Phosphorylation Coupled with Anaerobic Breakdown of Citrulline

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It has recently been demonstrated that preparations obtained from various micro-organisms are capable of converting citrulline into ornithine, ammonia and carbon dioxide. This reaction has been found in *Streptococcus faecalis* treated with toluene (Akamatsu & Sekine, 1951), or acetone (Oginsky & Gehrig, 1952), in freeze-dried cells of *Clostridium perfringens* (Schmidt, Logan & Tytell, 1952) and cell-free extracts of *Streptococcus lactis* (Korzenovsky & Werkman, 1952). With regard to the mechanism of the reaction, it is relevant that washed suspensions of *Strep. faecalis*, treated with cetyltrimethylammonium bromide do not attack citrulline until small amounts of adenosine diphosphate (ADP) or a larger quantity of adenosine triphosphate (ATP) is added (Knivett, 1952*a, b*); that cell-free extracts require inorganic phosphate and either muscle adenylate acid (AMP), ADP or ATP (Slade, 1953); and that citrulline breakdown by cell-free extracts is accompanied by the disappearance of inorganic phosphate together with the phosphorylation of ADP to ATP (Knivett, 1952*c*, 1953). Preliminary investigations in Cambridge (Knivett, 1952*c*) were concerned with the detection and isolation of ATP accompanying the breakdown of citrulline; whilst the present paper is concerned with a more detailed study of the phosphorylation of ADP to ATP. A procedure employing ^{32}P has been used which has the advantage of being independent of the action of dephosphorylating enzymes, and of making it possible to distinguish between ATP formed by the action of adenylate kinase (myokinase; see Colowick, 1951) and synthesis from ADP and inorganic phosphate.

METHODS

Preparation of the cell-free extract. *Strep. faecalis* (ST), National Collection of Type Cultures no. 6782, was grown in a medium containing 1.5% (w/v) L-arginine HCl, 1.0% (w/v) yeast autolysate (Marmite), 0.5% (w/v) tryptic digest of casein, 0.1% (w/v) K_2HPO_4 , 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% (w/v) $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, and 1.0% (w/v) glucose (sterilized separately). Satisfactory yields (5 mg. dry wt./ml.) of cells were obtained after incubation for 16 hr. at 30°.

The cells from 200 ml. of medium were harvested on the

Sorvall high-speed angle head centrifuge (Ivan Sorvall Inc., New York) at 20 000 *g* for 10 min. They were washed twice by resuspension in the same volume of distilled water and re-centrifuged.

The cells were disintegrated by shaking with glass beads (Ballotini no. 12, Chance Bros., Birmingham) at 50 cyc./sec. in the Mickle Sonic Disintegrator (Mickle, 1948). The cups containing 10 ml. of Ballotini beads and 10 ml. of a washed suspension of cells (approx. 20 mg. dry wt./ml.), were cooled at 0° and shaken for 15 min. The debris was filtered through a sintered glass funnel and the extract centrifuged for 20 min. at 20 000 *g* to remove unbroken cells and cell-wall debris. The clear supernatant was stored at -14° until required. The protein content of the extract was measured by the method of Kalkar (1947).

Adenosine diphosphate (ADP) and triphosphate (ATP). The barium salt of ATP was prepared from rabbit muscle according to the directions of LePage (1949). Barium ADP was supplied by Schwarz Laboratories Inc., 202 East 44th Street, New York 17. The barium compounds were dissolved in dilute HCl and barium was precipitated by the addition of the calculated quantity of 0.5 *M* Na_2SO_4 . After removal of the precipitate by centrifugation, the solutions were adjusted to pH 5.8 with NaOH. About 20% of the ADP and ATP was lost by adsorption on the precipitate (Bailey, 1949). The ultraviolet technique of Markham & Smith (1949) did not reveal any AMP or ATP in the chromatogram of the ADP preparation. The ATP preparation contained about 15% ADP, but contained no detectable quantities of AMP.

$\text{KH}_2^{32}\text{PO}_4$. Carrier-free $\text{KH}_2^{32}\text{PO}_4$ was obtained from the Atomic Energy Research Establishment at Harwell. The sample had an activity of about 1 mc and was diluted with stock saline to 10 ml. The radioactivity of the incubation mixture was about 2–3 $\mu\text{C}/\text{ml}$. solution.

Incubation mixtures. The incubation mixtures contained 0.3 ml. enzyme solution (35.2 mg. protein/ml.), 0.8 ml. 0.5 *M* potassium succinate buffer (pH 5.8), 0.1 ml. 0.12 *M* MgCl_2 , 0.2 ml. 0.09 *M* Na_2HPO_4 , 0.6 ml. 0.3 *M* L-citrulline, 0.1 ml. $\text{KH}_2^{32}\text{PO}_4$ (activity about 0.1 mc/ml.), and 0.9 ml. 0.016 *M* ADP or ATP. The CO_2 produced from citrulline was measured manometrically in a parallel sample in which $\text{KH}_2^{32}\text{PO}_4$ was omitted. Non-isotopic phosphate was also used for a control containing ATP but no citrulline.

The incubations were performed in air at 25°. ADP or ATP were added last. This initiated the reaction. Samples (0.5 ml.) were withdrawn at intervals, mixed with 30% (w/v) trichloroacetic acid (0.05 ml.) and stored at -14° until they were analysed. An initial sample withdrawn immediately after the addition of ADP and ATP.

Chromatography. Adenosine phosphates and inorganic phosphate were separated by the paper chromatographic methods of Hanes & Isherwood (1949) as modified by

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Eggleston & Hems (1952) and Krebs & Hems (1953). The second solvent consisted of 100 ml. *isobutyric acid*, 60 ml. $\text{n-NH}_4\text{OH}$, and 1.6 ml. 0.1M ethylenediaminetetraacetic acid. The solvent was allowed to descend for 16 hr. at room temperature. The phosphate determinations and radioactivity measurements were carried out in duplicate according to the methods of Eggleston & Hems (1952). Suitable volumes (10 μl .) were applied to the paper with the Agla micrometer syringe (Burrhoughs Wellcome and Co.) in quadruplicate. A separate control mixture of adenosine and inosine mono-, di-, and tri-phosphates was applied to assist identification of the phosphate fractions.

Interpretation of specific activity data. For the purpose of the following considerations the three adenosine phosphates are represented by the symbols AP, APP and APPP, and phosphate by the symbol P. Isotopic phosphorus is indicated by an asterisk. The ratio specific activity of adenosine phosphate/specific activity of phosphate is referred to as 'relative specific activity', of the adenosine phosphate, the specific activity being expressed in counts/min./m μ . mole.

If APP is phosphorylated by P* according to the reaction

and if no APPP is present initially the APPP* formed has the same specific activity as P*; the relative specific activity of APPP* is 1. If adenylate kinase is present APPP can also be formed without the participation of P:

The APPP formed by this reaction dilutes the APPP* formed by reaction (1) so that its relative specific activity is less than 1. When reaction (2) proceeds from right to left and if APPP* is the reactant APP* is formed. Radioactivity in APP therefore suggests the presence of adenylate kinase in the enzyme preparation, although the possibility that AP is phosphorylated directly by P* cannot be excluded. Phosphorylation of APP* by reaction (1) would yield APP*P*, and in this case the relative specific activity of APPP would be greater than 1.

RESULTS

The following substrate combinations were incubated: (1) citrulline, (2) ADP, (3) citrulline and ADP and a parallel series in which ATP (contaminated with about 15% ADP) replaced ADP. The evolution of carbon dioxide and the changes in the quantities and radioactivities of the various phosphate fractions were measured. Representative results are shown in Table 1. Citrulline alone was not changed in 60 min. At the same time a mixture containing ADP and citrulline released carbon dioxide, formed ATP and removed phosphate and ADP, the four changes being of the same order of magnitude though not exactly equivalent. For example, at 11.5 min. the changes are as follows: carbon dioxide, +1.11; phosphate, -1.85; ADP, -1.14; ATP, +1.29 $\mu\text{moles/ml}$. Some AMP was formed after 6 min. but partly disappeared later whilst the concentration of ATP increased. After 11.5 min. there was relatively little change in the concentrations of the various phosphate fractions, although carbon dioxide formation continued.

A steady state with regard to the concentrations of adenosine phosphates had been reached where the phosphorylating enzymes synthesizing ATP compensated the reaction of the dephosphorylating enzymes. This follows from the radioactivity data. The radioactivity of inorganic phosphate continually fell whilst that of ATP rose. The incorporation of isotopic phosphate into ATP steadily continued in spite of a decreased rate of accumulation of ATP.

The presence of adenylate kinase and the absence of adenosine diphosphatase in the enzyme preparation is shown by the controls from which citrulline had been omitted. When ADP alone was incubated, its disappearance was accompanied by the appearance of AMP and ATP, whilst the concentration and specific activity of inorganic phosphate remained unchanged. The labile phosphate fraction contained only traces of ^{32}P . Incubation of ATP alone caused an appearance of AMP and ADP and inorganic phosphate. This can be accounted for by the combined effects of adenosine triphosphatase and adenylate kinase. The specific activity of ADP increased steadily during incubation and was always much less than that of ATP. This observation suggests that the radioactivity was due to reaction (2) proceeding from right to left under the influence of adenylate kinase, and it is unlikely that AMP was phosphorylated directly by inorganic phosphate. In the earlier stages of incubation the relative specific activity of ATP was about 0.5. This suggests that half of the ATP was formed by the phosphorylation of ADP by isotopic phosphate and the remainder by the action of adenylate kinase (reaction 2). This would account for the formation of AMP, in the absence of adenosine diphosphatase. After 90 min. incubation, the relative specific activity of ATP had not exceeded two-thirds of the theoretical value of 2 (in the presence of adenylate kinase). This observation suggests that either adenylate kinase was rapidly destroyed or that small amounts were present. Eggleston & Hems (1952) give a value of 0.444 for the equilibrium constant, K , of the adenylate kinase system at 25°. After 75 min. incubation of the ATP preparation, a value of 0.32 for K was obtained, which suggested that an equilibrium state had not been reached. The presence of adenylate kinase has been reported in yeast (Kotel'nikova, 1949) but has not previously been reported in bacteria.

In the samples to which both citrulline and an ATP preparation containing about 15% ADP were added, the rate of carbon dioxide formation from citrulline was slower than when ADP was used, and no major changes in the amounts of adenosine phosphates occurred. No AMP was detected in these samples, in contrast to those containing ADP and citrulline or to those controls containing ATP

Table 1. *Changes in the phosphate fractions on incubation of cell-free extracts of Streptococcus faecalis with citrulline and adenosine di- and tri-phosphates*

For full details of conditions see text. The sample of ATP contained about 15% ADP. CO₂ measured manometrically (not corrected for retention). Relative specific activity of ADP and ATP is the ratio: specific activity of ADP or ATP/specific activity of phosphate.

Substrate added	...	...	ADP + Citrulline				ADP		ATP + Citrulline				ATP		Citrulline 60	
			1	6	11.5	88	11	90	1	6.5	16	35	76	1		75
Period of incubation (min.)																
Quantities of phosphates (μ moles/ml. incubated solution):																
Inorganic phosphate	6.43		5.36	4.58	4.26	6.25	6.11	7.00	—	7.14	—	6.78	7.42	9.05	—	
AMP	0.0		1.10	0.28	0.50	1.28	1.53	0.0	0.0	0.0	0.0	0.0	0.0	0.25	0.0	
ADP	2.48		1.76	1.34	1.12	1.92*	1.12	0.37	—	0.46	—	0.67	0.78	1.05	0.0	
ATP	0.31		0.97	1.60	2.39	0.55	0.77	2.67	—	2.87	—	2.79	2.58	1.41	0.0	
Radioactivity (% of total):																
Inorganic phosphate	97.9		92.3	82.6	53.7	99.8	99.3	98.5	94.7	88.0	82.8	75.9	No isotope added	100		
ADP	0.1		0.8	1.6	7.5	0.1	0.1	0.05	0.4	0.6	1.0	2.0	—	—	0	
ATP	2.0		6.9	14.7	37.0	0.1	0.4	1.5	4.9	10.6	12.7	20.8	—	—	0	
Other phosphates	0.0		0.0	1.1	1.8	0.0	0.2	0.0	0.0	0.8	3.5	1.3	—	—	0	
Specific activity (counts/min./ μ m. mole):																
Inorganic phosphate	80		91	103	70	87	90	80	—	67	—	64	—	—	—	
ADP	0.4		2.4	7.1	38.0	—	0.7	0.9	—	7.1	—	15.7	—	—	—	
ATP	33.6		37.9	54.5	87.1	0.9	2.7	3.2	—	20.1	—	40.0	—	—	—	
Relative specific activity:																
ADP	0.005		0.026	0.070	0.540	—	0.008	0.011	—	0.106	—	0.262	—	—	—	
ATP	0.42		0.42	0.53	1.24	0.01	0.03	0.04	—	0.30	—	0.67	—	—	—	
CO ₂ evolved (μ moles/ml. incubated solution)	—		0.6	1.1	4.06	0.0	0.0	—	0.4	1.1	2.0	3.6	0.0	0.0	0.0	

* Sample lost, estimated by difference.

but no citrulline. The amount of AMP present was calculated, assuming a value of 0.444 for the equilibrium constant, K , of adenylate kinase (Eggleson & Hems, 1952). The calculated amount of AMP present after 16 and 76 min. incubation was 0.03 and 0.07 μ mole/ml., respectively.

Although there were no major net changes, ^{32}P appeared in ATP and ADP. After 75 min. incubation, 20% of the radioactivity was present in ATP and 2% in ADP. The rate of incorporation was slower than in the corresponding samples to which ADP was added, one-third of the maximal incorporation being attained after 75 min. The presence of adenosine triphosphatase was confirmed by the fall in specific activity of the phosphate.

The radioactivity of AMP was never significantly greater than the background radiation. In the later stages of incubation, an unidentified radioactive substance appeared on the chromatogram travelling in a position between inosine di- and tri-phosphate. The ultraviolet absorption of this substance was indistinct, and when sprayed with molybdate reagent gave a darker shade of blue than the other phosphates. The phosphorus content of the spot was too small to be measured accurately, so that the substance must have had a high specific activity. It may have been either glucose 1-phosphate or glucose 6-phosphate formed by the interaction of traces of glucose in the enzyme preparation and radioactive ATP synthesized during the reaction. Hexokinase is known to be present in the extracts.

Chromatograms of incubation mixtures which contained citrulline and radioactive phosphate, but no ADP or ATP, were examined for the presence of a phosphorylated derivative of citrulline; only phosphate was detected, and all the radioactivity was located in this spot.

DISCUSSION

The results show that the breakdown of citrulline to ornithine is accompanied by the phosphorylation of ADP to ATP in approximately stoichiometric proportion. The overall change in the decomposition of citrulline may be represented by the scheme

Experimentally the expected stoichiometric relations are not strictly realized because other reactions, especially those catalysed by adenosine triphosphatase and adenylate kinase, introduce complicating factors. The steady incorporation of ^{32}P into the labile-phosphate fraction of the adenosine polyphosphate shows that ATP is continually formed, though its concentration does not rise.

The number of known substrate reactions which are coupled with the synthesis of ATP is small. Two occur in the course of the alcoholic and lactic acid

fermentations of glucose and a third is the oxidative dephosphorylation of α -keto acids (see Krebs, 1953). The conversion of citrulline into ornithine is the fourth substrate reaction of this type to be described.

The decomposition of citrulline under the experimental conditions, i.e. on the acid side near the neutral point may be formulated as follows:

The change of free energy of this reaction cannot be calculated owing to lack of basic data, but it is likely that it is not very different from that of the analogous reaction of urea

The free energy of formation under standard conditions (ΔG°_f) of urea (solid) at 25° is -47.1 kcal. (Ruehrwein & Huffman, 1946). On the basis of the data of Borsook & Huffman (1938) for the calculation of ΔG°_f of aqueous urea, a value of -48.7 is obtained. With this value, and the data for water, NH_4^+ (aq.), CO_2 (gas) given by Burton & Krebs (1953) ΔG of reaction (3) at 25° is -7.8 kcal. at pH 7 and -10.5 kcal. at pH 6. Under conditions more closely approximating to those of experiments (CO_2 pressure, 0.05 atm.; concentrations of urea and NH_4^+ , 0.01M), ΔG becomes considerably higher, namely -12.1 kcal. at pH 7 and -14.9 kcal. at pH 6. Under many physiological conditions, the concentration of NH_4^+ is likely to be much lower still, which would mean a higher ΔG value for reaction (3). These considerations indicate that sufficient energy is released in this type of reaction to make a synthesis of one pyrophosphate bond of ATP possible.

The coupling between the breakdown of citrulline and the synthesis of ATP appears to be obligatory, because citrulline is not broken down in the absence of phosphate and ADP, although arsenate can replace both ADP and phosphate (Knivett, 1954). It follows that unlike the hydrolysis of urea, the breakdown of citrulline is not a 'simple' hydrolysis.

SUMMARY

1. The phosphorylation of ADP to ATP coupled with the breakdown of citrulline to ornithine by a cell-free extract of *Streptococcus faecalis* has been studied quantitatively.

2. A mixture containing citrulline and ADP released CO_2 , formed ATP, and removed ADP and inorganic phosphate, the four changes being approximately equivalent. The results agree with the view that the overall change is

3. [^{32}P]Potassium dihydrogen phosphate was used to distinguish between ATP formed from ADP by the action of adenylate kinase (myokinase) and by synthesis involving inorganic phosphate. The appearance of ^{32}P in ADP, and in both of the labile phosphates of ATP, but not in AMP, indicates the presence of adenylate kinase in extracts of *Strep. faecalis*.

4. The fission of citrulline according to the reaction formulated in paragraph 2 represent a new type of coupled phosphorylation leading to a formation of pyrophosphate bonds of ATP.

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REFERENCES

- Akamatsu, S. & Sekine, T. (1951). *J. Biochem., Tokyo*, **38**, 349.
- Bailey, K. (1949). *Biochem. J.* **45**, 479.
- Borsook, H. & Huffman, H. M. (1938). *Chemistry of the Amino Acids and Proteins*, p. 860. Ed. Schmidt, C. L. A. Springfield: Charles C. Thomas.
- Burton, K. & Krebs, H. A. (1953). *Biochem. J.* **54**, 94.
- Colowick, S. P. (1951). *The Enzymes: Chemistry and Mechanism of Action*, vol. 2, pt. 1, p. 114. Eds. Sumner, J. B. & Myrback, K. New York: Academic Press.
- Eggleson, L. V. & Hems, R. (1952). *Biochem. J.* **52**, 156.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 461.
- Knivett, V. A. (1952a). *Biochem. J.* **50**, xxx.
- Knivett, V. A. (1952b). *2nd Int. Congr. Biochem., Abstr.*, p. 86.
- Knivett, V. A. (1952c). Ph.D. Thesis, Cambridge University.
- Knivett, V. A. (1953). *J. gen. Microbiol.* **8**, v.
- Knivett, V. A. (1954). *Biochem. J.* **56**, 606.
- Korzenovsky, M. & Werkman, C. H. (1952). *Arch. Biochem. Biophys.* **41**, 233.
- Kotel'nikova, A. V. (1949). *C.R. Acad. Sci. U.R.S.S.* **69**, 837. In *Chem. Abstr.* (1950), **44**, 4528c.
- Krebs, H. A. (1953). *Brit. med. Bull.* **9**, 97.
- Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
- LePage, G. A. (1949). *Biochem. Prep.* **1**, 5.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Mickle, H. (1948). *J. R. micro. Soc.* **68**, 10.
- Oginsky, E. L. & Gehrig, R. F. (1952). *J. biol. Chem.* **198**, 791.
- Ruehrwein, R. A. & Huffman, H. M. (1946). *J. Amer. chem. Soc.* **68**, 1759.
- Schmidt, G. C., Logan, M. A. & Tytell, A. A. (1952). *J. biol. Chem.* **198**, 771.
- Slade, H. D. (1953). *Arch. Biochem. Biophys.* **42**, 204.

The Effect of Arsenate on Bacterial Citrulline Breakdown

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It has previously been shown (Knivett, 1952a, b; see also Slade, 1953, and Korzenovsky & Werkman, 1953) that arsenate increases the rate at which carbon dioxide is released from arginine and citrulline by enzyme preparations of *Streptococcus faecalis*. The present paper is a detailed study of the arsenate effect.

EXPERIMENTAL

The organism, growth medium, preparation of suspensions, and of the cell-free extract (prepared by the Mickle disintegrator), adenosine diphosphate (ADP) and triphosphate (ATP) were as previously described (Knivett, 1954).

Preparation of cell-free extracts. In addition to the extract prepared with the Mickle disintegrator (designated 'M' extract), a cell-free extract having a greater enzymic

activity was prepared by crushing the cell-paste in the press described by Hughes (1951). The mucoid, crushed mass was taken up with water and centrifuged for 20 min. at 20000 g in a Sorvall high-speed angle head centrifuge (Ivan Sorvall Inc., New York). This extract is designated the 'P' extract.

Manometric methods. The evolution of CO_2 during citrulline breakdown was measured in Warburg manometers with conventional techniques. The activity of the washed cells was determined with arginine as substrate, as citrulline is attacked very slowly by intact cells. The rate of evolution is expressed as Q_{CO_2} ($\mu\text{l. CO}_2$ liberated/hr./mg. dry wt. of cells) calculated from the steady rate of gas output established after the first few minutes.

Experiments with cell-free extracts. Cell-free extracts were incubated at 37° with citrulline and a buffer of pH 5.8 (both sodium succinate and sodium arsenate buffers were used—see legends to tables); where necessary, CO_2 liberation was followed manometrically in parallel experiments. At suitable time intervals samples were removed and prepared for analysis. For the estimation of citrulline and ammonia a

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