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.

I wish to thank Dr E. F. Gale, F.R.S., for his generous help and encouragement, and Prof. H. A. Krebs, F.R.S., for his helpful advice and interest in this work. I am grateful to Mr R. Hems for advice and technical assistance, to the Medical Research Council for a research training grant, and to the Director, Chemical Research Laboratory, Teddington, for permission to undertake this investigation.

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The Effect of Arsenate on Bacterial Citrulline Breakdown

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It has previously been shown (Knivett, 1952*a*, *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} (μl. 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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1·0 ml. sample of the reaction mixture was transferred to 4 ml. 0·25 N-HCl; 0·5 ml. of the acidified mixture was used for the estimation of citrulline and 1·0 ml. for the estimation of ammonia. For the estimation of ornithine a 2·0 ml. sample was pipetted into 0·5 ml. N-HCl, warmed to 80° for 5 min., transferred quantitatively to a manometer flask and the pH was adjusted to 5·8 with N-NaOH.

Estimation of ammonia. The Conway micro-diffusion method was used for the distillation of the ammonia (Conway, 1950) which was then estimated by nesslerization.

Estimation of ornithine. Ornithine was estimated by the specific decarboxylase method of Gale (1945). The organism used was a strain of *Escherichia coli* (EC4) kindly supplied by Dr H. D. Slade.

Estimation of citrulline. The carbamido/diacetyl reaction is a sensitive test for citrulline (Fearson, 1939; Gornall & Hunter, 1941; Archibald, 1944), but does not give very reliable quantitative results. Kawerau (1946) found that colour fading occurs owing to over-oxidation and could be avoided by the use of (*a*) a mild oxidizing agent (arsenious oxide in conc. HCl instead of potassium persulphate), (*b*) an excess of diacetyl monoxime, (*c*) a boiling time of 15 min. Under these conditions neither colour fading nor light sensitivity was noted in 30 min. On testing Kawerau's method, however, considerable differences in colour intensity were noted between different sets of estimations. This was traced to the variations in the time elapsing between mixing the reagents with citrulline and boiling the solutions to develop the colour. The amount of colour produced was approximately doubled if the reagents were allowed to stand overnight at room temperature before boiling. Previous workers have noted that the colour produced is not proportional to the concentration of citrulline and that standards must be included.

The calibration curves did not pass through the origin, but cut the abscissa at an intercept of approximately 0·2 μmole citrulline. Therefore 0·25 μmole citrulline was added to each determination to ensure that sufficient colour would be produced to be measurable, and a correction was applied. A typical calibration curve had a slope of $\log I_0/I = 1{\cdot}040$ per μmole citrulline in 15 ml. at 492 mμ. with a 1 cm. light path. The method gave reliable results over a range 0–1·25 μmole. Reagents: 0·25 ml. 0·001 M L-citrulline; 3·75 ml. arsenious oxide (10%, w/v, in conc. HCl); 2·0 ml. diacetyl monoxime (1%, w/v, in 5%, v/v, acetic acid). The combined reagents (6·0 ml.) were added from a burette to 1·0 ml. of the test solution (containing not more than 1·25 μmoles citrulline) and kept overnight for the colour to develop. The covered tubes were heated in a boiling-water bath with frequent shaking for 15 min., cooled and distilled water was added to 15 ml. The colour produced was estimated in the Beckman Spectrophotometer at 492 mμ.; twelve estimations and four standards containing known amounts of citrulline were dealt with in a batch.

Estimation of protein. The protein content of the extracts was measured by the method of Kalckar (1947).

RESULTS

Effect of arsenate on the breakdown of arginine in washed bacterial suspensions. Table 1 illustrates the striking effect of arsenate on the rate of carbon dioxide formation from arginine by washed suspensions of *Strep. faecalis*. At a concentration of 0·33 M arsenate increased the rate more than fivefold and at 0·008 M about twofold. Phosphate (0·33 M) only increased the rate by about 25%. Addition of phosphate in the presence of arsenate reduced the stimulating effect of arsenate.

Effect of arsenate on the breakdown of citrulline in cell-free extracts. As previously shown (Knivett, 1954), citrulline is not attacked at an appreciable rate by cell-free extracts unless both ADP and inorganic phosphate are added. Table 2 shows that phosphate can be replaced by arsenate, and that the effect of higher concentrations of arsenate (0·016 M) is reduced by the addition of phosphate. In contrast to phosphate, arsenate stimulated the formation of carbon dioxide when no ADP has been added

Table 1. *The effect of arsenate and phosphate upon the release of* CO_2 *from arginine by washed suspensions of* Streptococcus faecalis

Total volume of fluid per Warburg cup, 3 ml. All cups contained 1·0 ml. M buffer (sodium phosphate, arsenate or phthalate), pH 5·8; 1·0 ml. of a washed suspension of cells (containing 2·34 mg. dry wt./ml.); and in the side compartment 0·5 ml. 0·03 M L-arginine HCl. Additions to the main compartment: 0·25 ml. 0·1 M sodium arsenate or sodium phosphate buffer, pH 5·8; water to volume. Gas: air. Temp. 37°.

	Final concentration of		
Main buffer	Phosphate (M)	Arsenate (M)	Q_{CO_2}
Phthalate	0	0	96
Phosphate	0·33	0	120
Arsenate	0	0·33	516
Phthalate	0·008	0	85
Phthalate	0	0·008	186
Phthalate	0·008	0·008	122

Table 2. *The effect of phosphate and arsenate upon the release of* CO_2 *from citrulline in a cell-free extract in the presence of ADP*

Total volume of fluid per cup, 3 ml. All cups contained 0·5 ml. 0·5 M potassium succinate buffer, pH 5·8; 0·1 ml. 0·12 M-$MgCl_2$; 0·9 ml. 0·008 M ADP; 0·6 ml. 0·3 M L-citrulline; and in the side compartment 0·3 ml. 'M' extract (35·2 mg. protein/ml.). Additions to the main compartment: varying quantities of 0·12 M sodium arsenate buffer, pH 5·8, and 0·18 M sodium phosphate buffer, pH 5·8; water to volume. Gas: air. Temp. 30°.

Expt. no.	Incubation time (min.)	Final concentration of Phosphate (M)	Final concentration of Arsenate (M)	CO_2 released (μmoles)
1	34	0	0	1·6
		0·006	0	8·1
		0	0·016	18·6
		0·006	0·016	12·6
		0·012	0·016	11·7
2	21	0	0	1·3
		0	0·004	7·2
		0	0·008	12·2
		0	0·016	16·3

(Table 3). This effect of arsenate is reduced by the addition of either phosphate, or fluoride, or iodoacetate.

The following experiment shows that the inhibition by phosphate is competitive. The rate of carbon dioxide production was measured over a range of arsenate concentrations, with and without phosphate. The reciprocal of the initial velocity was plotted against the reciprocal of arsenate concentration according to the method of Lineweaver & Burk (1934) and the line of closest fit calculated by the method of least squares (Fig. 1). The Michaelis constant (K_m) was 0·022 M in the absence of added phosphate. When phosphate (0·0067 M) was added, the apparent Michaelis constant was 0·093 M. The fourfold increase of apparent Michaelis constant on the addition of phosphate indicates that the inhibition is competitive. The rate of carbon dioxide release was measured over a range of citrulline concentrations (0–0·04 M) (Fig. 2). The Michaelis constant for citrulline in the presence of 0·029 M arsenate was 0·12 M.

Table 3. *Release of CO_2 from citrulline in a cell-free extract in the presence of arsenate (no adenosine phosphate added)*

Total volume of fluid per cup, 3 ml. All cups contained 0·5 ml. 0·5 M potassium succinate buffer, pH 5·8; 0·1 ml. 0·12 M-$MgCl_2$; 0·3 ml. 'M' extract (11·7 mg. protein/ml.), and in the side compartment 0·6 ml. 0·3 M L-citrulline. Other reagents added to side compartment. Gas: air. Temp. 30°. Incubation time 1 hr.

Additions	CO_2 released (μmoles)
None	0·0
Arsenate (0·004 M)	7·9
Arsenate (0·008 M)	12·0
Arsenate (0·008 M); phosphate (0·012 M)	3·6
Arsenate (0·008 M); NaF (0·01 M)	3·2
Arsenate (0·008 M); iodoacetate (0·01 M)	8·2

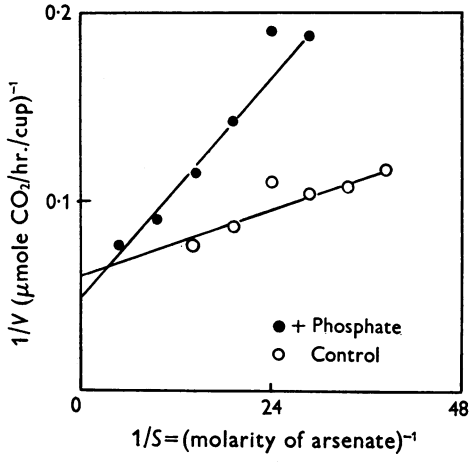

Fig. 1. Competitive inhibition by phosphate of the release of CO_2 from citrulline in the presence of arsenate. Total volume of fluid per cup, 1·5 ml. Main compartment: 0·3 ml. 0·5 M potassium succinate buffer, pH 5·8; 0·3 ml. 0·3 M L-citrulline; 0·5 ml. sodium arsenate buffer (0·026–0·208 M), pH 5·8; 0·1 ml. 0·1 M potassium phosphate buffer, pH 5·8, or water; and in the side compartment: 0·3 ml. of a mixture of 1·3 ml. 0·12 M-$MgCl_2$, 2·4 ml. 0·5 M potassium succinate buffer pH 5·8, and 0·2 ml. 'P' extract (4·6 mg. protein/ml.). Gas: air. Temp. 37°. CO_2 release was measured at 5 min. intervals after tipping, and the velocity calculated from the steady gas output in the first 20 min.

Products of citrulline breakdown in the presence of arsenate. Estimation of the amount of citrulline disappearing and the amount of ornithine, ammonia and carbon dioxide formed shows that the changes in the amounts of the four reagents are approximately equivalent (Table 4). The overall reaction of citrulline in the presence of arsenate is thus the same as in the presence of ADP and phosphate. The breakdown of citrulline was very rapid at first, but as would be expected from the high values of K_m for arsenate and citrulline, the rate of the reaction slowed down as the concentration of citrulline fell.

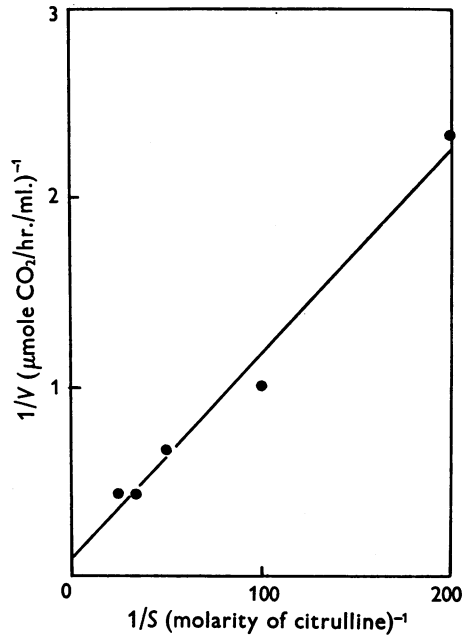

Fig. 2. The effect of citrulline concentration on the rate of release of CO_2 from citrulline in the presence of arsenate. Total volume of fluid per cup, 3 ml. All cups contained 1·0 ml. 0·5 M potassium succinate buffer, pH 5·8; 0·1 ml. 0·12 M-$MgCl_2$; 0·1 ml. 0·875 M sodium arsenate buffer, pH 5·8; 0·5 ml. 'P' extract (0·3 mg. protein/ml.); and in the side compartment varying quantities of 0·3 M L-citrulline, and water to volume. Gas: air. Temp. 37°. The rate of CO_2 evolution was constant during the first 100 min.

Table 4. *The products of citrulline breakdown in the presence of arsenate*

18 ml. M sodium arsenate buffer pH 5·8, 3·6 ml. 'P' extract, 3·6 ml. 0·12 M-$MgCl_2$, and 10·8 ml. 0·33 M L-citrulline. The reaction started after thermal equilibration by the addition of citrulline. Temp. 37°. For analytical procedures see methods. The ornithine samples were taken 0·5 min. later than the citrulline and ammonia samples. Quantities are corrected for control without citrulline.

Time (min.)	CO_2 released (μmoles/ml.)	NH_3 formed (μmoles/ml.)	Ornithine formed (μmoles/ml.)	Citrulline found (μmoles/ml.)	Citrulline removed (μmoles/ml.)
6·5	1·50	1·46	1·50	7·96	2·04
18·5	3·50	3·41	3·48	6·36	3·64
25·5	4·35	4·35	4·05	5·71	4·29
33·0	5·15	5·39	5·04	4·84	5·16
85·0	8·00	—	7·99	1·88	8·12
150·0	9·36	9·40	8·99	0·36	9·64
		Control without citrulline			
150·0	0·0	0·55	0·52	0·44	—

DISCUSSION

Accelerating effects of arsenate on enzymic reactions were first described by Harden & Young (1911), who found an increased rate of alcoholic fermentation in yeast extract. Similar effects have been observed by Needham & Pillai (1937) and Warburg & Christian (1939) in the phosphoglyceraldehyde dehydrogenase system (see also Bücher & Garbade, 1952*a*, *b*; Racker & Krimsky, 1952), by Doudoroff, Barker & Hassid (1947) in the sucrose phosphorylase system and by Stadtman & Barker (1950), see also Stadtman, Novelli & Lipmann (1951) in the transacetylase system, by Delwiche, Loomis & Stumpf (1951) in the glutamyl transphorase system, and by Kennedy & Barker (1951) in the fatty acid oxidation system. A common feature of the arsenate effects is that they occur in systems in which phosphorylated intermediates participate. Following the view of Warburg & Christian (1939) an effect of arsenate is taken to be due to the formation of an arsenate analogue of the phosphorylated intermediate which differs from the phosphate derivative in that it is readily hydrolysed.

The observations on the citrulline system are in accordance with this general concept of the action of arsenate. Thus at least two enzymes must be concerned with the decomposition of citrulline: (1) an enzyme bringing about the formation of a phosphorylated intermediary, possibly phosphocitrulline, (2) a phosphokinase which transfers phosphate from the phosphorylated intermediary to ADP. It must further be assumed that this compound is relatively stable in the absence of ADP and that the transfer of phosphate to ADP is coupled with the release of carbon dioxide and ammonia, also that the arsenate compound is unstable and, unlike the phosphorylated compound, decomposes spontaneously into ornithine, carbon dioxide, ammonia and arsenate. Since attempts to demonstrate phosphocitrulline in the reaction mixture have been unsuccessful, if it occurs at all its concentration must be very low; and the equilibrium in the system

$$\text{citrulline} + \text{phosphate} \rightleftharpoons \text{phosphocitrulline}$$

must be unfavourable for the accumulation of phosphocitrulline.

SUMMARY

1. The rate of the conversion of citrulline into ornithine, carbon dioxide and ammonia by a cell-free extract of *Streptococcus faecalis* in the presence of phosphate and adenosine diphosphate (ADP) is increased by the addition of arsenate.

2. In the presence of arsenate, citrulline is decomposed by extract without the addition of phosphate and ADP. The maximum rate in the presence of arsenate is about 5 times higher than that in the presence of ADP and phosphate.

3. The products formed from citrulline are the same with arsenate as with ADP and phosphate; the overall reaction in the presence of arsenate being

$$\text{citrulline} + H_2O = \text{ornithine} + CO_2 + NH_3.$$

4. The effect of arsenate is reduced by the addition of phosphate, or fluoride, or iodoacetate. The inhibition by phosphate is competitive.

5. The mechanism of the action of arsenate is discussed.

6. An improved method for the estimation of citrulline is described; it is based on the diacetyl reaction of Fearon (1939).

I wish to thank Dr E. F. Gale, F.R.S., for his generous help and encouragement, and Prof. H. A. Krebs, F.R.S., for his helpful advice and interest in this work. I am indebted to the Medical Research Council for a research training grant, and to the Director, Chemical Research Laboratory, Teddington, for permission to undertake this investigation.

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Interaction of Acids of High Molecular Weight with Albumin

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Sodium deoxyribonucleate has been shown to prevent the coagulation by heat of dialysed albumin solutions at about pH 5·5 (Carter & Greenstein, 1946; Greenstein & Hoyer, 1950). Evidence of such interactions between nucleic acids and proteins is of considerable interest, since one of the main problems of nucleoprotein structure is the way in which the protein and nucleic acid are bound together. In the experiments described here it is shown that polyacids in general are able to interact with albumin so as to prevent heat coagulation. A short account of this work has already been presented (Hamer, 1953*a*).

EXPERIMENTAL

Albumin solutions. Bovine plasma albumin (fraction V, Armour and Co.) was used throughout this work. A solution was made up containing approx. 2 g./100 ml. and the pH was adjusted to 6·7–6·8. The solution was then dialysed using cellulose casings (Visking Corp., Chicago) at 2–4° against distilled water. After 4 or 5 days the pH dropped to about 5·5–5·7 and the final protein concentration was then 1·4 g./100 ml., as estimated from the N content.

Other materials. Deoxyribonucleic acids were prepared from isolated calf-thymus nuclei: specimen 1 by saline extraction followed by treatment of the solution with chloroform:butanol mixtures and specimen 2 by saline extractions followed by deproteinization with sodium dodecyl sulphate (Hamer, 1953*b*). A commercial sample of yeast ribonucleic acid was used (British Drug Houses Ltd.). Heparin and alginic acid were also obtained from commercial sources. The heparin had an activity of 90 i.u./mg. (Evans, Lescher and Webb Ltd., batch N 10090) and a molecular weight of probably 17000 (Jensen, Snellman & Sylven, 1948). The alginic acid had a molecular-weight range of 10000–20000 (specimen HS/LD and details, including the mol.wt. which is based on viscosity determinations, supplied by Alginate Industries Ltd.). Dextran sulphate and dextran were supplied by Dr K. Walton. Both had molecular weights about 8000, as determined by measurements of osmotic pressure and intrinsic viscosity (see Walton, 1952; Ricketts, 1952). Polyglutamic acid was prepared from the capsular substance of *Bacillus subtilis* by the method of Hanby & Rydon (1946), the final product being dialysed and freeze-dried (supplied by F. W. Moore, Bacteriology Department, Birmingham University). The molecular weight of material obtained by this method was reported to be at least 15000.

Aqueous solutions (0·1%) of these compounds were prepared; where necessary to neutralize alginic and nucleic acids a trace of alkali was added, and the solutions were dialysed in the cold to remove salts. It may be noted here that the amounts of these solutions used in the tests described below were so small as to have no effect on the pH of the albumin solutions.

Heat coagulation. 2 ml. of albumin solution with 2 ml. of water (or 2 ml. of the solution under investigation) were heated in a boiling-water bath for 10 min. and chilled in cold water. Under these conditions the albumin coagulates to give a milky-white, colloidal solution which is quite stable