Further experiments are in progress on the nature of the block in M97-21, i.e. whether it is lacking an enzyme or is inhibited by a metabolite which it accumulates and which is involved in the valine-isoleucine equilibrium.

SUMMARY

1. The growth of four mutants of *Escherichia coli* requiring L-threonine is inhibited by the presence of L-serine in the culture medium.

2. The inhibition by DL-serine is competitive and is suppressed by D-threonine, α -aminobutyric acid, α -oxobutyric acid, α -oxo- β -hydroxybutyric acid and L-isoleucine.

3. All the above-listed compounds, except L-isoleucine, exert a sparing effect in the utilization of L-threonine.

4. A mutant requiring either D-threonine, α -aminobutyric acid or L-isoleucine is also inhibited by DL-serine, when grown on D-threonine or α -aminobutyric acid.

5. These results lead to the hypothesis that L-isoleucine synthesis depends on L-threonine utilization in these mutants.

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Stoichiometry of the Citrulline Phosphorylase Reaction of *Streptococcus lactis*

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It has recently been shown that the anaerobic degradation of the carbamido group of L-citrulline by *Streptococcus faecalis* (Knivett, 1953; Slade, 1953) and *Strep. lactis* (Korzenovsky & Werkman, 1953*a*, *b*) is coupled to the esterification of orthophosphate. Adenosine diphosphate (ADP), Mg^{++} and orthophosphate are the necessary components for the formation of ornithine, carbon dioxide and ammonia, as well as for the synthesis of acid-labile phosphate bonds (Knivett, 1953; Slade, 1953; Korzenovsky & Werkman, 1953*a*, *b*). Incorporation of radioactive phosphorus into adenosine triphosphate (ATP) (Knivett, 1953) or phosphorylation of glucose in the presence of iodoacetate (Korzenovsky & Werkman, 1953*a*, *b*) indicates that ADP functions as a phosphate acceptor resulting in the synthesis of ATP. Although adenosine 5-phosphate (AMP) can replace ADP (Korzenovsky & Werkman, 1953*b*) its role as a phosphate acceptor is not clear because the absence of enzymes capable of catalysing the reaction $2\text{ADP} \rightleftharpoons \text{AMP} + \text{ATP}$ has not been shown.

The purpose of the present paper is to establish the stoichiometry of the citrulline phosphorylase reaction according to the following equations:

METHODS

Conditions for the culture of *Strep. lactis* and preparation of cell-free extracts have been described (Korzenovsky & Werkman, 1953*b*). Extracts were dialysed against distilled water at 1° for 8 hr. and then diluted with 0.5M potassium phosphate buffer, pH 6.2-6.3, to contain about 5 mg. of protein per 0.1 ml. The diluted extracts contained approximately 5 μ moles of orthophosphate per 0.1 ml.

The reaction was conducted in 25 ml. Erlenmeyer flasks. Enzyme, orthophosphate (pH 6.2-6.3), maleate buffer (maleic acid brought to pH 6.2-6.3 with NaOH solution), $MgCl_2$ and iodoacetate were incubated for 10 min. Purified hexokinase and glucose, when used, were then added and the

mixture incubated for another 10 min. The reaction was started by the addition of ADP and citrulline or other substrate. Several methods were used to stop the reaction and deproteinize the mixture depending upon the product to be determined. Reference to these is made at appropriate places. Carbon dioxide was measured manometrically. For this purpose the reaction was carried out in Warburg flasks with three side arms permitting the addition of the above components in the same order. Acid was used to stop the reaction and release bound CO_2 . All experiments were made at 30.4° in air.

Glucose 6-phosphate (G 6-P) was determined manometrically with triphosphopyridine nucleotide (TPN), Zwischenferment and yellow enzyme. The analytical mixture (1.5 ml.) contained 0.15 M glycylglycine buffer (0.5 ml., pH 7.4); 0.2 mg. TPN; 4.0 mg. Zwischenferment; 0.4 ml. 25% (v/v) yellow enzyme solution; and the deproteinized unknown solution brought to pH 7.4 or the known solution of G 6-P. The reaction was conducted in Warburg flasks of about 6 ml. capacity with NaOH solution in the centre well; gas, O_2 . The procedure for the determination of G 6-P gave 85–90% recovery. Known solutions were always run with unknowns and appropriate corrections made. Protein-free filtrates to be used for analysis of G 6-P were made by adding 0.1 ml. 5N acetic acid to the reaction mixture followed by heating for 5 min. at 100° . Glucose was determined according to Somogyi (1945a) with the chromogenic reagent of Nelson (1944) on mixtures deproteinized with $\text{Ba}(\text{OH})_2 \cdot \text{ZnSO}_4$ (Somogyi, 1945b). These reagents also remove phosphoric esters (Colowick, Cori & Slein, 1947). The method of Roe (1934) was used for the determination of fructose. This procedure does not distinguish between fructose, fructose 6-phosphate or fructose 1, 6-diphosphate, hence results are reported simply as fructose. Analyses were made on mixtures deproteinized with 5% (w/v) trichloroacetic acid (TCA). Orthophosphate was determined by the method of Lowry & Lopez (1946) on reaction mixtures deproteinized with saturated $(\text{NH}_4)_2\text{SO}_4$ or with ice-cold 5% (w/v) TCA and immediately brought to pH 4.0 with 0.1 M sodium acetate. Ornithine was determined according to Chinard (1952) on TCA filtrates. For determination of NH_3 , protein-free filtrates were made alkaline with a saturated solution of sodium tetraborate and the NH_3 distilled into acid with a Parnas-Wagner apparatus. The distillate was nesslerized and the colour read in a photoelectric colorimeter. Methods for the determination of citrulline and protein have been cited (Korzenovsky & Werkman, 1953b).

Zwischenferment and yellow enzyme were prepared from yeast maceration juice according to the method of Warburg & Christian (1933). The preparation of yellow enzyme was carried through the first acetone precipitation stage. The precipitate was dissolved in a minimum of water, dialysed, and after dilution to a 25% (v/v) solution, used without further purification. Yeast hexokinase was prepared by Pabst Laboratories, Milwaukee, Wisconsin, according to the procedure of Berger, Slein, Colowick & Cori (1946). The purification was carried as far as Step 3-A followed by dialysis at 4° against 1% glucose. The enzyme was stored as a lyophilized powder and dissolved in cold distilled water immediately prior to use. The activity of the hexokinase is expressed in the units of Kunitz & McDonald (1946).

The purity of ADP was about 95% and of ATP about 97% according to chromatographic analysis. TPN was of about 60% purity. These three products were of yeast origin.

RESULTS

It is apparent from Fig. 1 that, under the experimental conditions specified, approximately one mole each of carbon dioxide and ammonia are produced from citrulline for each mole of orthophosphate utilized. The ratio is maintained for the entire time during which measurements were made, although the rate of reaction decreases. The decrease in reaction rate is caused by the inhibitory effect of ornithine (Korzenovsky & Werkman, 1953b).

The amount of ATP formed in the citrulline phosphorylase reaction may be determined by addition of purified yeast hexokinase and glucose to the reaction mixture. Either decrease in concentration of glucose or manometric determination of G 6-P is used as a measure of the ATP synthesized. The reliability of either procedure depends upon the absence of competing enzymes which may be present in crude preparations. Extracts of *Strep. lactis*

Fig. 1. The effect of time of incubation upon the relationship between CO_2 , NH_3 and orthophosphate in the citrulline phosphorylase reaction. The test system contained 0.1 ml. extract (4.3 mg. protein), 15 μmoles orthophosphate, 0.04 mole maleate buffer (pH 6.2), 20 μmoles MgCl_2 , 10 μmoles iodoacetate, 0.5 μmole ADP, 25 μmoles L-citrulline, 50 μmoles glucose and 56 k.m. units hexokinase (see text, this page). Total volume, 1 ml. The reaction mixture was deproteinized with 5% (w/v) TCA and brought to pH 4.0 with 0.1 M sodium acetate. $\bigcirc$ — $\bigcirc$, decrease in phosphate; $\square$ — $\square$, production of NH_3 ; $\triangle$ — $\triangle$, production of CO_2 .

catalyse the release of orthophosphate from ATP (Table 1, Expt. no. 1). However, under the experimental conditions chosen for the study of the citrulline phosphorylase reaction, i.e. excess of hexokinase and glucose, the hydrolytic destruction of ATP is markedly inhibited (Table 1, Expt. no. 2). Even greater inhibition is achieved by the addition of 5 μ moles ATP in 0.1 μ mole portions over a 30 min. period (Table 1, Expt. no. 3). Extracts of *Strep. lactis* also catalyse the release of orthophosphate from ADP (Table 1, Expt. no. 4). In the presence of hexokinase and glucose this does not occur (Table 1,

Expt. no. 5) but the concentration of glucose decreases (Table 2, Expt. no. 2). It is thus likely that ADP is not destroyed by hydrolysis but metabolized according to the reaction $2 \text{ ADP} \rightleftharpoons \text{AMP} + \text{ATP}$. Table 2, Expt. no. 1, indicates that the sample of ADP is sufficiently free of ATP for purposes of this test and the hexokinase is not contaminated with adenylate kinase. The small increase in orthophosphate which occurs in the absence of the hexokinase system (Table 1, Expt. no. 4) can be accounted for by hydrolysis of the ATP formed. It should be emphasized that the initial concentration

Table 1. Release of orthophosphate from ADP and ATP by extracts of *Streptococcus lactis*

Indicated additions were made to a test system which contained 0.1 ml. extract (4.5 mg. protein), 15 μ moles orthophosphate, 0.04 mole maleate buffer (pH 6.2), 20 μ moles MgCl_2 , and 10 μ moles iodoacetate. Total volume 1.1 ml.; incubated 30 min. Protein was precipitated with saturated $(\text{NH}_4)_2\text{SO}_4$. For definition of k.m. units hexokinase, see text p. 344.

Expt. no.	Additions	Orthophosphate in reaction mixture (μ moles)	Orthophosphate formed from ATP or ADP (μ moles)
1	ATP (5 μ moles)	26.81	4.97
	Without ATP	21.84	
2	ATP (5 μ moles), 56 k.m. units hexokinase, 50 μ moles glucose	22.74	0.75
	Without ATP	21.99	
3	Same as Expt. no. 2, but 5 μ moles ATP were added in 0.1 μ mole portions	22.17	0.21
	Without ATP	21.96	
4	ADP (5 μ moles)	22.73	0.89
	Without ADP	21.84	
5	ADP (5 μ moles), 56 k.m. units hexokinase, 50 μ moles glucose	21.84	0.00
	Without ADP	21.84	

Table 2. Conversion of ADP into ATP by extracts of *Streptococcus lactis*

Test system and conditions as described in Table 1. The amount of ATP formed is indicated by the decrease in glucose in the test system.

Expt. no.	Description of test system and additions	Glucose present at end of exp. (μ moles)	Decrease in glucose (μ moles)
1	Test system without extract, 5 μ moles ADP, 56 k.m. units hexokinase, 10 μ moles glucose	10.79	0.00
	Test system without extract or ADP	10.79	
2	Test system complete, 5 μ moles ADP, 56 k.m. units hexokinase, 10 μ moles glucose	8.47	2.32
	Without ADP	10.79	

Table 3. Recovery of glucose 6-phosphate

Indicated additions were made to a test system as described in Table 1.

Expt. no.	Additions to test system	Amounts present at the end of the reaction (μ moles)			
		Glucose	Fructose	G 6-P	Ortho-phosphate
1	G 6-P (6.5 μ moles)	0.34	—	6.7	21.9
	No G 6-P	0.24	—	0.4	21.9
2	G 6-P (6.5 μ moles)	—	0.26	—	—
	No G 6-P	—	0.26	—	—
3	G 6-P (6.5 μ moles) and ATP (7.5 μ moles)	—	0.27	—	29.4
	As above but no G 6-P	—	0.26	—	29.3

Table 4. *Determination of adenosine triphosphate*

Indicated additions were made to a test system described in Table 1. ATP was added in approximately 0.25 μ mole portions. The figures in parentheses give the decrease of glucose and increase of G 6-P, when ATP was added to the test system.

Expt. no.	Additions to test system	Glucose (μ moles)	G 6-P (μ moles)
1	Glucose (20 μ moles)	20.5	0.0
	Glucose (20 μ moles added after deproteinization)	20.5	0.0
2	Glucose (20 μ moles) and ATP (4.5 μ moles)	20.1	0.6
	As above but no ATP	20.5	0.1
		(0.4)	(0.5)
3	Glucose (20 μ moles) ATP (4.5 μ moles) and 56 K.M. units hexokinase	16.1	4.3
	As above but no ATP	20.5	0.1
		(4.4)	(4.2)

Table 5. *Stoichiometry of the citrulline phosphorylase reaction*

Test system as described in Fig. 1 except with 0.2 μ mole ADP. Total volume, 1.2 ml.; incubated 30 min. Experimental and control flasks were of identical composition except in that ADP and L-citrulline were added to the latter after the mixture was deproteinized. Citrulline and ornithine were determined on TCA filtrates and NH_3 on filtrates prepared with tungstic acid. Carbon dioxide was determined manometrically. The remaining compounds were determined on filtrates prepared as described in text. Values are in micromoles.

Compound	Initial	Final	Increase (+) or decrease (-)
Citrulline	24.99	21.09	-3.9
Orthophosphate	21.84	18.17	-3.7
Glucose	50.10	46.60	-3.5
Ornithine	0.0	3.8	+3.8
Carbon dioxide	0.2	3.8	+3.6
Ammonia	0.3	4.1	+3.8
Glucose 6-phosphate	0.1	3.7	+3.6

of ADP or ATP used to detect these activities greatly exceeds the concentration present at any moment during the citrulline phosphorylase reaction.

When G 6-P is incubated in a test system either in the presence or absence of ATP, neither glucose, fructose nor extra phosphate can be detected and recovery of the G 6-P is within experimental error (Table 3).

The data presented in Table 4, Expt. no. 3, show that ATP is satisfactorily determined in terms of either glucose or G 6-P. Some formation of G 6-P occurs in the absence of added hexokinase indicating that extracts of *Strep. lactis* contain a hexokinase system (Table 4, Expt. no. 2). The enzymic determination of G 6-P is specific; no compound present in the reaction mixture other than G 6-P gives a measurable O_2 uptake.

A complete chemical balance of products appears in Table 5. The data show that for each mole of citrulline degraded one mole of ATP is formed. The

analyses given represent the sum of the following equations:

(Citrulline phosphorylase enzyme)

(Hexokinase)

Sum of reactions 1 and 2:

DISCUSSION

The results presented in this paper make it possible to write a balanced equation for the degradation of L-citrulline by cell-free extracts of *Strep. lactis*. Of possible importance for the formulation of a mechanism is the observation that the ratio of ammonia (or carbon dioxide):phosphorus remains essentially constant with time. This may be interpreted to indicate that the carbamyl group of citrulline is split-off by a phosphoroclastic type of reaction. The products of the phosphoroclastic reaction are released from the enzyme surface simultaneously with the transfer of high-energy phosphate to a suitable acceptor. The nature of the immediate phosphate acceptor is not certain, although the evidence so far available indicates that it is ADP. Adenosine 5-phosphate can replace ADP. However, the presence of an adenylate kinase system does not permit a clear interpretation of its role.

SUMMARY

1. A complete chemical balance of products of the citrulline phosphorylase reaction of *Strep. lactis* is presented.

2. The data show that one mole of orthophosphate is required to degrade an equivalent of citrulline to ornithine, carbon dioxide and ammonia. An equivalent of ATP is synthesized.

3. Extracts of *Strep. lactis* release orthophosphate from ATP but not from ADP. The presence of an adenylate kinase system is indicated.

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A Simple Method for the Preparation of Horseradish Peroxidase

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Of the two available methods for the preparation of highly purified horseradish peroxidase, that of Theorell (1942) involves the use of electrophoresis apparatus, and there are many steps in that of Keilin & Hartree (1951). A simpler method has now been devised which avoids the use of electrophoresis and considerably reduces the number of fractionation procedures. The method depends on the removal of much inactive material from the crude extract by precipitation with a mixture of chloroform and ethanol (the reagent of Tsuchihashi, 1923). Following this treatment, highly purified peroxidase preparations have been obtained by fractional precipitations with ammonium sulphate and ethanol. The product obtained is a stable dry preparation which can be crystallized from ammonium sulphate solution.

EXPERIMENTAL

Methods

Activity of the peroxidase preparations. The 'Purpurogallinzahl' (P.Z.) (Willstätter & Stoll, 1918) of the preparations, i.e. mg. of purpurogallin formed by 1 mg. of the enzyme preparation in 5 min. from pyrogallol and H_2O_2 under fixed conditions, was estimated by the method of Keilin & Hartree (1951) using an EEL (Evans Electro-selenium Ltd.) colorimeter with Ilford Bright Spectrum Blue filter 622.

Peroxidase units. The number of peroxidase units (E.U.) in the various fractions and final preparations is given by the total weight of purpurogallin (g.) which these would give under the conditions of the above method.

Dry weights. Samples were dried to constant weight at 105° .

Absorption data. These were obtained using the Unicam Model S.P. 500 quartz spectrophotometer with 1 cm. cells. **Haemin.** The haemin content of the peroxidase preparations was estimated at a concentration of 0.4 mg. preparation/ml. by measuring the light absorption at 557 m μ . after conversion into pyridine haemochromogen (Paul, Theorell & Åkeson, 1953). The value for the molar absorption of the α -band of the pyridine ferroprotoporphyrin (8.0×10^7 cm.² mol.⁻¹) given by Paul *et al.* (1953) was used for calculation of the haemin content.

Isolation of peroxidase from horseradish root

Preparation of crude extract. Roots of wild horseradish (*Cochlearia armoracia* L.) were gathered in spring when interference by root polysaccharides during the fractionation is at a minimum (Keilin & Hartree, 1951). Washed root (5 kg.) was cut into small pieces and put through a large domestic meat mincer. The mince was mixed thoroughly with 7 l. of water and then macerated in batches in a Waring Blender. Each batch was macerated for 2 min. The macerate was squeezed by hand in strong cotton cloth and the extract (9 l.) was stored overnight at $0-2^\circ$ (P.Z. 1.1; E.U. 598).

Treatment with ethanol:chloroform. A 2:1 (v/v) ethanol:chloroform mixture (2.7 l.) was cooled to 0° and added to the extract at 0° with brisk mechanical stirring. The stirring was continued for 20 min. The mixture was then centrifuged at 3000 rev./min. for 15 min. The clear yellow supernatant (10.42 l.; E.U. 537) was adjusted to pH 5.5 with 2N-NaOH and concentrated by distillation under reduced pressure to 2.18 l. (E.U. 488). The temperature of the solution was kept below 30° and the distillation rate was about 0.9 l./hr.

Fractional precipitation with ammonium sulphate. The relatively inactive precipitate obtained by adding $(\text{NH}_4)_2\text{SO}_4$ (250 g./l. concentrate) was centrifuged off and discarded (E.U. 29). A further 250 g. of $(\text{NH}_4)_2\text{SO}_4$ for each litre of original concentrate was added to the supernatant. The suspension was kept overnight in the cold room ($0-2^\circ$) and then the precipitate, which contained most of the