THE METABOLISM OF ARGININE IN *SERRATIA MARCESCENS*

II. CARBAMYL–ADENOSINE DIPHOSPHATE PHOSPHOFERASE

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Summary

Carbamyl–adenosine diphosphate (ADP) phosphoferase, an enzyme which catalyses the synthesis and breakdown of carbamyl phosphate (CAP), has been purified 43-fold and obtained free of carbamyl phosphate phosphatase activity. ADP, but not adenosine monophosphate, has been shown to be a substrate for the enzyme. Magnesium or manganous ions are required for activity. Inhibition by heavy metal cations and *p*-chloromercuribenzoate indicate that a sulphydryl group is involved in catalysis.

The mechanisms of carbamyl–ADP phosphoferase action and some aspects of citrulline metabolism are discussed.

I. Introduction

In a study of *Serratia marcescens* (Glasziou 1956), no urease, arginase, or arginine desmidase activity could be detected. Evidence was given that the conversion of citrulline to arginine proceeded via argininosuccinic acid. Phosphorolysis and arsenolysis of citrulline were demonstrated. Unlike bacteria such as *Streptococcus faecalis* and *Staphylococcus aureus*, the rate of phosphorolysis of citrulline was not increased by the addition of adenosine phosphates to the reaction mixture. In this respect the system in *S. marcescens* is similar to that found in mammalian liver (Krebs, Eggleston, and Knivett 1955).

Jones, Spector, and Lipmann (1955) showed carbamyl phosphate (CAP) to be an intermediate in the biosynthesis of citrulline from ornithine by extracts from *Strep. faecalis* and also from liver mitochondria. CAP was dissimilated in the presence of acetone-dried cells of *S. marcescens* under conditions which give rise to phosphorolysis of citrulline in bacterial and animal systems. The reaction was followed by measurement of CO_2 output, and a marked stimulation of the reaction rate was observed in the presence of adenosine diphosphate (ADP). Thus it appeared that there were two pathways for the breakdown of CAP, one a hydrolysis, and the other a transfer of energy-rich phosphate to ADP. This paper reports the preparation of carbamyl–ADP phosphoferase from *S. marcescens* free of detectable phosphatase activity, and describes some of the properties of the enzyme.

II. Materials

Acetone-dried cells of an arginine-requiring mutant from a laboratory strain of *S. marcescens* were used as the starting material for all the experiments reported. This mutant grows on arginine or citrulline but not ornithine or CAP. Arsenolysis of citrulline does not occur so that carbamyl-ornithine kinase is inactive. The methods

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of preparation of acetone-dried cells, CAP, and *N*-acetyl glutamic acid were described in Part I of this series (Glasziou 1956). Adenosine-5-monophosphate (AMP), ADP, and adenosine triphosphate (ATP) were commercial preparations (Pabst Laboratories Ltd.).

III. Methods

(*a*) *Cell-free Extracts*

Acetone-dried cells were extracted by grinding in a mortar in either buffer solution or distilled water. The suspension was kept at 0–1°C and stirred at intervals. The time and medium for extraction were varied as required. In some experiments the extraction was made with distilled water and with dialysis at 0–1°C. In such cases it was necessary to add buffer before centrigufation to facilitate packing of the residues. Centrifugation was carried out in an International refrigerated centrifuge, model PR2, at 25,000 *g* for 10–15 min. The clear supernatant was withdrawn and kept at 0–1°C until required.

(*b*) *Preparation of Carbamyl–ADP Phosphoferase*

Acetone-dried cells (400 mg) were extracted with 10 ml of 0·25M Sorensen's phosphate buffer, pH 6·1, for 2 hr at 0–1°C. After centrifugation, aliquots of the cell-free extract were removed for protein determination and for enzyme assays. To 6·5 ml of the extract an equal volume of sat. ammonium sulphate was added. The precipitated protein was collected by centrifugation at 25,000 *g* for 15 min, the supernatant being discarded. The precipitate was dissolved in 3 ml of 0·01M phosphate buffer, pH 6·4. The temperature throughout the ammonium sulphate fractionation was maintained at 0–1°C.

To the 3 ml of enzyme solution, absolute ethanol at 0°C was added dropwise and with constant stirring. The temperature during this step was between 0 and 3°C. At lower temperatures (ethanol at −10°C) carbamyl phosphate phosphatase (CAP-ase) activity was preserved, and contaminated the carbamyl–ADP phosphoferase fraction. Fractions were collected at concentrations of 55 per cent. (v/v) ethanol and from 55–62 and 62–67 per cent. (v/v) ethanol. The precipitated material of each fraction was collected by centrifugation and taken up in 5 ml of 0·25M phosphate buffer, pH 6·1. Of these three fractions, the major part of the enzyme activity was in the 62–67 per cent. fraction. The data for this fractionation are given in Table 1. The enzyme assays and protein determinations were completed within 3 hr of commencing centrifugation of the suspension.

From this procedure a standardized preparation was developed in which 500 mg cells were extracted overnight in 10 ml of buffer. The precipitate from half saturation with ammonium sulphate was taken up in 3 ml of 0·01M buffer as before and 5 ml ethanol added. The precipitate was discarded and a further 1·5 ml ethanol added to the supernatant. The precipitate from this step was collected and taken up in 5 ml buffer or water as required. This solution is subsequently referred to as the carbamyl–ADP phosphoferase preparation.

(c) *Enzyme Assay*

The two enzymes were assayed by the manometric measurement of CO_2 evolution using Warburg manometers. The enzyme unit of CAP-ase activity was defined as that amount of enzyme which catalysed the formation of 1 μl CO_2 per min in a system which contained 15 μmoles CAP, 20 μmoles $MgSO_4$, and 125 μmoles phosphate buffer, pH 6·1, in a total vol. of 3·3 ml at 30°C and in an initial gas phase of nitrogen. The unit of carbamyl–ADP phosphoferase activity was similarly defined except that 20 μmoles ADP were included in the reaction mixture. In both cases corrections were made for the spontaneous breakdown of CAP. The rates were measured under conditions which closely approximated zero order reactions.

TABLE 1

SEPARATION OF CARBAMYL–ADP PHOSPHOFERASE (PHOSPHOFERASE) FROM CARBAMYL PHOSPHATE PHOSPHATASE (CAP-ASE)

Step	Volume (ml)	Enzyme Activity (units/ml)		Protein (mg/ml)	Ratio of Absorption (280/260 mμ)	Specific Activity		Percentage Yield of Phosphoferase	Purification
		Phosphoferase	CAP-ase			Phosphoferase	CAP-ase		
Original extract	6·5	8·0	0·4	6·2	0·84	1·3	0·065	100	—
Ethanol fraction* (62–67%)	5·0	4·1	—	0·073	0·93	56	—	40	43×

* See text for fractionation procedure.

(d) *Determinations*

Methods for deproteinization, measurement of CAP, and orthophosphate have been cited previously (Glasziou 1956). Ammonia was determined by a Conway method modified from the method described by Braganca, Quastel, and Schuchen (1954). Protein was determined by measurement of the optical density of the solutions at 260 and 280 mμ (Hilger Uvispek) and applying the formula derived by Kalckar (1947). CO_2 was measured using standard Warburg manometric technique.

IV. RESULTS

(a) *The Separation of Carbamyl–ADP Phosphoferase from CAP-ase*

The two activities towards CAP found in acetone-dried cells of *S. marcescens* could conceivably be catalysed by one enzyme. Preliminary experiments, in which the ratios carbamyl–ADP phosphoferase/CAP-ase were measured using a variety of extraction conditions, gave values ranging from 2·5 to 24. Fractionation procedures resulted in the preparation of carbamyl–ADP phosphoferase free of CAP-ase activity indicating that two enzymes are involved for the reactions catalysed (Table 1).

Recently Marshall, Hall, and Cohen (1955) showed that compound *X*, an intermediate in citrulline synthesis by rat liver preparations (Grisolia and Cohen 1952), was identical with CAP. The enzyme reported by Grisolia and Marshall (1954) which split compound *X* was presumably a CAP-ase.

(*b*) *The Stoichiometry of the Reaction Catalysed by Carbamyl–ADP Phosphoferase*

The results presented in Table 2 show that there is a stoichiometric relationship between the amount of CO_2, NH_3, and ATP formed during decomposition of CAP in the presence of a cell-free extract from *S. marcescens*.

TABLE 2

THE FORMATION OF ADENOSINE TRIPHOSPHATE, CO_2, AND NH_3, FROM CARBAMYL PHOSPHATE (CAP)

Extract (1·7 ml) buffered with 85 μmoles succinate buffer pH 6·1, 20 μmoles $MgSO_4$, 9·6 μmoles CAP (which contained 0·3 μmole NH_3), 15·5 μmoles ADP; total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C

Experiment	Treatment	CO_2 Formed (μmoles)	Labile Phosphorus* (μmoles)	Increase in Labile Phosphorus (μmoles)	NH_3 Formed (μmoles)
1	Stopped at zero time	—	15·5	—	
2	Stopped at zero time	—	15·5	—	
3	Incubated at 30°C	6·1	21·4	5·9	
4	Incubated at 30°C	6·3	21·5	6·0	
5	Incubated at 30°C	5·4			5·7
6	Incubated at 30°C	6·0			5·5

* That amount of orthophosphate hydrolysed in 8 min at 100°C in the presence of N HCl. The figures quoted are corrected for inorganic and CAP orthophosphate.

(*c*) *The Synthesis of CAP from ATP, CO_2, and NH_3*

The synthesis of CAP from ATP, CO_2, and NH_3 was demonstrated by Jones *et al.* (1955) using extracts from *Strep. faecalis* or liver mitochondria. This synthesis had previously been demonstrated by Grisolia and Cohen (1952) although the reaction product was not identified. The synthesis of CAP in the presence of carbamyl–ADP phosphoferase from *S. marcescens* proved difficult to demonstrate due to the increase in orthophosphate, which was not diminished by potassium fluoride (Table 3). The addition of *N*-acetyl glutamic acid did not increase the amount of CAP formed as it does in the rat liver system reported by Marshall *et al.* (1955).

(*d*) *The Effect of Adenosine Phosphates on the Breakdown of CAP*

No conclusive evidence has been presented to show which of the adenosine phosphates accepts the energy-rich phosphate group produced during the phosphorolysis of citrulline. Oginsky and Gehrig (1953) using acetone-dried cells of *Strep. faecalis* found that AMP was the best phosphate acceptor under the conditions of their experiments. The addition of Mg^{++} stimulated both ATP breakdown to yield AMP (apyrase

was present in the system) and the acceptance of an energy-rich phosphate group by AMP.

TABLE 3

THE SYNTHESIS OF CARBAMYL PHOSPHATE FROM ADENOSINE TRIPHOSPHATE (ATP), CO_2, AND NH_3

The complete system contained 1·0 ml carbamyl–ADP phosphoferase preparation containing 500 μmoles tris(hydroxymethyl)aminomethane buffer pH 7·8, 20 μmoles $MgSO_4$, 20 μmoles ATP, 100 μmoles $(NH_4)_2CO_3$; final vol. 3·0 ml. System incubated for 60 min at 30°C

System	Inorganic Phosphate (μmoles)	Unstable Phosphate* (μmoles)
No enzyme	1·94	0·18
No ATP	0·73	0·06
Complete	4·25	0·6
Complete + 20 μmoles *N*-acetyl glutamate	3·85	0·5

* That amount of orthophosphate hydrolysed in 1 min at 100°C in the presence of 0·01N HCl. This value defines CAP (Jones *et al.* 1955).

The breakdown of CAP in the presence of partially purified carbamyl–ADP phosphoferase from *S. marcescens* takes place with ADP but not AMP or ATP as phosphate acceptor (Table 4).

TABLE 4

THE EFFECT OF ADENOSINE PHOSPHATES ON THE BREAKDOWN OF CARBAMYL PHOSPHATE

The complete system contained 0·5 ml carbamyl–ADP phosphoferase preparation buffered with 125 μmoles phosphate buffer pH 6·1, 20 μmoles $MgSO_4$, 20 μmoles adenosine phosphate (AMP, ADP, or ATP), 10 μmoles CAP; total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C

System	CO_2 Produced (μl)		
	10 Min	30 Min	60 Min
No enzyme	2	7	12
No adenosine phosphate	2	6	12
Complete (AMP)	2	7	12
Complete (ADP)	55	123	143
Complete (ATP)	6	12	16

(*e*) *The Effect of* N-*Acetyl Glutamic Acid on Carbamyl–ADP Phosphoferase*

The catalytic effect of derivatives of glumatic acid on the synthesis of compound *X* (CAP) has been well established for liver preparations. Grisolia and Cohen (1953) showed that *N*-acetyl glutamate, *N*-chloroacetyl glutamate, carbamyl gluta-

mate, and *N*-formyl glutamate were effective in that order. Krebs *et al.* (1955) found that the phosphorolysis of citrulline in the presence of *Eschericia coli* and liver enzymes was accelerated by carbamyl glutamate, carbamyl glutamine, acetyl glutamate, and

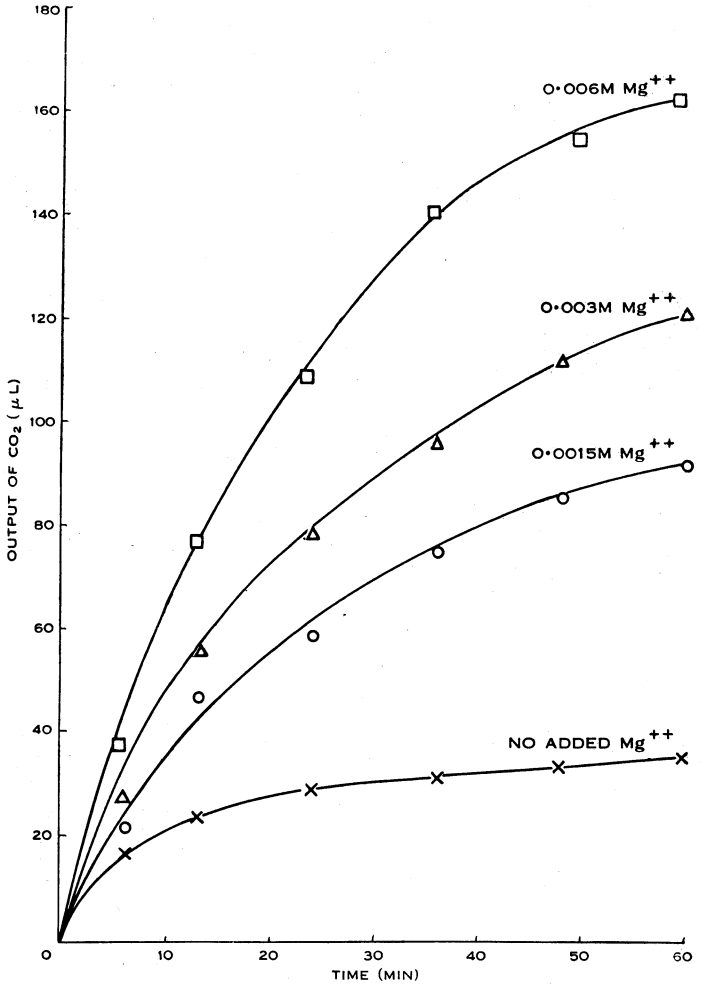

Fig. 1.—The effect of Mg^{++} on the breakdown of CAP. In addition to $MgSO_4$, the reaction mixtures contained 0·5 ml carbamyl–ADP phosphoferase preparation buffered with 125 μmoles phosphate buffer pH 6·1, 20 μmoles ADP, 10 μmoles CAP, total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C.

carbamyl alanine. The concentrations of amino acid derivatives used by Krebs *et al.* (1955) were much greater than those required for catalysis of CAP formation. The most likely role of these substances appears to be as cofactors of carbamyl–ADP phosphoferase or as precursors of a cofactor.

Attempts to demonstrate an effect by *N*-acetyl glutamate, the most effective of these catalysts, on carbamyl–ADP phosphoferase prepared from *S. marcescens* have not met with any success either on the synthesis or breakdown of CAP. Dialysis of the enzyme in dialysis tubing which had been soaked for 24 hr in distilled water

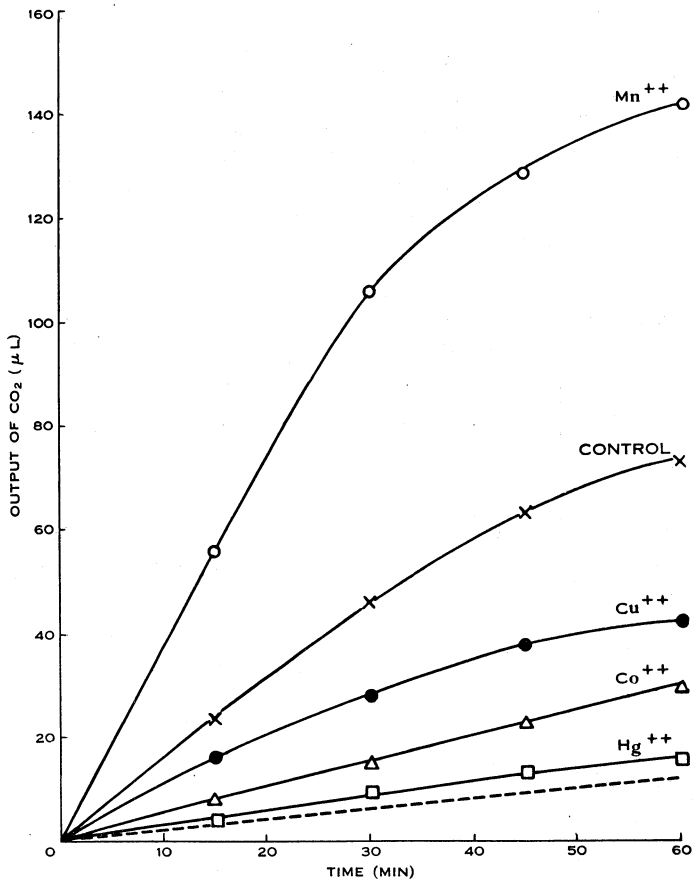

Fig. 2.—The effect of divalent cations on the breakdown of CAP. The reaction mixtures contained 0·5 ml carbamyl–ADP phosphoferase preparation buffered with 125 μmoles phosphate buffer pH 6·1, 20 μmoles ADP, 10 μmoles CAP, 5 μmoles $MgSO_4$, total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C. Added cations were at a final concn. of 0·006M. The dotted line gives the spontaneous breakdown of CAP under the conditions of the experiment.

resulted in partial or complete inactivation. The activity was not restored by *N*-acetyl glutamate but was restored by either cysteine, glutathione, or boiled liver extract. Further attempts are being made to split the enzyme into an apoenzyme and a prosthetic group.

(*f*) *The Effect of Divalent Cations and* p-*Chloromercuribenzoate on Carbamyl–ADP Phosphoferase*

Mg^{++} is required for the synthesis of CAP (compound *X*) by liver preparations (Grisolia and Cohen 1952; Jones *et al.* 1955) or by extracts of *Strep. faecalis* (Jones *et al.* 1955). Oginsky and Gehrig (1953) reported a requirement for Mg^{++} or Mn^{++} in the phosphorolysis of citrulline by *Strep. faecalis*, and observed inhibition by Cu^{++} and

TABLE 5

THE EFFECT OF *p*-CHLOROMERCURIBENZOATE ON CARBAMYL–ADP PHOSPHOFERASE

The complete system contained 0·5 ml carbamyl–ADP phosphoferase preparation buffered with 125 μmoles phosphate buffer pH 6·1, 30 μmoles $MgSO_4$, 10 μmoles CAP, 20 μmoles ADP; total vol. 3·3 ml. System incubated in an atmosphere of N_2 at 30°C

System +		Output of CO_2 (μl)		
p-Chloromercuribenzoate (final concn.)	Glutathione (final concn.)	10 Min	35 Min	78 Min
—	—	40	78	109
—	1×10^{-3}M	35	69	102
2×10^{-5}M	—	17	21	31
2×10^{-5}M	1×10^{-3}M	32	66	98
2×10^{-6}M	—	16	22	35
2×10^{-6}M	1×10^{-3}M	37	72	103
2×10^{-7}M	—	38	70	104
2×10^{-7}M	1×10^{-3}M	34	68	100

Hg^{++}. The inhibition by Hg^{++} was reversed by glutathione and British anti-lewisite (1, 3-dimercaptopropanol). The reaction was also inhibited by *p*-chloromercuribenzoate and this effect could be reversed by glutathione. This suggests that an –SH group is functional in one of the enzymes of the "citrullinase" system.

The necessity for either Mg^{++} or Mn^{++} in the breakdown of CAP by carbamyl–ADP phosphoferase from *S. marcescens* is shown in Figures 1 and 2. Inhibition by Hg^{++}, Co^{++}, and Cu^{++} is shown in Figure 2.

The effect of *p*-chloromercuribenzoate was tested (Table 5). The inhibition by this substance was reversed by glutathione. These data suggest that the –SH group is important for the catalytic activity of the enzyme.

V. DISCUSSION

The proposal that the reaction catalysed by carbamyl-ornithine kinase is rate-limiting in the phosphorolysis of citrulline was put forward to explain the lack of stimulation of the reaction rate by added adenosine phosphates in *S. marcescens* and liver preparations (Glasziou 1956). The marked stimulation by phosphate acceptors

in bacteria such as *Strep. faecalis* and *Staph. aureus* could be due to a greater content of carbamyl-ornithine kinase. If this explanation is correct then gross metabolic differences exhibited by different organisms are due to alteration in relative enzyme content rather than a change in enzyme pattern. It has been shown that ADP and not AMP will accept energy-rich phosphate from CAP in the presence of carbamyl–ADP phosphoferase from *S. marcescens*. Purification of the corresponding enzyme from *Strep. faecalis* may show that AMP is the acceptor in this system as claimed by Oginsky and Gehrig (1953). However, if this were so it would not invalidate the argument that the carbamyl-ornithine kinase reaction controls the overall properties of the citrullinase system.

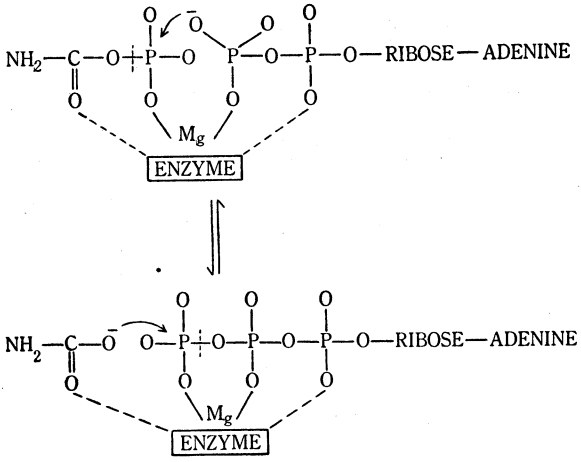

Fig. 3.—The possible role of metal ions in the reaction catalysed by carbamyl–ADP phosphoferase.

The role of glutamate derivatives remains obscure. The best hypothesis that can be proposed at present appears to be that a glutamic acid derivative with a *N*-carbonyl grouping is functional in the active centre of carbamyl–ADP phosphoferase, and that the cofactor is readily dissociated from the liver enzyme but not from the bacterial enzyme.

The transfer of energy-rich phosphate to ADP during phosphorolysis of citrulline may involve the participation of an enzyme-phosphoryl linkage (Krebs *et al.* 1955; Glasziou 1956). Alternatively, the mechanism of action proposed for pyruvate–ADP phosphoferase by Boyer and Harrison (1954) would equally well explain the reaction catalysed by carbamyl–ADP phosphoferase and at the same time account for the incorporation of ^{18}O from orthophosphate into CO_2 during phosphorolysis of citrulline (Stulberg and Boyer 1954). The participation of Mg^{++} or Mn^{++} in the forward and reverse reaction is best explained by assuming complex formation with the enzyme and both substrates, the high charge density of the metal ion mediating an electronic shift and thereby increasing the residual positive charge on the terminal phosphorus atom of ATP or CAP (Fig. 3). In the reaction mechanism postulated the metal ion is also functional in binding ATP to the enzyme. Cohn (1953), using the paramagnetic

absorption resonance technique, has demonstrated that Mn^{++} forms chelate compounds with ATP and other organic phosphates.

VI. Acknowledgments

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VII. References

Boyer, P. D., and Harrison, W. W. (1954).—"A Symposium on the Mechanism of Enzyme Action." (Ed. W. D. McElroy, and B. Glass.) (The John Hopkins Press: Baltimore.)
Braganca, B. M., Quastel, J. A., and Schucher, R. (1954).—*Arch. Biochem. Biophys.* **52**: 18.
Cohn, M. (1953).—*J. Biol. Chem.* **201**: 735.
Glasziou, K. T. (1956).—*Aust. J. Biol. Sci.* **9**: 117.
Grisolia, S., and Cohen, P. P. (1952).—*J. Biol. Chem.* **198**: 561.
Grisolia, S., and Marshall, R. O. (1954).—*Biochem. Biophys. Acta* **14**: 446.
Jones, M. E., Spector, L., and Lipmann, F. (1955).—*J. Amer. Chem. Soc.* **77**: 819.
Kalckar, H. (1947).—*J. Biol. Chem.* **167**: 461.
Krebs, H. A., Eggleston, L. F., and Knivett, V. A. (1955).—*Biochem. J.* **59**: 185.
Marshall, R. O., Hall, L. M., and Cohen, P. P. (1955).—*Biochem. Biophys. Acta* **17**: 279.
Oginsky, E. L., and Gehrig, R. H. (1953).—*J. Biol. Chem.* **204**: 721.
Stulberg, M. P., and Boyer, P. D. (1954).—*J. Amer. Chem. Soc.* **76**: 5569.