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The Limitation of Glycolysis in Adenine-Deficient *Escherichia coli*

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1. In many instances, there was no change in the rate of oxygen consumption per cell when adenine was withdrawn from purine auxotrophs of *Escherichia coli* and *Salmonella typhimurium*. 2. However, adenine deficiency inhibited the metabolism of glucose, mannitol or glycerol in a *purA*$^-$ strain, in *purB*$^-$ or *purH*$^-$ strains in the absence of histidine and in *purB*$^-$ mutants supplied with hypoxanthine. These are all instances where reactions occur to consume adenine nucleotides. 3. The inhibition of glucose oxidation is accompanied by the accumulation of fructose 1,6-diphosphate and dihydroxyacetone phosphate. 4. Insufficiency of ADP for phosphoglycerate kinase is the most probable cause of the inhibition.

Since bacteria grow rapidly and are rich in nucleic acids the utilization of purine nucleotides for nucleic acid synthesis must compete for adenine nucleotides with other processes such as energy metabolism. The available information on this question is both meagre and contradictory. Pardee (1954, 1955) showed that adenine starvation of a purine-requiring auxotroph of *Escherichia coli* did not affect the respiration rate per cell even though the deficiency was sufficient to stop net synthesis of nucleic acids. Moses & Prevost (1966) have, however, reported large inhibitions of respiration in somewhat similar experiments with another auxotroph and glycerol as substrate.

The present paper is concerned with a study of energy metabolism in various purine auxotrophs under different conditions. An inhibition of energy metabolism has been discovered in instances where severe decreases of adenine nucleotide concentrations are either known or expected to occur. Evidence is presented for the inhibition being due to a lack of sufficient ADP for phosphoglycerate kinase.

EXPERIMENTAL

Bacteria and media. Details of *E. coli* strain AB-1325 (*pur B*$^-$ *pro A*$^-$ *his*$^-$) and the growth medium were given by Thomas, Varney & Burton (1970). Carbon sources were used at a concentration of 1%, glycerate and lactate being used as sodium salts and the sodium chloride then being omitted. Supplements were added when required for growth or as otherwise indicated. Except where stated otherwise, adenine, hypoxanthine, L-arginine hydrochloride or L-histidine hydrochloride were used at 10mg/l; L-proline at 30mg/l and thiamine hydrochloride at 1mg/l. All bacteria were grown and studied in aerated medium except for the experiment in Fig. 2. Strain G-26 is a *pur*$^-$ mutant of strain A.T.C.C. 9723 given by Dr R. G. Tucker. *Salmonella typhimurium* ad-1 and three purine-requiring *E. coli* (B-96, B-94 and M-55) were given by Dr J. S. Gots. Strain 202 (from Dr E. C. Lin) is a derivative of *E. coli* HfrC lacking both the anaerobic and aerobic L-α-glycerophosphate dehydrogenase. It was used for the phosphoglycerate kinase studies to obviate any possible interference in the enzyme assays due to the glycerophosphate dehydrogenases.

Oxygen consumption. Warburg manometers were used with air as the gas phase and alkali in the centre well. When a medium permitted normal growth, the respiration rate was corrected for growth. If a is the rate of O_2 consumption per cell and N the number of cells at zero time, the amount of O_2 consumed between times t_1 and t_2 is $aN(e^{kt_2}-e^{kt_1})/k$ where $k = 0.695/D$ and D is the generation time which is measured independently. In the absence of growth, the amount consumed would be $aN(t_2-t_1)$. Hence the observed consumption is divided by the factor $(e^{kt_2}-e^{kt_1})/k(t_2-t_1)$.

Measurement of glycolytic intermediates. Cultures were mixed with 0.1vol. of 55% (w/v) trichloroacetic acid or 0.25vol. of 1M-$HClO_4$, chilled and centrifuged. The supernatants were stored at −15°C until assayed. Up to 0.4ml of supernatant was added to the appropriate buffer plus sufficient NaOH to neutralize the acid in the sample. The remaining reagents were then added for the enzymic assay in a total volume of 2.5ml. Dihydroxyacetone phosphate was measured by the L-glycerol 1-phosphate dehydrogenase system and fructose 1,6-diphosphate by the same system with the use of aldolase and triose phosphate isomerase (Bücher & Hohorst, 1965). There was little interference from the ClO_4^- anion but trichloroacetate inhibited the dehydrogenase. The oxidation of NADH was determined fluorimetrically with exciting light at 340nm in a Unicam SP. 800 spectrophotometer with its fluorimeter attachment. In the experiment of Table 4, the same compounds were determined by using aldolase and isomerase together with glyceraldehyde 3-phosphate dehydrogenase to reduce NAD^+ in the presence of 0.2M-sodium arsenate and 0.01M-EDTA at pH8.5. Similar results were given by both systems. The enzymes

were supplied by Boehringer Corp. (London) Ltd., London W.5, U.K.

A radioactive labelling procedure was used to confirm the changes in the amounts of fructose 1,6-diphosphate. Growing cultures were washed and resuspended at 3×10^8 cells per ml in glucose-free medium. A sample of 0.1 ml of cell suspension was added to [U-^{14}C]glucose (87 nmol; 115 Ci/mol) in a silicone-treated test-tube shaken at 37°C. At the required time, 10 μl of 55% (w/v) trichloroacetic acid was added and the contents of the tube were applied as a single spot to Whatman no. 1 paper (46 cm × 57 cm). Electrophoresis [3 kV for 70 min in 7% (w/v) formic acid under white spirit] was used to separate the phosphorylated intermediates from glucose and its non-phosphorylated metabolites. This separation was followed by descending chromatography in the second, shorter dimension by using butan-1-ol–propionic acid–water (46:23:31, by vol.) for 48 h. The solvent reached the end of the paper in about 24 h and was allowed to drip off the end. R_F values in this solvent have been reported for a large number of metabolites (Benson *et al.* 1950; Bassham, Shibata, Steenberg, Bourdon & Calvin, 1956; Moses 1959). Radioactivity was measured in these areas with an efficiency of about 40% after cutting them out and immersing in a toluene liquid scintillant.

RESULTS AND DISCUSSION

Effects of purine deficiency on the rates of energy metabolism. The observation (Pardee, 1954, 1955) that withdrawing adenine from a purine auxotroph can cause little change in the rate of oxygen consumption per cell is confirmed by many of the examples shown in Table 1. However, inhibitions were observed with a *purA*$^-$ auxotroph (Table 2), with *purB*$^-$ or *purH*$^-$ mutants in the absence of histidine and with *purB*$^-$ mutants supplied with hypoxanthine (Table 1 and Fig. 1).

In these instances the rate of respiration could be restored promptly by adding adenine but restoring histidine to a *purH*$^-$ strain in the absence of adenine did not restore the respiration.

Thomas *et al.* (1970) described that supplying hypoxanthine in the absence of adenine to a *purB*$^-$ strain caused a severe fall in the intracellular concentrations of ATP, ADP and AMP because RNA synthesis was stimulated by the hypoxanthine thus consuming adenine nucleotides. Severe falls of adenine nucleotides would also be expected on withdrawing adenine in the absence of histidine from *purB*$^-$ or *purH*$^-$ mutants since the first step of histidine biosynthesis would be active and would consume ATP which would not, in these mutants, be replaced by biosynthesis (Magasanik & Karibian, 1960; Ames, Martin & Garry, 1961; Shedlovsky & Magasanik, 1962). This phenomenon should not occur in mutants blocked before the first of the two stages blocked in *purB*$^-$ mutants. Since the *purA*$^-$ mutation does not stop the synthesis of guanine nucleotides, there would still be a marked synthesis of RNA which would consume adenine nucleotides in the same manner as in a *purB*$^-$ strain supplied with hypoxanthine. Thus it appears that energy

Table 1. *Respiration rates of several purine auxotrophs in various conditions*

Respiration rates were measured in air at 37°C on 3 ml of culture ($3–7 \times 10^{11}$ cells/l). Rates in the presence of adenine were corrected for the increase expected from growth, except for the values marked with an asterisk where there was no increase in the respiration rate during the measurement period, presumably because the medium was otherwise changed from that of the growing culture. In almost all other instances, the rate was constant for at least 40 min; where this was not so it was measured over the period of 10–25 min after transfer to the new medium. See Fig. 1 for other details. Abbreviations: ade, adenine; his, histidine; hyp, hypoxanthine.

		Growth medium		Rate of respiration (μl of O_2/min)					
Strain	Locus of *pur* mutation	C-source	Histidine	C-source	ade + his	ade	his	his + hyp	hyp	no addition
B-94	*purB*	Glucose	Absent	Glucose†	3.3	—	2.9	—	—	1.7
		Glucose	Absent	Lactate†	2.8*	2.8*	2.8	2.8	2.8	2.8
		Mannitol	Absent	Mannitol	3.7	3.5	3.6	2.1	—	2.85
		Mannitol	Present	Mannitol	5.0	5.05	4.65	3.0	2.55	3.1
AB-1325	*purB*	Glucose	Present	Glucose	3.0	—	2.5	1.8	—	—
B-96	*purH*	Glucose	Present	Glucose	—	3.5	3.6	—	—	1.5
		Mannitol	Present	Mannitol	5.5	—	4.0	—	—	1.75
		Mannitol	Absent	Mannitol	—	3.3	3.2	—	—	1.6
		Lactate	Absent	Lactate	3.4	3.4	3.7	—	—	3.4
		Glycerol+	Present	Glycerol†	19	—	6.1	—	—	3.5
		DL-glycerate	Present	Glycerate†	9.3*	—	8.7	—	—	8.6
M-55	before *purB*	Mannitol	Present	Mannitol	3.1	3.2	3.1	—	—	3.2
G-26	*purC* or *E*	Mannitol	Absent	Mannitol	3.0	3.0	2.5	—	2.5	2.5

† A single washed culture was simultaneously examined with two carbon sources.

Table 2. *Respiration of a* purA⁻ *auxotroph*

S. typhimurium ad-1 was grown at 25°C and respiration measured at the same temperature. For expt. 1, glucose was the carbon source and histidine was present in the growth medium; in expt. 2, glycerol was the carbon source and histidine was not added to the growth medium.

Addition	Rate of respiration (μl of O_2/min) Expt. 1	Expt. 2
Adenine + histidine	2.3	3.1
Adenine	2.3	—
Histidine	2.2*	0.7
None	0.9	0.7

* In this instance, the rate fell progressively after about 20 min.

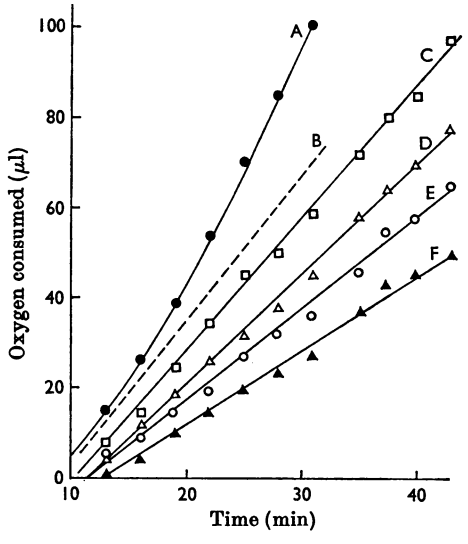

Fig. 1. Effects of adenine, histidine and arginine on oxygen consumption by a *purB⁻ arg⁻* strain. An exponential culture of strain B-94 in glucose medium supplemented with adenine and arginine was washed and resuspended in unsupplemented glucose medium. Portions (3 ml) were immediately added to manometer vessels which already contained supplements in the main compartment: A (●), adenine, arginine and histidine; B (----) is curve A corrected for growth (division time = 56 min); C (□), histidine and arginine; D (△), adenine and histidine; E (○), no addition; F (▲), arginine. Temperature: 37°C.

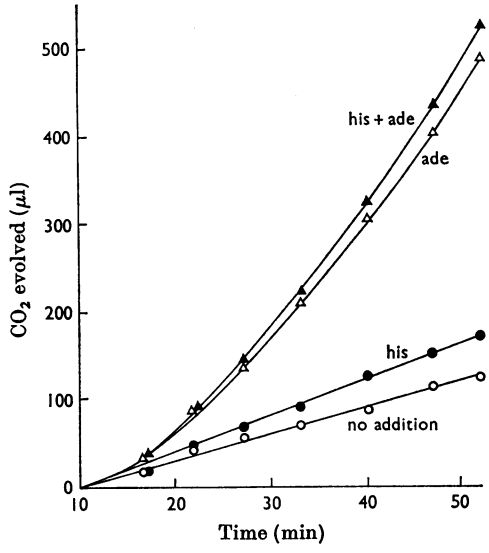

Fig. 2. Effects of adenine starvation on the anaerobic evolution of CO_2. Strain B-96 (*purH⁻*) was grown anaerobically on glucose medium containing adenine. The culture was washed and suspended in unsupplemented glucose medium. Portions (3 ml) were immediately added to manometer vessels which already contained adenine (ade) or histidine (his) as indicated. CO_2 evolution was measured at 37°C in N_2 with yellow phosphorus in the centre well.

metabolism is inhibited when there is severe depletion of adenine nucleotides.

Large inhibitions due to adenine deficiency were seen on both the oxidation of glycerol (Table 1) and on the anaerobic production of carbon dioxide from glucose (Fig. 2). There was, however, little or no effect on the oxidation of glycerate or lactate even though the rates of oxygen consumption were comparable with those given by glucose or glycerol (Table 1). These findings suggest that the inhibition occurs before the formation of 3-phosphoglycerate since this compound is on the route by which glycerate is oxidized in *E. coli* (Ornston & Ornston, 1969).

It is not likely that the inhibition of respiration in severe adenine deficiency is directly due to the general inhibition of macromolecular synthesis since uncomplicated adenine starvation is itself enough to prevent net synthesis of nucleic acids and proteins and it does not affect the respiration rate per cell. However, inhibition of respiration has been reported during amino acid starvation of stringent strains (Fields & Luria, 1969; Sokawa, Nakao-Sato & Kaziro, 1970). An inhibition of 27–30% was found on proline starvation of strain AB-1325 or on arginine starvation of strain B-94, both of which are stringent strains. The experiment with strain B-94 is shown in Fig. 1, where it will be seen that the inhibition due to removal of histidine in the absence of adenine (compare curves C and F) is greater than the effect of arginine starvation. The fact that

starvation for arginine, histidine and adenine (curve E) has a smaller effect than starvation for just histidine and adenine can be ascribed plausibly to a conservation of histidine in the absence of arginine. Evidently therefore, the effect of severe adenine deficiency is superimposed on the smaller one due to amino acid starvation.

Absence of killing effect. No effects were observed on the number of colonies that could be grown on nutrient agar after strains B-96 and B-94 had been aerated at 37°C for up to 5h in various types of adenine-deficient media with or without histidine and, in the case of strain B-94, with or without hypoxanthine.

Accumulation of fructose 1,6-diphosphate and dihydroxyacetone phosphate. By enzymic assay, there was at least a threefold increase of fructose 1,6-diphosphate and dihydroxyacetone phosphate in the *purH*$^-$ strain B-96 after incubation in a glucose medium lacking both histidine and adenine (Table 3). Fructose 1,6-diphosphate was also found to accumulate in a *purB*$^-$ strain when adenine nucleotides were depleted by supplying hypoxanthine or by starvation for both histidine and adenine (Table 4). No appreciable accumulation occurred during starvation for both proline and adenine. Much of the fructose diphosphate eventually leaked out into the culture medium but the results show clearly that the fructose diphosphate first accumulated within the cells (Table 3). Glyceraldehyde 3-phosphate and 3-phosphoglycerate were not found in amounts sufficient for reliable comparisons to be made.

In other experiments, intermediates of glycolysis in strain B-96 were examined by two-dimensional paper chromatographic separations after supplying [U-^{14}C]glucose. The principal radioactive spot corresponding to a phosphorylated glycolytic intermediate occupied the same position as authentic fructose 1,6-diphosphate and the radioactivity of this spot showed the greatest changes, there being in strain B-96 a fourfold increase after a period of 15–30min in the absence of adenine and histidine (Table 5). The identity of the radioactive fructose diphosphate was further confirmed by elution, treatment with prostatic phosphomonoesterase (see Burton & Petersen, 1960) and paper chromatography in aq. 85% (w/w) phenol–conc. aq. NH_3 (200:1, v/v). Most of the radioactivity moved as a single spot which coincided with authentic fructose.

Measurements of enzyme activities. The accumulation of fructose diphosphate and dihydroxyacetone phosphate from glucose and the large inhibition of glycerol oxidation without a comparable effect on glycerate oxidation limit the site of inhibition to triose phosphate isomerase, triose phosphate dehydrogenase or phosphoglycerate kinase. The activity of aldolase has been reported as being relatively low in *E. coli* (Wang & Morse, 1968), but inhibition of this enzyme would not explain the accumulation of dihydroxyacetone phosphate or the effects on the utilization of glycerol.

Direct tests did not indicate any effect of adenine starvation on the activities of aldolase, triose phosphate isomerase or glyceraldehyde phosphate dehydrogenase in extracts prepared from strain

Table 3. *Glycolytic intermediates in cultures of a* purH$^-$ *strain*

Strain B-96 growing on glucose medium at 37°C supplemented with adenine and histidine was washed and transferred to fresh medium at 1.2×10^{12} cells/l with supplements as indicated. Analyses were performed either on the total culture or on the medium after removing the cells by centrifuging.

Time (min)	Supplements	Fructose diphosphate (μM)	Dihydroxyacetone phosphate (μM)
		In total culture	
20	Adenine, histidine	4	2
60	Adenine, histidine	4	5
3	Histidine	4	0
15	Histidine	6	2
30	Histidine	4	8
60	Histidine	16	18
3	None	12	1
7	None	15	3
15	None	46	6
30	None	65	14
60	None	50	30
		In medium	
60	Histidine	6	8
15	None	3	2
30	None	10	7
60	None	21	23

Table 4. *Accumulation of fructose* 1,6-*diphosphate in a* purB$^-$ *strain*

Washed cells of strain AB-1325 at 37°C on glucose medium at 4.2×10^{11} cells/l and supplemented with thiamine and other compounds as indicated: ade, adenine 20 mg/l; his, 15 mg of L-histidine hydrochloride/l; hyp, 20 mg of hypoxanthine/l; pro, 50 mg of proline/l. Values are the concentration of fructose 1,6-diphosphate in the culture (μM) as measured by the aldolase, isomerase and glyceraldehyde phosphate dehydrogenase reactions.

	Fructose 1,6-diphosphate (μM)				
Time (min)	Supplements ... ade+his+pro	pro	his+pro	his	hyp+his+pro
3	—	—	—	—	0.7
10	0	—	—	—	0.7
20	0.7	—	—	—	2.0
40	—	0.8	0.1	0.7	2.8
60	—	1.4	0.6	0.5	3.77

Table 5. *Radioactivity incorporated into fructose* 1,6-*diphosphate from* [U-^{14}C]*glucose*

For details see the Experimental section. Strain B-96 (*purH*$^-$) grown on glucose medium supplemented with adenine and histidine, transferred to media containing [U-^{14}C]glucose and no adenine at 0 min.

	$10^{-4} \times$ Radioactivity in fructose diphosphate spot (c.p.m.)	
Time (min)	Histidine present	Histidine absent
5	2.6	2.9
10	2.0	8.1
20	2.4	9.4

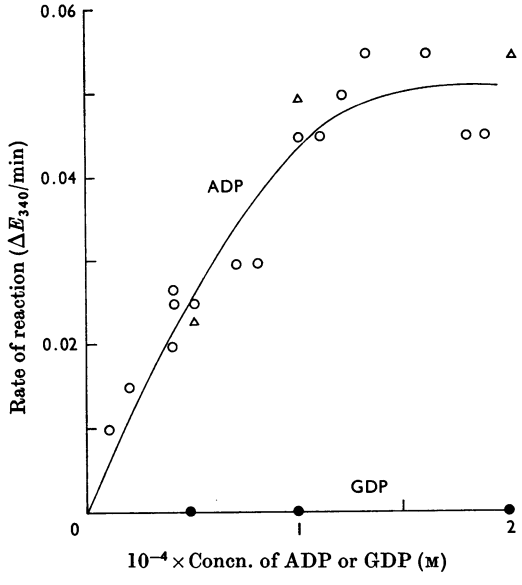

Fig. 3. Reaction of *E. coli* phosphoglycerate kinase with ADP and GDP. Strain 202 was grown on glucose medium, washed with water, cells were suspended in 0.1 M-triethanolamine buffer, pH 7.6 (2.4×10^9 cells/ml), broken in a French press and centrifuged. A portion of the extract (10 ml) was dialysed with stirring for a total of 6 h against three 100 ml portions of 0.02 M buffer at 4°C. The kinase activity in 10 μl of this extract was measured at 20°C by the reduction of NAD (56 μmol) in a total volume of 0.90 ml containing 1 μg of glyceraldehyde phosphate dehydrogenase, 50 μmol of P_i, pH 7.6, 0.25 mg of glutathione, 2 μmol of $MgSO_4$, ADP or GDP as indicated and glyceraldehyde 3-phosphate: ○ or ●, 0.15 μmol; △, 0.30 μmol.

B-96 after adenine starvation in the presence or absence of histidine. In other experiments, various amounts of AMP, ADP and ATP were added to the assay systems (Bücher & Hohorst, 1965) and no stimulation of activity was seen.

Since ADP is a substrate for the forward action of phosphoglycerate kinase during glycolysis, a decrease in the concentration of ADP might well inhibit at this stage. However, GDP accumulates when adenine nucleotides are depleted in *purB*$^-$ strains supplied with hypoxanthine in the absence of adenine (Thomas *et al.* 1970) and phosphoglycerate kinase from yeast can use GDP readily in place of ADP. It is therefore relevant that there is no appreciable reaction of GDP with the *E. coli* enzyme (Fig. 3). (In fact the guanine nucleotides can cause small inhibitions of the reaction with ADP.) Thus it is very probable that a low concentration of ADP may decrease the activity of phosphoglycerate kinase during severe adenine deficiency of *E. coli*. Presumably, 1,3-diphosphoglycerate accumulates, inhibits the glyceraldehyde phosphate dehydrogenase and in turn causes an accumulation of triose phosphates and fructose 1,6-diphosphate, the latter being in greater amount because of the equilibrium of the aldolase reaction. It is conceivable that the same mechanism may cause the accumulation of

glycolytic intermediates after killing by certain colicins (Fields & Luria, 1969).

Even though phosphofructokinase is commonly regarded as being the principal controlling stage in glycolysis, there are indications that phosphoglycerate kinase may also be important, for example in erythrocytes (Parker & Hoffman, 1967). This enzyme and glyceraldehyde phosphate dehydrogenase are closely linked by the ability of 1,3-diphosphoglycerate to inhibit the dehydrogenase (Hess, 1965; Williamson, 1965). Control or limitation of the glycolytic pathway at these stages has been reported in *E. coli* (Hempfling, 1965; Hempfling, Höfer, Harris, & Pressman, 1967) and in ascites tumour cells (Maitra & Chance, 1965), even though the increased flux of the Pasteur effect is apparently due to an activation of phosphofructokinase (Hempfling *et al.* 1967; Harrison & Maitra, 1969). An action at phosphoglycerate kinase is consistent with the view that there is more than one site at which glycolysis can be controlled (Maitra & Chance, 1965; Hempfling *et al.* 1967) and that different sites may be primarily concerned in different situations.

In conclusion, therefore, the behaviour of severely adenine-deficient *E. coli* indicates that glycolysis is limited between fructose 1,6-diphosphate and 3-phosphoglycerate and most probably at the phosphoglycerate kinase reaction. However, electron transport to molecular oxygen is not affected. It is noteworthy that the inhibition can be brought about *in vivo* by the consumption of ATP in the biosynthetic reactions of RNA polymerase and phosphoribosyl-ATP pyrophosphorylase. Even after sudden nutritional changes, such severe depletion of adenine nucleotides should either be prevented or quickly restored in wild-type bacteria by the allosteric controls of the pathways for the biosynthesis of purine nucleotides and histidine.

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REFERENCES

Ames, B. N., Martin, R. G. & Garry, B. J. (1961). *J. biol. Chem.* **236**, 2021.

Bassham, J. A., Shibata, K., Steenberg, K., Bourdon, J. & Calvin, M. (1956). *J. Am. chem. Soc.* **78**, 4120.

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W. (1950). *J. Am. chem. Soc.* **72**, 1710.

Bücher, Th. & Hohorst, H. J. (1965). In *Methods of Enzymatic Analysis*, p. 246. Ed. by Bergmeyer, H.-U. London: Academic Press (Inc.) Ltd.

Burton, K. & Petersen, G. B. (1960). *Biochem. J.* **75**, 17.

Fields, K. L. & Luria, S. E. (1969). *J. Bact.* **97**, 64.

Harrison, D. E. F. & Maitra, P. K. (1969). *Biochem. J.* **112**, 647.

Hempfling, W. (1965). In *Control of Energy Metabolism*, p. 360. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.

Hempfling, W. P., Höfer, M., Harris, E. J. & Pressman, B. C. (1967). *Biochim. biophys. Acta*, **141**, 391.

Hess, B. (1965). In *Control of Energy Metabolism*, p. 196. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.

Magasanik, B. & Karibian, D. (1960). *J. biol. Chem.* **235**, 2672.

Maitra, P. K. & Chance, B. (1965). In *Control of Energy Metabolism*, p. 157. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.

Moses, V. (1959). *J. gen. Microbiol.* **20**, 184.

Moses, V. & Prevost, C. (1966). *Biochem. J.* **100**, 336.

Ornston, M. K. & Ornston, L. N. (1969). *J. Bact.* **97**, 1227.

Pardee, A. B. (1954). *Proc. natn. Acad. Sci. U.S.A.* **40**, 263.

Pardee, A. B. (1955). *J. Bact.* **69**, 233.

Parker, J. C. & Hoffman, J. F. (1967). *J. gen. Physiol.* **50**, 893.

Shedlovsky, A. E. & Magasanik, B. (1962). *J. biol. Chem.* **237**, 3731.

Sokawa, Y., Nakao-Sato, E. & Kaziro, Y. (1970). *Biochim. biophys. Acta*, **199**, 256.

Thomas, G. A., Varney, N. F. & Burton, K. (1970). *Biochem. J.* **120**, 117.

Wang, R. J. & Morse, M. L. (1968). *J. molec. Biol.* **32**, 59.

Williamson, J. R. (1965). In *Control of Energy Metabolism*, p. 333. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.