

Table 3. *Inactivation and reactivation of glucose 6-phosphate dehydrogenase*

Period at 4°	...	...	...	...	Activity (ΔE /mg./min.)					
					0	30 min.	60 min.	24 hr.	1 week	3 weeks
(1) Reprecipitated 60% $(\text{NH}_4)_2\text{SO}_4$ ppt. (C)					4.40	—	—	—	—	—
(2) The same, dialysed					1.32	—	—	1.30	2.20	—
(3) Dialysed, freeze-dried resuspended in										
(a) water					0.65	—	—	0.85	1.05	—
(b) Tris, 0.2M, pH 8.0					1.08	—	—	3.75	—	—
(c) Tris + G 6-P					1.68	2.79	3.21	3.00	2.73	2.20
(d) Tris + G 6-P + Mg					1.38	2.40	—	2.70	2.82	2.55
(e) glycylglycine pH 7.4 + G 6-P					—	—	—	2.1	2.32	0.45
(f) albumin					—	—	—	1.23	0.52	0

Table 4. *Effect of dilution of extracts*

Extract	Dilution	Diluent	Substrate	Activity (ΔE /mg. prot./min.)
Water extract 1	0	—	Glucose 6-phosphate	1.08
	100	Tris, 0.2M, pH 8.7		0.81
Water extract 2	0	—	Glucose 6-phosphate	1.68
	10	Glycylglycine, 0.2M, pH 7.4		1.09
Water extract 3	0	—	Phosphogluconate	0.75
	10	Glycylglycine, 0.2M, pH 7.4		0.80
	100	Glycylglycine, 0.2M, pH 7.4		0.94
	100	Water		0.78
Water extract 4	0	—	Glucose 6-phosphate	0.40
	10	Glycylglycine, 0.2M, pH 7.4		0.87
Glycylglycine extract 5	0	—	Glucose 6-phosphate	1.24
Tris, pH 7.4, extract 6	5	Tris, 0.2M, pH 8.7 + G 6-P + Mg^{++}		1.72
	0	—		1.82
	5	Tris, 0.2M, pH 8.7 + G 6-P + Mg^{++}		1.85

tracts was measured with samples diluted as much as was practicable.

For maintenance of activity, Tris buffer was best for glucose 6-phosphate dehydrogenase. This enzyme in Tris, pH 8.0, lost only 7% of its activity after 6 weeks at 4°. Phosphogluconate dehydrogenase did not maintain its activity as well and was more stable in glycylglycine buffer than in Tris. In Tris at pH 8.0, two-thirds of the activity was lost in 6 weeks while in glycylglycine buffer at pH 7.4, one-third was lost in 10 weeks at 4°.

Fig. 1. Concentration of enzyme. G6-P: Reaction mixtures contained 1 μ mole G 6-P, 0.15 μ mole TPN, 0.02M-Mg and enzyme (ppt. B, Table 2, dialysed) in 0.04M glycylglycine at pH 7.4. Total vol. 1 ml. PG: reaction mixtures contained 1 μ mole PG, 0.15 μ mole TPN, 0.02M- Ca^{++} and enzyme (ppt. G, dialysed) in 0.04M glycylglycine at pH 7.4. Total vol. 1 ml.

Effect of ions on enzyme activities

Dialysed glucose 6-phosphate dehydrogenase prepared from *Esch. coli* was inactive without the addition of divalent cations. The dehydrogenase from yeast (zwischenferment) prepared by the method of Kornberg (1950) had 33% of its maximum activity without the addition of any ions. Adsorption on tricalcium phosphate and elution must have left residual calcium ions attached to the enzyme which were not removed by subsequent dialysis. Dialysed phosphogluconate dehydrogenase from *Esch. coli* always had some activity in the absence of any salts except those present in the buffer and substrate.

Both enzymes were activated by bivalent cations as chlorides (see Figs. 2 and 3). While magnesium

ions produced maximal activity at concentrations of 0.015 and 0.02M, calcium ions caused greater activation at a slightly higher concentration and were active over a wider range (Figs. 4 and 5). As will be discussed below, in experiments with varying TPN concentration, magnesium and calcium ions must differ somewhat in their mode of activation. The activating cation must be present in the buffer mixture when the enzyme is added. The order of addition of the other component was without effect on the rate of reduction of TPN.

Table 5. Inhibition of enzymes by bivalent cations

Cation	Molarity	Glucose 6-phosphate* dehydrogenase inhibition (%)	Phosphogluconate† dehydrogenase inhibition (%)
Cupric	2×10^{-3}	90†	100†
	1×10^{-3}	75	75
	1×10^{-4}	7	—
Zinc	1×10^{-3}	100†	75
	1×10^{-4}	93	28
Cadmium	1×10^{-3}	30	72
	1×10^{-4}	—	45
Cobaltic	1×10^{-2}	—	57
	1×10^{-3}	0†	28
Beryllium	1×10^{-3}	0†	44
Manganous	1×10^{-2}	—	10
Mercuric	2×10^{-3}	—	84
	1×10^{-3}	100	—
	1×10^{-4}	75	—

* Reaction mixture for glucose 6-phosphate contained 0.018 mg. dialysed enzyme, 0.04M Tris, pH 8.7, 0.015M- MgCl_2 , 2.5 μ mole G 6-P and 0.3 μ mole TPN.

† Reaction mixture for phosphogluconate contained 0.043 mg. dialysed 100% saturated $(\text{NH}_4)_2\text{SO}_4$ ppt., 0.04M-glycylglycine, pH 7.4, 0.02M- CaCl_2 , 1 μ mole PG and 0.15 μ mole TPN.

‡ Denotes precipitation.

Univalent cations, as chlorides at 10 times the concentration of the bivalent ions, could activate phosphogluconate dehydrogenase but not glucose 6-phosphate dehydrogenase (Fig. 6). However, both enzymes in extracts of eggs of the polychaete, *Chaetopterus*, were activated by 0.15M-sodium chloride or potassium chloride (Cohen, 1951c).

Sodium phosphate, bicarbonate and citrate activated glucose 6-phosphate dehydrogenase to about 10% of the activation with calcium or magnesium ions, but were inhibitory in the presence of calcium or magnesium ions. Sodium sulphate and phosphate activated phosphogluconate dehydrogenase to the extent of 88 and 65% of the activation by calcium ions, and were inhibitory in the presence of calcium ions only in high concentrations.

In systems containing calcium or magnesium ions the addition of other bivalent cations, copper, cadmium and zinc especially, caused marked inhibition (Table 5). Inhibition was less with

Fig. 2.

Fig. 2. Activation of glucose 6-phosphate dehydrogenase by bivalent cations. Reaction mixtures contained 1 μ mole G 6-P, 0.15 μ mole TPN, and 0.02M-salt as chloride in 0.04M-glycylglycine buffer at pH 7.4 and 36° in total vol. 1 ml., and enzyme as follows: A, 0.02 ml. of *Esch. coli* extract (O). B, 0.016 mg. protein of dialysed ppt. (B). C, 0.0075 mg. protein of yeast 'zwischenferment'.

Fig. 3.

Fig. 3. Activation of phosphogluconate dehydrogenase by bivalent cations. Reaction mixtures contained 1 μ mole PG, 0.15 μ mole TPN, 0.039 mg. protein of dialysed ppt. (G) and 0.02M-salt as chloride in 0.04M-glycylglycine buffer at pH 7.4 and 36°.

Fig. 4.

Fig. 4. Activation of glucose 6-phosphate dehydrogenase by calcium and magnesium. The reaction mixtures contained 2 μ mole glucose 6-phosphate, 0.15 μ mole TPN, 0.04M-Tris, pH 8.17 and 0.06 mg. enzyme in 1 ml. total vol. at 36°. The enzyme was ppt. (C), dialysed, freeze-dried, and dissolved in albumin solution.

Fig. 5.

Fig. 5. Activation of phosphogluconate dehydrogenase by calcium, magnesium and sodium. The reaction mixtures contained 0.72 μ mole PG, 0.15 μ mole TPN and 0.044 mg. protein of ppt. (F), dialysed, in 0.04M-glycylglycine buffer pH 7.4 at 36°.

extracts than with purified enzymes, owing to the higher concentration of protein in the extracts, which removed these ions from solution. The buffer used also removed these ions. Zinc ions, however, precipitated the proteins. Inhibition of phosphogluconate dehydrogenase by copper sulphate was also noted by Horecker & Smyrniotis (1951).

The effect of variation of concentration of substrate

The activity of partially purified enzymes at various substrate concentrations is shown in Figs. 7 and 8. The Michaelis constant for glucose 6-phosphate combination with enzyme was of the order of $3-5 \times 10^{-4}$. For the phosphogluconate-enzyme complex K_m was 2.2×10^{-5} in the presence

Fig. 6. Effect of sodium, ammonium and sulphate ions on enzyme activity. Glucose 6-phosphate dehydrogenase: the reaction mixtures contained 1 μ mole G 6-P, 0.2 μ mole TPN, and 0.016 mg. protein of dialysed ppt. (C) in 0.04M-glycylglycine buffer at pH 7.4 and 36°. Phosphogluconate dehydrogenase: the reaction mixtures contained 1 μ mole PG, 0.15 μ mole TPN and 0.045 mg. protein of dialysed ppt. (G) in 0.04M-glycylglycine buffer at pH 7.4 and 36°.

Fig. 7.

Fig. 7. Effect of substrate concentration, glucose 6-phosphate. The reaction mixtures contained 0.3 μ mole TPN, 0.032 mg. protein of ppt. (C), 0.015M-MgCl₂ or 0.025M-CaCl₂ in 0.04M-Tris at pH 8.7 and 36°.

Fig. 8.

Fig. 8. Effect of substrate concentration, phosphogluconate. The reaction mixtures contained 0.15 μ mole TPN, 0.044 mg. protein of dialysed ppt. (F), 0.02M-MgCl₂ or 0.03M-CaCl₂ in 0.04M-glycylglycine at pH 7.4 and 36°. K_m , Mg²⁺ = 2.5×10^{-6} ; K_m , Ca²⁺ = 3.3×10^{-6} .

of magnesium ions and 3.3×10^{-5} with calcium ions. These are of the same order as the K_m given by Horecker & Smyrniotis (1951). It would seem that magnesium ions hold the substrate more tightly to the latter enzyme than calcium ions.

Fig. 9. Effect of TPN concentration on phosphogluconate dehydrogenase. The reaction mixtures contained 1 μmole PG, 0.086 mg. protein of dialysed ppt. (G), 0.02 M-MgCl₂ or 0.02 M-CaCl₂ in 0.04 M-glycylglycine at pH 7.4 and 36°.

Fig. 10. Effect of pH on enzyme activity. Glucose 6-phosphate dehydrogenase: the reaction mixtures contained 1 μmole G 6-P, 0.3 μmole TPN, 0.01 M-MgCl₂ and 0.032 mg. protein of dialysed ppt. (B) in 0.04 M-Tris or glycylglycine or mixtures of buffers at indicated pH and 36°. Phosphogluconate dehydrogenase: the reaction mixtures contained 0.15 μmole TPN, 0.36 μmole PG, 0.02 M-MgCl₂ and 0.038 mg. protein of dialysed ppt. (G) in 0.04 M-Tris or glycylglycine buffer or mixtures, at indicated pH and 36°.

These enzymes may be used for assay of substrate concentration. Separated and partially purified enzymes in amounts sufficient to oxidize all the substrate within 20 min., and 0.4 μmole TPN are necessary for assay of either substrate.

Effect of variation of concentration of TPN

The effect of variation of TPN concentration on the activity of these enzymes is not easily interpreted. Dissociation constants are only approximations, for the state of the enzymes, whether fully activated or not, the ions present, and the presence of non-specific proteins, have effects on the enzyme-coenzyme complex formation.

Fig. 11. The effect of temperature on enzyme activity. Glucose 6-phosphate dehydrogenase: the reaction mixtures contained 0.3 μmole TPN, 0.76 μmole G 6-P, 0.015 M-MgCl₂ and 0.022 mg. protein of dialysed ppt. (B) in 0.04 M-Tris buffer at pH 7.8. Phosphogluconate dehydrogenase: the reaction mixtures contained 0.3 μmole TPN, 0.36 μmole PG, 0.02 M-MgCl₂ and 0.034 or 0.068 mg. protein of dialysed ppt. (G) in 0.04 M-glycylglycine buffer at pH 7.2.

Glucose 6-phosphate dehydrogenase in extracts had a K_m for TPN of about 4×10^{-5} . For the dialysed enzyme K_m for TPN was 3×10^{-4} with calcium ions and 2×10^{-4} with magnesium ions.

The difference between the action of calcium and magnesium was much more marked with phosphogluconate dehydrogenase (see Fig. 9). The dialysed enzyme reached a peak of activity at 8×10^{-5} M-TPN and fell rapidly as the concentration was increased above this, if calcium was the activating ion. In the presence of 0.02 M-magnesium, however, activity was constant over a range of TPN concentration from 8×10^{-5} to 2×10^{-4} M.

Effect of pH

From Fig. 10, it can be seen that glucose 6-phosphate dehydrogenase was optimally active from pH 7.7 to 8.6, and phosphogluconate dehydrogenase from pH 6.6 to 7.7. The medium in which the bacteria were grown had pH 7.8, at which pH both enzymes have within 5–10 % of their maximum activity.

Fig. 12. Heat inactivation of enzymes and protection by substrate. Glucose 6-phosphate dehydrogenase: the reaction mixtures contained 2 μ mole G 6-P, 0.15 μ mole TPN, 0.025 M-CaCl₂ in 0.04 M-Tris buffer at pH 8.7 at 36°. The enzyme was 0.06 mg. freeze-dried, dialysed, ppt. (C), dissolved in 0.06 % serum albumin and held at indicated temperatures for varying lengths of time. Phosphogluconate dehydrogenase: the reaction mixtures contained 0.2 μ mole TPN, 0.70 μ mole PG, 0.03 M-CaCl₂ in 0.04 M-glycylglycine buffer pH 7.4, at 36°. The enzyme was the precipitate formed on dialysis of ppt. (G) dissolved in glycylglycine buffer pH 7.4. The enzyme was held at temperatures indicated for indicated times with or without the presence of substrate or salts.

Effect of temperature

Activation. As shown in Fig. 11, both enzymes had maximal activity at 37°. Between 22 and 36°, glucose 6-phosphate dehydrogenase had a temperature coefficient of 1.55 and phosphogluconate dehydrogenase, 2.2. At higher temperatures inactivation of the enzymes occurred rapidly.

Inactivation. Extracts and partially purified enzymes lost activity rapidly when unprotected by the substrate. Glucose 6-phosphate dehydrogenase was 40 % inactivated in 12 min. at 37° and about 10 % inactivated in 24 hr. at 4°. Phosphogluconate dehydrogenase lost 15 % of its activity in 12 min. at 37°. At 53° for 2 min., almost all phosphogluconate activity was lost, but 30 % of glucose 6-phosphate activity remained. However, the enzyme could be protected very markedly by substrates, by buffer or

albumin solutions somewhat, and by magnesium ions, but not by calcium ions. In the reaction mixture, complete except for TPN, both enzymes were stable at all temperatures up to 36° (Fig. 12).

The enzymes were not protected by TPN, but rather the activity of both enzymes was decreased about 50 % by 5×10^{-4} M-TPN added to dilutions of the extract in Tris. Separated phosphogluconate

Fig. 13. The reaction mixtures contained 0.025 M-CaCl₂ and 0.20 mg. protein of ppt. (C) in 0.04 M-glycylglycine at pH 7.4. Cuvette (1) contained 0.03 μ mole G 6-P. Cuvette (2) contained 0.0196 μ mole PG. Cuvette (3) contained 0.03 μ mole G 6-P + 0.0196 μ mole PG. At zero time, 0.28 μ mole TPN was added. At 13 min. 0.18 mg. of phosphogluconate dehydrogenase, ppt. (F), was added. ●—●, 0.030 μ mole G 6-P; △—△, 0.0196 μ mole PG; x—x, 0.030 μ mole G 6-P + 0.0196 μ mole PG; ○—○, the sum of curves (1) and (2).

dehydrogenase was precipitated from glycylglycine solution by 2.8×10^{-3} M-TPN and could not be redissolved or reactivated.

Oxidation by phosphogluconate dehydrogenase of the product of glucose 6-phosphate dehydrogenase

Fig. 13 shows the action of the separated enzymes. The glucose 6-phosphate dehydrogenase caused no reduction of TPN in the presence of phosphoglu-

conate. Phosphogluconate dehydrogenase was equally unreactive in the presence of glucose 6-phosphate. The latter enzyme reduced the phosphogluconate, which had been produced by the first enzyme, at the same rate as it reduced synthetic phosphogluconate. This would seem to indicate that, if the primary product of the glucose 6-phosphate dehydrogenase action is the phosphogluconolactone (Cori & Lipmann, 1952), either there was a lactonase present in one or both of the enzyme preparations, or the lactone was hydrolysed chemically.

Methods of enzyme assay

By selecting the proper conditions, the activity of either enzyme can be determined in extracts where both are present. The order of addition of components is immaterial except that the bivalent ion and buffer must be in the mixture when the enzyme is added. The chosen methods of assay are shown in Table 6. Under these conditions of assay of glucose 6-phosphate dehydrogenase, reduction of TPN by the action of the other enzyme on the phosphogluconate produced was minimized.

Table 6. Conditions for assay of enzymes

Buffer	Glucose 6-phosphate dehydrogenase Tris, 0.04 M	Phosphogluconate dehydrogenase Glycylglycine, 0.04 M
pH of buffer	8.7	7.4
pH of mixture	8.5	7.2
μ mole substrate	2.0	0.7
μ mole TPN	0.3	0.15
Salt concentration	0.015 Mg^{++} or 0.025 Ca^{++}	0.020 Mg^{++} or 0.025 Ca^{++}
Temperature	36°	36°
Total volume	1 ml.	1 ml.
Enzyme*	0.01–0.1 ml.	0.01–0.1 ml.

* Cf. Table 1.

These conditions for assay of glucose 6-phosphate dehydrogenase are quite different from those given by Kornberg (1950) for *zwischenferment*. Values obtained by the latter method may be multiplied by a factor of 2.4 to give values corresponding to those obtained by the method in Table 6 at 26°. Including the temperature coefficient, the factor is 3.9 for assay at 36°.

Our conditions for assay of phosphogluconate dehydrogenase are, except for temperature, approximately the same as those employed by Horecker & Smyrniotis (1951) and Seegmiller & Horecker (1951), and give the same values for activity of enzymes.

DISCUSSION

The two dehydrogenases of the oxidative pathway of glucose 6-phosphate obtained from extracts of *Esch. coli* have been separated and partially purified.

Both enzymes were comparatively labile when separated from each other and from some of the other bacterial protein, especially in dilute solution. They could, however, be stabilized by mixture with buffer or non-specific protein, or even better by the presence of substrate and magnesium ion in the buffer. Both enzymes were specific for reduction of TPN and had no action with DPN. Both enzymes were activated by calcium and magnesium though the effect of different concentration of the ions was somewhat different.

In other properties, the two enzymes differed. The pH optima and the temperature coefficients were different. The greater concentration of substrate and of TPN needed for the activity of the glucose 6-phosphate enzyme and the greater sensitivity of this enzyme to inhibition by ions other than the bivalent-cation chlorides are noteworthy. On the other hand, the phosphogluconate enzyme showed a sensitivity to concentration of TPN not so evident in the former enzyme. Concentrations of TPN above $1 \times 10^{-4} M$ were inhibitory to the reaction, and incubation of the enzyme with TPN in higher concentration may inactivate the enzyme entirely.

Whether these differences in specificity and sensitivity reflect the natural state of the enzyme in the intact cell it is impossible to say at the present time. It might be that conditions of temperature, pH, TPN and ion concentration in the cell determine what part of the metabolism of glucose 6-phosphate is to be carried over the oxidative route and what part over the glycolytic route. The phosphogluconate dehydrogenase was active under a wide variety of environmental conditions.

SUMMARY

1. Glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase were obtained from extracts of *Escherichia coli*, and were separated and partially purified.

2. Optimal conditions for the action of these enzymes in reducing triphosphopyridine nucleotide in the presence of their substrates and for their assay were determined.

3. Both enzymes were activated by bivalent cations as chlorides. Magnesium and calcium ions were most active at about $2 \times 10^{-2} M$ concentration.

4. Michaelis constants for the enzyme-substrate complex were of the order of 3×10^{-4} for glucose 6-phosphate dehydrogenase and 3×10^{-5} for phosphogluconate dehydrogenase.

5. Optimal pH was 7.7–8.6 for glucose 6-phosphate dehydrogenase and 6.6–7.7 for phosphogluconate dehydrogenase, and maximal activity of both enzymes occurred at 37°.

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The Oxidative Pathway of Carbohydrate Metabolism in *Escherichia coli*

2. QUANTITATIVE STUDIES OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE

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In this paper data are presented on the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase extracted from *Escherichia coli* in an effort to estimate the proportion of carbohydrate which might be metabolized by the enzymes during bacterial growth under aerobic conditions. Enzymes were extracted which accounted for approximately 40 % of the total carbohydrate metabolized by the cell.

METHODS

As will be shown, the amounts of these enzymes in *Esch. coli* varied according to the conditions of growth. The following procedure was followed in all experiments presented in this paper. *Esch. coli*, strain B, from the stock culture in synthetic medium (Cohen, 1949), was used to inoculate 100 ml. of synthetic medium containing 1 mg. glucose/ml. After 17 hr. aerobic growth at 36° the glucose was exhausted and the culture contained 9×10^8 cells/ml.; 50 ml. were then inoculated into each of two roller flasks containing 250 ml. of synthetic media with the desired concentration of carbohydrate as sole carbon source. The flasks were rotated at 300–400 rev./min. in the incubator at 36°. While spinning at this rate the fluid formed a band completely around the flask, thus assuring maximal aeration of the culture. The

growth of the culture was followed by measurement of the turbidity at 420 m μ . in the Klett photometer and sometimes by plate count. Fig. 1 shows the correlation of turbidity with plate count during growth of the bacteria on glucose or potassium gluconate.

The cells were harvested either during exponential growth or when the carbon source, or the nitrogen source, or both, were exhausted. The culture was immediately chilled to 4°. Centrifugation, washing, and extraction were carried out at 2–4°. The bacteria were disrupted by grinding with alumina (McIlwain, 1948) and extracted two or three times with water or tris(hydroxymethyl)aminomethane buffer (Tris) at pH 8.0. Determination of the enzyme activities was made by measurement of the reduction of triphosphopyridine nucleotide (TPN) in the presence of glucose 6-phosphate (G 6-P) or 6-phosphogluconate (PG) by the methods described in the preceding paper (Scott & Cohen, 1953).

RESULTS

At the beginning of growth on glucose in the roller flask there was either no lag phase or it was very short. With gluconate there was usually a lag of about 20–40 min., presumably for formation of the adaptive enzyme, gluconokinase (Cohen & Scott, 1950; Cohen, 1951a).