Hexokinase of *Escherichia coli*. Assay of Enzyme Activity and Adaptation to Growth in Various Media

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SUMMARY

Hexokinase was determined in extracts of *Escherichia coli* by measurement of the reduction of nicotinamide-adenine dinucleotide phosphate by glucose-6-phosphate dehydrogenase, and the glucose-6-phosphate formed by the action of hexokinase. This method gave reproducible results. Extracts of *E. coli* contained low hexokinase activity when the organism had been grown in the absence of glucose and this enzyme activity was increased after adaptation of the organism to growth on glucose. The *E. coli* needed 2–2.5 divisions to reach the degree of hexokinase activity of glucose-grown bacteria when transferred to salts + glucose from media not containing glucose.

INTRODUCTION

Previous studies (Scott & Chu, 1959) showed that glucose-6-phosphate (G-6-P) dehydrogenase activity was higher in extracts of *Escherichia coli* when the bacteria had been grown in the presence of glucose than when grown in media containing other sources of carbon. Phosphogluconate (PG) dehydrogenase activity was relatively constant in the extracts from bacteria grown in different media. In further studies of the uptake and utilization of glucose by *E. coli*, the activity of hexokinase was determined to see whether it behaved like G-6-P or PG dehydrogenase (see below). It was found that certain changes in the media involving carbon sources or other additions produced changes in the degree of activity of the hexokinase in extracts which were not, however, as great as the changes in G-6-P dehydrogenase.

METHODS

The method for cultivation of 'weekly' and 'overnight' cultures was described previously (Scott & Chu, 1959). The cultures of *Escherichia coli* strain B were grown at 37° in an aerated medium of Bacto-beef extract and Bacto-peptone (Difco

Laboratories, Detroit, Michigan, U.S.A.) or salts media (Cohen & Arbogast, 1950), with glucose 2 mg./ml. medium. Casein hydrolysate (an acid hydrolysate of casein; General Biochemicals, Inc., Chagrin Falls, Ohio, U.S.A.) 4 mg./ml. medium, or Difco yeast extract (the water-soluble portion of autolysed fresh yeast) 2 mg./ml. medium, was added to the salts media instead of glucose as indicated.

The 'weekly' culture, which was a salts + glucose culture, was used to inoculate two 'overnight' cultures of either one form of salts medium or nutrient broth. One culture was harvested to be extracted. The other was centrifuged and the bacteria were resuspended in salts medium to inoculate the final cultures, which were allowed to grow 1, 1.5 or 2 divisions as noted in Table 1. The harvested organisms were washed in saline, recentrifuged, the bacterial pellet weighed, ground with alumina ($2.5 \times$ wet weight) in the cold and extracted with 0.05M-tris buffer adjusted to pH 7.4 with 2N-HCl. The extracts were centrifuged 1 hr at 10,000g at 4° in an International Refrigerated Centrifuge PR-2. The amounts of protein in the extracts were determined by light extinction after precipitation with 10% (w/v) perchloric acid.

The methods of measurement of G-6-P and PG dehydrogenases were as reported previously (Scott & Cohen, 1953). The direct method for the hexokinase reaction was done in 1 ml. of a mixture containing 0.05M-tris chloride buffer (pH 7.4), 1 μ mole glucose, 10 μ moles MgSO_4 , 0.02N-nicotinamide and extract containing 0.05–1 mg. protein. NADP^+ was added and the endogenous reaction recorded. With the addition of 1 μ mole ATP, the G-6-P formed was oxidized by the G-6-P dehydrogenase, permitting the rate of NADPH formation to be measured at 340 m μ at 37° in a Beckman DU spectrophotometer. Two other methods were also used for comparison; these were measurement of glucose removed and measurement of G-6-P formed. The mixture described above that was used for the direct measurement was incubated in a 37° water bath and 1 ml. samples removed at selected times into cold HCl (0.04 M). After centrifugation at 4°, the supernatant fluid was neutralized with NaOH (0.04 M). Glucose and G-6-P concentrations were measured in the samples. The glucose was determined by the 'Glucostat', a preparation of glucose oxidase (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.). The assay for G-6-P was done in 1 ml. of a mixture of 0.05M-tris chloride buffer (pH 7.4), 10 μ moles MgSO_4 , 0.4 μ mole NADP^+ and the sample. Purified G-6-P dehydrogenase was added and the total formation of NADPH was measured in a Beckman DU spectrophotometer at 340 m μ at 37°. The G-6-P dehydrogenase was obtained either from Sigma Chemical Co. (St Louis, Missouri, U.S.A.) or was purified in our laboratory from extracts of *Escherichia coli* by methods previously reported (Scott & Cohen, 1953).

RESULTS

Figure 1 shows the rate of reduction of NADP^+ by G-6-P formed when ATP was added to mixtures of glucose and an extract of *Escherichia coli* grown in salts + glucose medium. The rate of reaction with G-6-P indicated that G-6-P dehydrogenase was present in large excess in the extract and was not limiting in the measurement of the rate of the hexokinase reaction. In the hexokinase assay during the first few minutes the G-6-P formed was limiting; then a constant rate was attained which was taken as the measure of the hexokinase activity. There was no NADPH oxidase activity in these extracts when magnesium ions were present unless oxidized

glutathione was added. There was slight or no activity with ATP or glucose omitted. The presence of nicotinamide inhibited NADP⁺ase. It was not necessary to make corrections for NADPH produced by PG dehydrogenase since the NADPH was in high enough concentration to inhibit PG dehydrogenase activity. The presence of some activator in the extracts was indicated by the lack of proportionality of activity to protein content at low dilutions of extracts, as is illustrated in Fig. 2.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Hexokinase of extracts of *Escherichia coli* grown in salts + glucose medium. Each 1.5 ml. cuvette contained 10 μ moles MgSO_4 , 0.01 ml. extract (equiv. 81 μ g. protein). $\blacktriangle$, G-6-P dehydrogenase reaction: 1 μ mole G-6-P, 0.05 M-tris chloride buffer (pH 8.7). $\bullet$, Hexokinase reaction: 0.05 M-tris chloride buffer (pH 7.4), 1 μ mole glucose, 1 μ mole nicotinamide; 0.03 ml. NADP^+ (10 mg./ml.) was added to each cuvette. After 3 min. 1 μ mole ATP was added. Total volume was 1 ml. $\blacksquare$, Hexokinase reaction with either ATP or glucose omitted. Extinction, E, at 340 $m\mu$ was read on a Beckman DU spectrophotometer at 37°.

Fig. 2. Effect of sample size on hexokinase activity. The sample used was an extract from a salts + glucose culture of *Escherichia coli* that contained equiv. 8.1 mg. protein/ml. The other contents of the cuvette were as in Fig. 1.

Fig. 3. Comparison of three methods to determine hexokinase of *Escherichia coli* extracts. The extract used was from bacteria grown on salts + glucose medium and contained equiv. 9.82 mg. protein/ml. The methods are explained in the Method section. The direct method (*) was a measurement of μ mole NADPH/ml. reaction mixture. The glucose uptake method (●) was a measurement of μ mole glucose removed/ml. reaction mixture and the G-6-P measurement (■) was a measurement of μ mole G-6-P found/ml. reaction mixture.

The comparison of the three methods used for determination of hexokinase is shown in Fig. 3. The initial rates of glucose disappearance and appearance of G-6-P were the same for the first 2 min. After 2 min. the G-6-P was metabolized by other pathways, so that it became unavailable for measurement. The glucose was taken up at a decreasing rate because of the inhibition of hexokinase by G-6-P formed and not removed (Crane & Sols, 1954).

Table 1. *Effect of transfer of Escherichia coli to different media on enzyme activities**

Medium	Number of divisions	Hexokinase		G-6-P dehydrogenase		PG dehydrogenase	
		Units/mg. protein	Units/10 ⁹ bacteria	Units/mg. protein	Units/10 ⁹ bacteria	Units/mg. protein	Units/10 ⁹ bacteria
I. Inoculum nutrient broth (overnight)	—	100	100	100	100	100	100
A. $\frac{1}{2}$ vol. nutrient broth + $\frac{1}{2}$ vol. salts + glucose	1	285	400	110	137	92	124
	2.5	232	164	116	76	104	70
B. Salts + glucose	1	92	86	112	112	93	86
	1	100	212	132	274	96	200
	1	52	59	218	227	97	105
	1.5	206	271	132	158	78	100
	1.5	150	350	120	313	78	186
II. Inoculum salts + glucose	2	100	100	100	100	100	100
A. $\frac{1}{2}$ vol. nutrient broth + $\frac{1}{2}$ vol. salts + glucose	1	74	—	56	—	88	—
B. Nutrient broth	1	34	6	32	6	109	19
	2	44	—	57	—	82	—
	2	52	10	76	14	106	19
	3	54	—	60	—	—	—
	3	56	12	54	10	96	19
III. Inoculum $\frac{1}{2}$ vol. nutrient broth + $\frac{1}{2}$ vol. salts + glucose	1	100	100	100	100	100	100
Nutrient broth	1	67	—	62	—	108	—
IV. Inoculum salts + casein hydrolysate (overnight)	1	100	100	100	100	100	100
Salts + glucose	1	191	300	190	314	84	132
	1	89	94	157	170	114	120
	1	207	300	207	278	79	106
	1.5	228	238	228	245	100	102
	1.5	126	144	184	206	102	174
	1.5	230	550	322	733	72	171
V. Inoculum salts + casein hydrolysate + yeast extract (overnight)	1.5	100	100	100	100	100	100
Salts + glucose	1	162	150	182	170	105	100
	1	170	200	126	158	91	113
	1.5	218	225	300	290	110	107
	1.5	168	230	167	239	114	160
VI. Inoculum salts + yeast extract (overnight)	1.5	100	100	100	100	100	100
Salts + glucose	1	107	325	186	590	118	338
	1	67	125	114	216	108	219
	1.5	110	400	161	620	106	375
	1.5	106	350	163	426	118	333

* The enzyme activities of the inoculum were set at 100.

Table 1 lists the results from experiments testing the effects of growth in different media on values of hexokinase activity. The activity of the original inoculum is taken as 100 %. In the first series (I), the overnight inoculum was grown in nutrient broth. When the bacteria were transferred to media of equal parts of nutrient broth and salts + glucose, in only one division striking increases (3-fold) were found in hexokinase activity. The *Escherichia coli* grown on nutrient broth were smaller in size than those organisms grown on defined media with glucose. This difference in size was reflected in differences in enzyme activity/10⁹ bacteria as compared to activity/mg. protein. The G-6-P dehydrogenase showed some fluctuation but the PG dehydrogenase (/mg. protein) remained in the same range for all the series with a mean

Table 2. Average division times of growing cultures of *Escherichia coli*

Inoculum	Medium	Division	Time (min.)
Salts + glucose	Salts + glucose	1	73
		2	60
		3	60
Nutrient broth	Salts + glucose	1	175
		1.5	48
Salts + casein hydrolysate	Salts + glucose	1	108, 78*
		1.5	48
		2	67*
Salts + yeast extract	Salts + glucose	1	105
		1.5	40
Salts + casein hydrolysate + yeast extract	Salts + glucose	1	142
		1.5	52
Salts + glucose	Nutrient broth	1	62
		2	58
		3	46
$\frac{1}{2}$ vol. salts + glucose + $\frac{1}{2}$ vol. nutrient broth	Nutrient broth	1	30
Nutrient broth	Nutrient broth $\frac{1}{2}$ vol. nutrient broth + } $\frac{1}{2}$ vol. salts + glucose }	1	65
		1	70
		2	88
Salts + glucose	$\frac{1}{2}$ vol. nutrient broth + } $\frac{1}{2}$ vol. salts + glucose }	1	62
		1	62
Salts + casein hydrolysate	Salts + casein hydrolysate	1	95
		2	70
Salts + yeast extract	Salts + yeast extract	1	75
		2	60
Salts + yeast extract + casein hydrolysate	Salts + yeast extract + } casein hydrolysate }	1	90

* With 8 mg. casein hydrolysate/ml.

of 97.5 % $\pm$ 11 (s.d.). When the overnight inoculum grown in nutrient broth was put into salts + glucose containing no nutrient broth, the first division was delayed. Division occurred at 175 min. instead of 73 min. when the inoculum was grown in salts + glucose. The hexokinase ranged from 50 to 100 % of control but, at $\frac{1}{2}$ division time (48 min.) later, the activity increased sharply. Table 2 shows a comparison of division times in different media.

In series II (Table 1) where the bacteria were grown in salts + glucose for two divisions and then were transferred to media made up of equal parts salts + glucose

and nutrient broth for 1 division, the hexokinase activity was decreased 25 % and the G-6-P dehydrogenase was decreased 50 %. When bacteria grown in salts + glucose + nutrient broth media for one division (Table 1, series III) were transferred to nutrient broth for 1 division, the activity of both enzymes fell 40 % from their previous values or 80 % from the original salts + glucose values. Series II (Table 1) shows the greater decreases when bacteria grown on salts + glucose (2 divisions) were transferred to nutrient broth for 1 division. Both hexokinase and G-6-P dehydrogenase activities were only 30 % of original values/mg. protein. When the *Escherichia coli* organisms were allowed to grow 2 or 3 divisions in nutrient broth, the activity of both enzymes levelled off at 50 % of original activity of the salts + glucose inoculum. The activity of both enzymes/ 10^9 bacteria decreased to 15 % of those grown in glucose because the bacteria were so much smaller.

Bacteria grown in salts + casein hydrolysate overnight and transferred to salts +

Fig. 4

Fig. 5

Fig. 4. Growth and enzyme values of *Escherichia coli* grown in salts + casein medium and transferred to salts + glucose medium. Two towers each containing 200 ml. of salts + casein medium were grown overnight. One was harvested to be extracted; the other was centrifuged, the organisms washed and suspended in salts medium. This suspension was used to inoculate two towers each containing total 200 ml. salts medium + glucose (2 mg./ml.). Turbidity curves are shown as - - - and viable cell counts as —. Arrow ↓ indicates time of harvesting. Enzyme activities of the extracts of the washed pellets expressed in μ mole NADP^+ /min./mg. protein are shown in the histograms, shaded for hexokinase (H), clear for G-6-P dehydrogenase and cross-hatched for PG dehydrogenase. The turbidity was measured with a 420 $m\mu$ filter in the Klett photometer at the time of harvest is indicated by $\log_2 T/T_0$.

Fig. 5. Growth and enzyme values of *Escherichia coli* grown in salts + casein + yeast-extract medium and transferred to salts + glucose medium. Two towers each containing 200 ml. of salts + casein + yeast-extract were grown overnight. One was harvested for extraction and the other was centrifuged, washed and suspended in salts medium. This was used to inoculate two towers each containing total 200 ml. salts medium with glucose (2 mg./ml.). Symbols are as in Fig. 4.

glucose showed a two- to threefold increase in activity of hexokinase and G-6-P dehydrogenase as seen in series IV (Table 1). When the overnight cultures also had yeast extract added (Table 1, series V), the same pattern resulted. However, when the overnight inoculum was grown in salts + yeast extract (Table 1, series VI), there was essentially no gain in hexokinase activity/mg. protein and a 1.5–4-fold gain/ 10^9 bacteria. The G-6-P dehydrogenase showed a gain of 1.5-fold/mg. protein and of 2–6-fold/ 10^9 bacteria. Figures 4 and 5 show typical experiments with turbidity and enzyme activities plotted.

DISCUSSION

The experiments showed that the spectrophotometric method for determining hexokinase was reproducible. Cochrane, Peck & Harrison (1953) showed with extracts of a *Streptomyces* a reduction of NADP⁺ by glucose and ATP and by G-6-P dehydrogenase and PG dehydrogenase, indicating the presence of hexokinase. Changes in hexokinase activity in *Escherichia coli* could be induced by changes in media. The hexokinase activity was increased after changes from media not containing glucose to media containing glucose. It was reported by Damodaran, Jagannathan & Singh (1955) for an *Aspergillus* that most of the enzymes of the glycolytic pathway and the hexosemonophosphate shunt, including hexokinase, decreased as the culture aged while growing on glucose and accumulating citrate. Hamilton & Dawes (1959, 1960, 1961) reported that *Pseudomonas aeruginosa*, when grown on medium containing half glucose and half citric acid from an inoculum grown on citric acid, used first the citric acid and then the glucose, so that a 'diauxie' was shown. However, when the inoculum organism was grown on glucose, both citric acid and glucose were metabolized simultaneously. In our experiments when *Escherichia coli* grown on nutrient broth was used to inoculate a medium containing half salts + glucose medium and half nutrient broth, there was a lag while the hexokinase built up, and then growth began. The *E. coli* needed 2.5 divisions to reach the degree of hexokinase activity of glucose-grown bacteria when transferred to salts + glucose from any medium tested, except casein hydrolysate + yeast extract, which needed only 2 divisions. The mechanism of adaptation must be the same as or similar to that reported by Hamilton & Dawes. There does not seem to be a substance in the casein hydrolysate, yeast extract or nutrient broth which acts as a repressor of hexokinase because glucose induced hexokinase in their presence. In an attempt to find the activator in the extracts a whole series of amines and other compounds were tested in our system. A report of these results is in preparation.

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REFERENCES

- COCHRANE, V. W., PECK, JR., H. D. & HARRISON, A. (1953). The metabolism of species of *Streptomyces*. VII. The hexosemonophosphate shunt and associated reactions. *J. Bact.* **66**, 17.
COHEN, S. S. & ARBOGAST, R. (1950). Chemical studies in host-virus interactions. *J. exp. Med.* **91**, 619.

- CRANE, R. K. & SOLS, A. (1954). The non-competitive inhibition of brain hexokinase by glucose-6-phosphate and related compounds. *J. biol. Chem.* **210**, 597.
- DAMODARAN, M., JAGANNATHAN, V. & SINGH, K. (1955). Carbohydrate metabolism in citric acid fermentation. III. The variation in glycolytic enzyme content of *Aspergillus niger* during citric acid fermentation. *Enzymol.* **17**, 199.
- HAMILTON, W. A. & DAWES, E. A. (1959). A diauxic effect with *Pseudomonas aeruginosa*. *Biochem. J.* **71**, 25 p.
- HAMILTON, W. A. & DAWES, E. A. (1960). The nature of the diauxic effect with glucose and organic acids in *Pseudomonas aeruginosa*. *Biochem. J.* **76**, 70 p.
- HAMILTON, W. A. & DAWES, E. A. (1961). Further observations on the nature of the diauxic effect with *Pseudomonas aeruginosa*. *Biochem. J.* **79**, 25 p.
- SCOTT, D. B. M. & CHU, E. (1959). The oxidative pathway of carbohydrate metabolism in *Escherichia coli*. 6. Adaptation of glucose-6-phosphate dehydrogenase to growth in complex media. *Biochem. J.* **72**, 426.
- SCOTT, D. B. M. & COHEN, S. S. (1953). The oxidative pathway of carbohydrate metabolism. 1. The isolation and properties of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. *Biochem. J.* **55**, 23.