of insulin in Freund's adjuvant. Animals producing antibodies were resistant to insulin. Serum from these animals neutralized insulin, as shown by inoculation into normal mice. This neutralization took place whether the insulin and serum were mixed together before injection or were injected separately at different times.

3. Endogenous guinea pig and sheep insulins were not neutralized by homologous antibodies.

4. Two types of guinea pig insulin have been prepared which were immunologically distinct. One type, classed as altered, was neutralized by immune guinea pig serum. The other, like endogenous guinea pig insulin, was not neutralized. The sheep insulins tested, which included one commercial preparation, were neutralized by immune sheep serum and therefore may be classed as altered.

5. This study indicates that endogenous guinea pig insulin is immunologically distinct from altered guinea pig insulin, from endogenous mouse insulin, and from preparations of pig, ox, sheep and rabbit insulin; and that endogenous sheep insulin is immunologically distinct from altered guinea pig insulin and from preparations of pig, ox, sheep and rabbit insulin.

6. Mice were rendered diabetic by injection of guinea pig anti-insulin serum. These diabetic mice showed abnormally high blood-glucose levels, loss of

weight, and acetone bodies in the urine during the period of administration of serum.

We acknowledge with thanks financial help given by the J. B. Bickell Foundation.

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Arsenolysis and Phosphorolysis of Citrulline in Mammalian Liver

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(Received 11 August 1954)

It has been shown by Knivett (1952*a, b*, 1953*a, b*, 1954*a, b, c*), Slade (1953), Slade, Doughty & Slamp (1954), Oginsky & Gehrig (1952, 1953) and Korzenovsky & Werkman (1953, 1954) that (a) citrulline can react in *Streptococcus faecalis* and other organisms according to the reaction

where ADP and ATP represent adenosine diphosphate and adenosine triphosphate, respectively; (b) citrulline can decompose in the presence of arsenate (by arsenolysis) according to the reaction

phosphate being required in this case; (c) citrulline can be split in the presence of phosphate (by phosphorolysis) according to the reaction

provided that the ornithine formed is removed, e.g. by addition of ornithine decarboxylase.

The present experiments are concerned with analogous reactions in animal tissues. They show that reactions (2) and (3) readily occur in mammalian liver. They are absent from avian liver. The evidence suggests that the reactions are connected with the enzyme system responsible in the intact liver for the synthesis of citrulline from ornithine, CO_2 and ammonia.

MATERIALS AND METHODS

Adenosine triphosphate was prepared according to LePage (1949) and contained 72.5% ATP, 13.5% ADP, and 14% inorganic phosphate. In some experiments a commercial preparation, referred to in the text as ATP-ADP mixture, was used; it contained 34.6% ATP, 30.5% ADP, 7.2% AMP, 11.3% inorganic phosphate and 16.4% unknown organic phosphate. The data are based on chromatographic analysis (Eggleson & Hems, 1952; Krebs & Hems, 1953) and are expressed as percentage of total phosphate.

Arsenate buffers were made by mixing $m\text{-Na}_2\text{HAsO}_4$, $n\text{-HCl}$ and water. The approximate pH was calculated, the pK of the second ionization constant being taken as 6.7. The actual pH was measured in many cases with a glass electrode after dilution. Phosphate buffers consisted of mixtures of 0.5 $m\text{-NaH}_2\text{PO}_4$ and 0.5 $m\text{-Na}_2\text{HPO}_4$. Succinate buffers were mixtures of m succinic acid and $2n\text{-NaOH}$.

N -Acetyl-L-glutamic acid was prepared according to Nicolet (1930). Carbamyl derivatives of amino acids were prepared in this laboratory by Dr W. Bartley by the following method which has the advantage over other procedures of avoiding prolonged heating and risk of racemization.

The L-amino acid or amide was dissolved in the minimal amount of water and to this was added slightly more than one equivalent of nitro-urea, prepared by the method of Davis & Blanchard (1929). With stirring and heating to 70° , a measured quantity of dry $\text{Ba}(\text{OH})_2$ was added to bring the pH to 8–8.5 and was maintained for 30–60 min. Barium was precipitated by gradually adding the calculated amount of H_2SO_4 . After cooling and centrifuging, the supernatant solution was concentrated *in vacuo*, adjusted to pH 5.5–6 with NaOH , and treated with excess basic lead acetate, the pH being maintained by the addition of acetic acid. After standing overnight the lead salt was separated and suspended in warm water, and the lead precipitated with H_2S . The clear filtrate was concentrated *in vacuo* until crystals separated. Recrystallization was from water or from ethanol-ether mixtures.

Liver suspensions. Two types of liver preparations were used. Fresh liver suspensions were prepared by homogenizing or blending ice-chilled liver with four parts of water or a saline medium containing 9 parts 0.9% (w/v) KCl and 1 part 0.1 $m\text{-MgCl}_2$. The stainless-steel homogenizer of the Potter-Elvehjem type and a Waring Blender, both chilled, were used. The suspensions were stored at -15° . To prepare acetone-dried liver, ice-chilled tissue was disintegrated in a Waring Blender with 10 vol. acetone at -10° . After filtration on a filter funnel the liver was dried *in vacuo* over H_2SO_4 and stored at -15° . In some cases the acetone treatment was repeated two or three times. The dried material was shaken with 10 or 25 parts of water, or the KCl-MgCl_2 solution described above, for 2 hr. at 4° and then centrifuged. The supernatant was stored at -15° . In some experiments it was dialysed against large volumes of ice water for 4–16 hr.

Ornithine decarboxylase. *Escherichia coli* (E:C:4) (Nat. Coll. Ind. Bact., 8571) was grown on a medium containing 3% enzymic casein digest (Pronutrin), 1% glucose, 0.5% 'Difco' yeast extract, 2.5% agar and 0.1% L-ornithine (all w/v). After incubation for 16–20 hr. at 37° , cells were washed three times in water and then suspended in 0.1 $m\text{-KCl}$ or water. On storage at 2° the ornithine decarboxylase activity was maintained for several weeks.

Manometric measurement of CO_2 evolution from citrulline. Conical Warburg flasks with one or two side arms were used, a second side arm containing 0.5 ml. 10% (v/v) H_2SO_4 . The acid was added at the end of the incubation to release bound CO_2 . The total volume of reagents in the flasks was between 2 and 4 ml. The gas space contained N_2 , the centre well yellow phosphorus. Citrulline was usually added from the side arm after thermal equilibration. The temperature of incubation was 40° .

Paper chromatography. To identify ornithine as a breakdown product of citrulline the incubated material was deproteinized with about 0.15 vol. of 30% (w/v) trichloroacetic acid (TCA). Of the filtrate 20–50 μl . were dried on to chromatogram papers in a 1 cm. square area with a current of warm air. The papers were developed with a formic acid-isopropyl ether solvent of Hanes & Isherwood (1949) for 4 hr. with ascending flow, or alternatively by descending flow, for 16–18 hr., with a mixture of 4 parts liquid phenol and 1 part NH_4OH , sp.gr. 0.880, or phenol saturated with water at 20° . After drying in a current of warm air, the papers were sprayed with 0.4% (w/v) ninhydrin in water-saturated n -butanol, and then heated 15 min. at 60° . L-Citrulline and L-ornithine reference spots were run simultaneously.

To test for the presence of carbamyl derivatives of amino acids, a spray containing 4% (w/v) p -dimethylaminobenzaldehyde in $n\text{-HCl}$ was used (Phillips, 1954). Yellow spots appeared on heating the paper when these compounds were present.

RESULTS

Effect of phosphate and arsenate on CO_2 production from citrulline

When citrulline was added to rat-liver preparations, together with ATP or ADP and inorganic phosphate, no appreciable evolution of CO_2 took place under conditions where bacterial extracts cause a rapid decomposition of citrulline. A very small increase in CO_2 production which just exceeds the limit of error was observed when the phosphate and adenosine phosphate concentrations were 0.01 m , and definitely measurable rates of CO_2 evolution were observed when higher concentrations of phosphate were added (0.025–0.25 m). This is shown in Table 1. The addition of ATP or ADP did not affect the rate of CO_2 evolution, although adenosine phosphates were fairly stable in extracts of acetone-dried liver.

Table 1. *The effect of phosphate concentration on CO_2 production from citrulline*

Guinea pig liver homogenate, 1 in 20 final dilution; 0.05 m L-citrulline; pH 6.5; total vol., 2 ml.; 40° ; N_2 .

Additions	CO_2 formed in 60 min. (μl .)
None	6
Phosphate, 0.025 m	18.5
Phosphate, 0.05 m	29
Phosphate, 0.125 m	35
Phosphate, 0.25 m	44
Arsenate, 0.25 m	386
NaCl , 0.25 m	3

In contrast with phosphate, arsenate was found to accelerate greatly the rate of decomposition of citrulline, both in fresh liver suspensions and in acetone-dried liver extracts (Tables 1 and 2). The stimulating effect of arsenate was diminished by the addition of phosphate. A plot according to Lineweaver & Burk (1934) indicates that the inhibition is competitive.

The formation of ornithine was established by paper chromatography, the formation of ammonia by Nessler's reagent.

Enzymic characteristics of arsenolysis

The pH optimum of the arsenolysis was found to be at about pH 6.7. The optimum is fairly sharp, the activities at pH 6.3 and 7.1 being 72–75% of the maximal activity under the test conditions (Table 3). The rate of arsenolysis increased with the citrulline concentration up to 0.1M citrulline (Table 4). The Michaelis constant as calculated from Table 4 was about 0.014M. It varied with the experimental conditions between 0.01 and 0.05, being higher at the lower pH values. The time course of the arsenolysis indicated that the reaction did not go to completion even when low concentrations of substrates with relatively large amounts of enzyme were used (Table 5). The rate of CO₂ evolution rapidly decreased, and after about 90% of the expected amount had been liberated the gas evolution became almost nil.

Storage of fresh liver suspensions or extracts of acetone-dried liver at –15° for 3 months did not reduce the enzyme activity. Extracts made with distilled water were as active as those containing KCl and MgCl₂. Prolonged dialysis (16 hr. at 0°) against tap water or distilled water, or addition of boiled liver extract had no effect on the rate of arsenolysis and in none of the conditions tested could

arsenate be effectively replaced by phosphate and ADP. The relative reaction rates in the presence of arsenate and phosphate remained of the order shown in Table 1.

Table 2. The effect of arsenate concentration on CO₂ production from citrulline

Expt. 1. Aqueous extract of acetone-dried guinea pig liver, 0.5 ml.; 0.025M L-citrulline; pH 6.7; total volume 2 ml. Expt. 2. Rat-liver homogenate, 1 in 20 final dilution; 0.05M L-citrulline; pH 6.3; total vol., 2 ml. Buffer concentrations are final; 40°; N₂.

Expt. no.	Arsenate (M)	Phosphate (M)	CO ₂ formed in 60 min. (μl.)
1	0	0	0
	0.0625	0	132
	0.25	0	175
2	0	0.02	6
	0.25	0	409
	0.25	0.02	268
	0.125	0	389
	0.125	0.02	233
	0.0625	0	314
	0.0625	0.02	151

Table 3. The effect of pH on the arsenolysis of citrulline

Rat-liver homogenate, 1 in 20 final dilution; 0.05M L-citrulline; 0.25M arsenate buffer; total vol., 2 ml.; 40°; N₂.

pH	CO ₂ formed in 60 min. (μl.)		
	Free	Bound	Total
5.59	8	0	8
6.02	49	0	49
6.27	216	10	226
6.70	260	54	314
7.14	148	88	236
7.63	59	104	163

Table 4. Effect of citrulline concentration on the rate of arsenolysis of citrulline

Each flask contained 0.5 ml. dialysed guinea pig liver homogenate (1 part liver, 4 parts water); 0.5 ml. 0.5M arsenate buffer, pH 6.7; 0.05–1.0 ml. 0.5M DL-citrulline. Total vol., 2 ml.; N₂. Readings began after 10 min. equilibration. Free CO₂ was about 80% of total CO₂.

	Final citrulline concn. (M)					
	0.125	0.10	0.05	0.025	0.0125	0.00625
μl. CO ₂ (free) after 10 min.	70	70	56.5	47.5	35	22.5
μl. CO ₂ (free) after 60 min.	288	283	232	192	140	91.5

Table 5. Time course of arsenolysis of citrulline

Guinea pig liver homogenate, 1:13; 0.25M arsenate buffer, pH 6.7; 0.0031M L-citrulline. Total vol., 4 ml.; N₂.

Time (min.)	Total CO ₂ formed (μl.)	% of CO ₂ expected for complete hydrolysis
30	110	42
60	172	62
90	213	76
120	237	85
150	249	89

Table 6. *The relative arsenolytic activities of fractions obtained from sheep-liver mitochondria*

For details see text. Enzymic assay carried out at 40° in N₂. Q_{CO₂} = μ l. CO₂ liberated/mg. dry wt. of enzyme/hr.

Enzyme fraction	Whether dialysed	Q _{CO₂}
Whole suspension (in water) of washed mitochondria	No	10.5
Lysed mitochondria, sediment	No	9.8
Lysed mitochondria, supernatant	No	33.1
Lysed mitochondria, supernatant	Yes	57.8
Ppt. from 20% saturation of supernatant with (NH ₄) ₂ SO ₄	Yes	13.3
Ppt. from 40% saturation of supernatant with (NH ₄) ₂ SO ₄	Yes	16.1
Ppt. from 60% saturation of supernatant with (NH ₄) ₂ SO ₄	Yes	156.8
Ppt. from 80% saturation of supernatant with (NH ₄) ₂ SO ₄	Yes	40.6
Ppt. from final acidification with TCA to pH 2.0	Yes	6.2

Table 7. *Inhibition of the arsenolysis of citrulline by ornithine*

Guinea pig liver homogenate, 1 in 20 final dilution; 0.0375 M L-citrulline in Expt. 1, 0.025 M in Expt. 2; 0.25 M arsenate buffer in Expt. 1, 0.125 M in Expt. 2; pH 6.7; total vol., 2 ml.; 40°; N₂.

Expt. no.	Additions	CO ₂ liberated in 60 min. (μ l.)
1	None	287
	L-Ornithine, 0.02 M	133
	L-Ornithine, 0.02 M, NH ₄ Cl, 0.02 M	131
	NH ₄ Cl, 0.02 M	276
	NH ₄ Cl, 0.02 M	276
2	None	316
	L-Ornithine, 0.05 M	62
	L-Ornithine, 0.0125 M	167
	L-Ornithine, 0.00625 M	226

Purification of the enzyme

Liver mitochondria prepared by the method of Bartley & Davies (1954) were found to contain the enzyme, whilst the supernatant was inactive. After treatment of the washed mitochondria with 5 vol. of water, alternate freezing at -15° and thawing at 2° (twice) and centrifuging, most of the enzyme was in the clear supernatant. Treatment with ammonium sulphate led to precipitation when the salt concentration reached 60–80% saturation. The relative enzymic activity of various fractions is shown in Table 6. For assaying, the material was dissolved in 0.125 M arsenate buffer, pH 6.7, containing 0.1 M DL-citrulline. It will be seen that the enrichment in the most active samples was about 15-fold. There was a loss of about 50% of the total activity during the ammonium sulphate fractionation.

Effects of various substances on the arsenolysis and phosphorolysis of citrulline

The following substances were found to be without effect on the arsenolysis of citrulline (pH 6.7, 0.25 M arsenate and 0.025 M L-citrulline): D-citrulline (0.05 M), L-glutamate (0.01 M), carbamyl-L-glutamate (0.05 M), succinate (0.02 M), *sec*-octanol

(50 mg.), cetyltrimethylammonium bromide (0.1%, w/v), hydroxylamine (0.01 M), BeCl₂ (0.001 M), FeSO₄ (0.01 M) and KCN (0.001 M). Iodoacetate (0.01 M) and mercaptoacetate (0.01 M) caused small inhibitions (about 10%); *p*-chloromercuribenzoate (0.01 M) inhibited almost completely. 0.05 and 0.10 M BAL (2:3-dimercaptopropanol) were also inhibitory.

A specific inhibition is caused by one of the end products of the reaction, L-ornithine. The magnitude of the effect is shown in Table 7; at 0.0125 M the inhibition was about 50%. The other end products, ammonium ions and CO₂, had no effect. The inhibition by ornithine provides an explanation for the incomplete fission of citrulline already mentioned.

Attempts to replace arsenate by other agents were unsuccessful. Among those tried were 2:4-dinitrophenol (10⁻³–10⁻⁴ M) and NaF (0.05 M). Attempts were also made to increase the rate of CO₂ production from citrulline in the presence of phosphate and ADP and absence of arsenate. The following substances were tested and none of them was found to be effective: Ca²⁺ or Mg²⁺ (0.0025–0.02 M), ethylenediaminetetra-acetate (0.005–0.01 M), L-lysine (0.01 M), L-histidine (0.01 M), L-cysteine (0.005–0.02 M), glutathione (0.003 M), L-ascorbic acid (0.01 M), BAL (0.0025–0.05 M), BeCl₂ (0.001 M), Mn²⁺ (0.005 M), Fe²⁺ (0.01 M), NH₄Cl (0.05 M), cetyltrimethylammonium bromide (0.1%, w/v), coenzyme A (~10⁻⁴ M), flavin-adenine dinucleotide (~10⁻⁵ M), flavin mononucleotide (~10⁻⁵ M), L-glutamate (0.05 M), L-glutamine (0.05 M), L-aspartate (0.05 M), L-asparagine (0.05 M), carbamyl-L-glutamate (0.05 M), carbamyl-L-glutamine (0.05 M), carbamyl-L-asparagine (0.05 M), α -oxoglutarate (0.05 M), pyruvate (0.05 M), oxaloacetate (0.05 M) and glucose (0.05%, w/v). Various crude enzyme preparations were added to the phosphate-buffered system as it was thought that the slow rate of reaction might be due to the absence or low concentration of some factor in the liver enzyme required to split a hypothetical citrulline-phosphate postulated by previous authors. The preparations which were tried and were without effect included: intestinal phos-

phatase, brewer's yeast extract (Lebedev juice), rat-kidney homogenate, aqueous extract of soya bean, and cobra venom.

Nature of the inhibition by ornithine

The fact that ornithine only among the three end-products is inhibitory suggests that the inhibition is not due to a mass-action effect but to a competition for the enzyme between ornithine and citrulline, analogous to the cases described by Frantz & Stephenson (1947), Huang & Niemann (1951) and Thomas, MacAllister & Niemann (1951) where one of the end-products of peptide hydrolysis was a competitive inhibitor. The data given in Table 8 confirm that the inhibition by ornithine is competitive. At a given ornithine concentration the inhibition increased the lower the citrulline concentration. A plot of the reciprocal of the substrate concentration (abscissa) against the reciprocal of the velocity (ordinate), according to Lineweaver & Burk (1934) gave the same intersect on the ordinate with and without ornithine, but the two curves had significantly different slopes.

Effect of ornithine decarboxylase

The inhibition of the arsenolysis by ornithine suggested that ornithine might also be responsible for the very low rate of phosphorolysis of citrulline. To test this, suspensions of *Esch. coli* containing an L-ornithine decarboxylase were added to the system (see Knivett, 1954c). Preliminary experiments showed that fresh liver suspensions and aqueous extracts of acetone-dried liver rapidly yield CO₂ on addition of *Esch. coli* in the absence of citrulline, due to the decarboxylation of amino acids in the liver extract and to the fermentation of carbohydrate. Dialysis of the liver extract was found to reduce these side reactions to a low value. As seen in Table 9, suspensions of *Esch. coli* greatly increase the rate of CO₂ evolution from citrulline. Allowing for the blank, the increase was about 20-fold and approached the rates observed in the presence of arsenate. When comparing the rates shown in Table 9 it must be borne in mind that ornithine decarboxylase doubled the total CO₂ production from citrulline.

Ornithine decarboxylase is known to be inhibited by hydroxylamine (Taylor & Gale, 1945). The last three horizontal lines of Table 9 show that although 0.01M hydroxylamine inhibits the ornithine decarboxylase activity almost completely, the acceleration of the hydrolysis of citrulline by *Esch. coli* is not completely inhibited by hydroxylamine. This suggests that apart from ornithine decarboxylase *Esch. coli* suspensions contain a second substance, henceforth referred to as 'factor' which promotes the decomposition of citrulline. As heated

suspensions of *Esch. coli* have no effect, the 'factor' is probably an enzyme.

Phosphate is an essential component in this system as illustrated by Table 10, whilst ATP and ADP have no effect. In control experiments it was noted that the decarboxylation of L-ornithine and L-lysine by *Esch. coli* was also increased by

Table 8. *Competitive inhibition of the arsenolysis of citrulline by ornithine*

Dialysed guinea pig liver suspension (stored for 6 weeks at -15°), 1 in 20 final concentration; 0.125M arsenate buffer, pH 6.7; 40°; N₂; total vol., 4 ml. The data present the initial reaction rates.

Concentration of DL-citrulline (M)	Total CO ₂ formed in 10 min. (μl.)	
	No ornithine	0.0125M L-ornithine
0.1	121	61
0.05	109	39
0.025	78	21.8
0.0125	43.5	10.5
0.0063	23.7	5.3
0.0031	11.8	2.6

Table 9. *The effect of washed cells of Esch. coli on citrulline breakdown in the presence of guinea pig liver and phosphate*

Dialysed guinea pig liver homogenate, 1 in 20 final dilution; 0.125M phosphate, pH 6.5, present throughout, unless otherwise stated; L-citrulline, 0.05M; L-ornithine, 0.025M; hydroxylamine, 0.01M; *Esch. coli* ≡ 15 mg. dry wt; total vol., 2 ml.; 40°; N₂; 60 min.

Additions	CO ₂ evolution (μl.)
No liver, <i>Esch. coli</i> , citrulline	9
Liver, <i>Esch. coli</i>	28.5
Liver, citrulline	30
Liver, <i>Esch. coli</i> , citrulline	430.5
Liver, <i>Esch. coli</i> (heated 10 min. at 100°), citrulline	28
Liver, no phosphate, citrulline, arsenate 0.125M	312
Liver, <i>Esch. coli</i> , ornithine	754
Liver, <i>Esch. coli</i> , ornithine, hydroxylamine	11.5
Liver, <i>Esch. coli</i> , citrulline, hydroxylamine	122.5

Table 10. *Effect of phosphate on CO₂ evolution from citrulline in the presence of liver and Esch. coli*

Dialysed aqueous extract of acetone-dried sheep liver. 0.05M L-citrulline; pH 6.8; *Esch. coli* ≡ 15 mg. dry wt.; total vol., 2 ml.; 40°; N₂.

Phosphate concentration (M)	CO ₂ formed in 60 min. (μl.)
0	28
0.005	67
0.04	172.5
0.04 (no <i>Esch. coli</i>)	3
0.04 (no liver)	2

phosphate (Table 11), but this effect is relatively small compared with the effect on the decomposition of citrulline.

Effects of amino acid derivatives

The phosphorolysis of citrulline in the presence of *Esch. coli* and liver enzymes is accelerated by the addition of carbamyl and acetyl derivatives of amino acids (0.04M). These substances were tested because Cohen & Grisolia (1950) and Grisolia & Cohen (1951, 1952, 1953) have shown that this type of compound can accelerate the aerobic conversion of ornithine into citrulline in mammalian liver. The acceleration of phosphorolysis is shown in Table 12. Effects were obtained with carbamylglutamate, acetylglutamate, carbamylglutamine, and carbamylalanine. They increased with time and were much larger during the second hour than during the first. No effects were observed with carbamylphenylalanine, carbamylleucine, acetylleucine, acetylalanine, carbamylaspartate, carbamylasparagine, or with glutamate, glutamine, aspartate and asparagine. It should be emphasized that no effects of the amino acid derivatives were found when either liver extract, or *Esch. coli* was omitted (see also

Slade, 1953). Arsenolysis was not affected by the substances.

The effects recorded in Table 12 were obtained in the presence of hydroxylamine, i.e. in the absence of ornithine decarboxylase activity. They are therefore related to the action of the *Esch. coli* factor. Effects also occur in the absence of hydroxylamine (Table 13), but their relative magnitude is smaller, as they are superimposed on the large effect of ornithine decarboxylase.

Search for phosphorylated intermediates

Phosphorolysis or arsenolysis involves in some cases (Warburg & Christian, 1939; Bücher & Garbade, 1952) the intermediate formation of a phosphorylated or arsenolated compound. In an attempt to detect a phosphorylated intermediate, experiments of the type described in Tables 9 and 10 were repeated with radioactive phosphate ($10 \mu\text{C } ^{32}\text{P}/\text{flask}$). After various periods of incubation the medium was analysed chromatographically for phosphorylated compounds but no radioactive spot, apart from that for inorganic phosphate, appeared. When ATP and ADP were added no incorporation of radioactive phosphate into ATP or ADP was found. The latter experiments were carried out on washed liver mitochondria where the rate of decomposition of adenosine phosphates was slow.

Distribution of the enzyme systems responsible for the arsenolysis of citrulline

Brain, kidney, testis, lung, spleen, cardiac and striated muscle of the rat, pancreas and small intestine of the sheep, as well as pigeon liver were found to be completely inactive when tested under the conditions stated in Tables 1 and 2. The three mammalian livers tested (rat, guinea pig and sheep) as well as toad liver contained the enzyme system.

Table 11. *Effect of phosphate on the decarboxylation of L-ornithine and L-lysine by washed cells of Esch. coli*

Succinate buffer 0.1M, pH 6.5; Na phosphate, pH 6.5; 0.025M amino acid; *Esch. coli* $\equiv$ 15 mg. dry wt.; total vol., 2 ml.; 40°; N₂.

Amino acid	Phosphate concentration (M)	CO ₂ formed in 60 min. ($\mu\text{l.}$)
L-Ornithine	0	692
L-Ornithine	0.005	766
L-Ornithine	0.04	809
L-Lysine	0	726
L-Lysine	0.04	925

Table 12. *The acceleration by carbamyl- and acetyl-amino acids of citrulline breakdown in the presence of liver, phosphate, Esch. coli and hydroxylamine*

Aqueous extract of lysed sheep-liver mitochondria in Expts. 1-3; dialysed guinea pig liver suspension in Expt. 4; 0.1M phosphate, pH 6.8; *Esch. coli* $\equiv$ 15 mg. dry wt.; 0.01M hydroxylamine; 0.04M substrates; total vol., 2.5 ml.; 40°; N₂.

Expt. no.	Substrates	CO ₂ liberated ($\mu\text{l.}$)		
		0-60 min.	60-120 min.	120-150 min.
1	L-Citrulline	81.5	47.5	18
	Carbamyl-L-glutamate	19.5	8	2
	Carbamyl-L-glutamate; L-citrulline	127	82	32
2	L-Citrulline	71	45.5	22.5
	Carbamyl-L-glutamine	8	3	6
	Carbamyl-L-glutamine; L-citrulline	124	87	41
3	L-Citrulline	44	24.5	11
	Acetyl-L-glutamate	3	4	2
	Acetyl-L-glutamate; L-citrulline	92	63	28
4	L-Citrulline	91	59	28
	Carbamyl-L-alanine	16	0	0
	Carbamyl-L-alanine; L-citrulline	133	89	38

Table 13. *Effect of acetyl-L-glutamate on the phosphorolysis of citrulline in the presence of liver extract, phosphate and Esch. coli, and absence of hydroxylamine*

Dialysed guinea pig liver homogenate; 0.143M phosphate, pH 6.8; 0.029M L-citrulline; *Esch. coli* $\equiv$ 30 mg. dry wt.; total vol., 3.5 ml.; 40°; N₂.

Acetyl-L-glutamate concentration (M)	CO ₂ liberated (μ l.) after	
	60 min.	180 min.
0	188	439
0.0145	209.5	490
0.029	215.5	510
0.058	227.5	555

Arsenolysis was also found in micro-organisms for which 'arginine dihydrolase' activity has already been reported, e.g. *Clostridium welchii*, *Proteus vulgaris* and *Pseudomonas fluorescens*. Dried baker's yeast and brewer's yeast showed distinct, but weak arsenolysis (about 1 μ l. CO₂/mg. dry weight/hr.).

The presence of the factor which accelerates the phosphorolysis of citrulline was tested in a system containing liver extract, phosphate buffer, citrulline and hydroxylamine (at concentrations and conditions as stated in Table 9). No activity was found in washed suspensions of *Lactobacillus arabinosus*, *Streptococcus faecalis*, *Bacillus proteus vulgaris*, *Clostridium welchii* SR 12, *Bacillus subtilis*, *Sarcina lutea* and *Bacterium faecalis alkaligenes*, when these organisms were grown on the same medium in which *Esch. coli* produced the factor. When the amino acid hydrolysate was omitted from the medium *Esch. coli* failed to produce the factor.

DISCUSSION

Comparison of citrulline decomposition in bacteria and in liver

The experiments reported in this paper demonstrate the occurrence in mammalian liver of an enzyme system which can decompose citrulline to ornithine, carbon dioxide and ammonia by arsenolysis or phosphorolysis. The enzyme system is very similar to that previously found in micro-organisms. Of the three reactions (1), (2) and (3), which take place in bacteria (to be regarded as different manifestations of the same basic enzyme system), so far only (2) and (3) have been found in liver.

The fact that the distribution of the enzyme system in animal tissues parallels the occurrence of the urea-synthesizing enzymes suggests that the two are associated, the degrading enzymes being components of the systems which under physiological conditions are responsible for the synthesis. The conditions required for arsenolysis and phos-

phorolysis are usually not realized in living cells, as arsenate is absent and the physiological concentrations of the reactants are too low for phosphorolysis. Another factor favouring synthesis of citrulline from ornithine under physiological conditions is the inhibition of phosphorolysis by ornithine.

Arginine is known in micro-organisms to be synthesized from ornithine via citrulline, e.g. in *Neurospora crassa* (Srb & Horowitz, 1944), in *Ophiostoma multiannulatum* (Bergström & Sjöbeck, 1950) in *Esch. coli* (Davis, 1953) and *Lb. arabinosus* (Hood & Lyman, 1950; Ory, Hood & Lyman, 1954). The bacterial enzymes decomposing citrulline may be looked upon as being connected with the synthesis of arginine in the same manner as the corresponding hepatic enzyme system is connected with the formation of urea. In those micro-organisms which possess the arginine dihydrolase component, reactions of this system have evolved to serve as an energy-giving process (reaction 1) if the substrate is available in the medium.

Mechanism of arsenolysis and phosphorolysis

Warburg & Christian (1939), who studied arsenolysis in the triosephosphate dehydrogenase system, assumed the formation of an arsenolated intermediate, arsenophosphoglycerate, replacing the normal phosphorylated intermediate, diphosphoglycerate (see also Racker & Krimsky, 1952). The formation of arsenolated intermediates (glucose 1-arsenate and acetylarsenate) was also assumed by Doudoroff, Barker & Hassid (1947) and Stadtman & Barker (1950) to explain the arsenolysis of glucose 1-phosphate and acetylphosphate. Recently the view has gained ground that effects of phosphate in some cases may be due to the phosphorylation of an enzyme rather than of an intermediate (Webster & Varner, 1954; Lipmann, 1954; Boyer & Harrison, 1954; Snoke, 1953). In such cases arsenolysis is expected to be due to combination of arsenate with the enzyme.

The facts which are to be taken into consideration by any hypothesis attempting to account for the arsenolysis and phosphorolysis of citrulline are as follows.

(a) Arsenolysis requires only substrate, enzyme and arsenate, in both liver and bacteria.

(b) Phosphorolysis, in order to proceed at an appreciable rate, requires not only phosphate, substrate and enzyme but also removal of ornithine by ornithine decarboxylase (in liver and bacteria) or removal of phosphate by transfer to ADP (in bacteria).

(c) Ornithine inhibits competitively both arsenolysis and phosphorolysis.

(d) Arsenolysis is competitively inhibited by phosphate.

(e) High concentrations of citrulline, phosphate and arsenate are required for appreciable reaction rates.

The following inferences must also be taken into consideration in formulating a hypothesis.

(f) The competition between ornithine and citrulline indicates that the two substances react with the same group of the enzyme.

(g) Competition between phosphate and arsenate indicates that these two substances also react with a common group of the enzyme. The points of attachment to the enzyme of the pairs phosphate-arsenate and citrulline-ornithine are not the same.

The following reaction mechanism, which is in some respects analogous to that proposed by Webster & Varner (1954) for the synthesis of glutamine and of Snoke (1953) for the synthesis of glutathione, is in accordance with these facts and inferences. It is assumed that the enzyme (*E*) can combine reversibly with citrulline, and possibly also with ornithine:

The enzyme-citrulline complex, by reaction with phosphate or arsenate is assumed to be split as follows:

The centre stages of (5a) and (5b), it should be noted, are written as being irreversible, although

$E \begin{array}{l} \text{phosphate} \\ \text{citrulline} \end{array}$ and $E \begin{array}{l} \text{phosphate} \\ \text{ornithine} \end{array}$ may be interconvertible by the exchange reaction

The competitive inhibition by ornithine is either due to (4b) or to (6). The reactions (5a), though not reversible as a whole, can be brought to a standstill either by the competitive inhibition of ornithine, or by blocking the enzyme in the form of *E*-phosphate. Hence either the removal of ornithine by ornithine decarboxylase or the transfer of phosphate from *E*-phosphate to ADP

promotes (5a) from left to right.

To explain the phosphorolytic effect of high concentrations of phosphate (Table 1) and of ornithine decarboxylase (Table 9) it must be assumed that *E*-phosphate is capable of undergoing hydrolysis at a rate increasing with its concentration. Removal of ornithine by ornithine decarboxylase, or high concentrations of inorganic phosphate both cause the postulated increase in the concentration of *E*-phosphate.

To shift reaction (5b) from left to right a rapid decomposition of *E*-arsenate is postulated, in accordance with the general experience that arsenate esters of physiological substances are less stable than the corresponding phosphate esters.

The virtual non-occurrence of reactions (4a) and (5a) under physiological conditions is accounted for by the fact that the relative affinity of the enzyme for citrulline is low, and by the inhibition by ornithine.

The formation of a phosphorylated substrate (Oginsky & Gehrig, 1953; Knivett, 1954b) according to the scheme

is unlikely (though not ruled out) because of the failure to detect a phosphorylated substrate.

As the phosphorolysis of citrulline can be coupled (in bacteria) with the synthesis of ATP from ADP and phosphate, an energy-rich phosphate group must arise at some intermediate stage. The energy-rich phosphate is taken to be the phosphorylated enzyme (*E*-phosphate) formed at stage (5a).

Since the reactions discussed here do not occur under physiological conditions, those in which phosphate takes part are assumed to be stages of citrulline synthesis in the living cell. This synthesis is initiated by reaction (7) and followed by a modified form of (5a) and completed by (4a), all three reactions proceeding from right to left. The modification of (5a) occurs at the irreversible centre step. This is replaced by other reactions in which the carbamyl group is built up and transferred to the enzyme-bound ornithine. The carbamyl donor is presumably compound X of Grisolia (1951). The arsenolysis or phosphorolysis do not require an acceptor for the carbamyl group which is taken as indicating that the mechanism of the fission of the carbamyl group of citrulline differs from that of the synthesis.

SUMMARY

1. Citrulline, whilst stable in suspensions of fresh liver or extracts of acetone-dried liver, is readily split into ornithine, CO₂ and ammonia if arsenate is added. This reaction is analogous to the arsenolysis of citrulline in *Streptococcus faecalis* (Knivett, 1954b).

2. A slow fission of citrulline occurs in the presence of high concentrations (0.05M and above) of orthophosphate.

3. Some properties of the enzyme system responsible for the arsenolysis have been studied. The pH optimum of the arsenolysis is at 6.7. The Michaelis constant varies with experimental conditions between 0.01 and 0.05.

4. Phosphate inhibits competitively the action of arsenate.

5. The arsenolytic ability of liver preparations is not reduced by prolonged dialysis. Ornithine specifically inhibits arsenolysis and phosphorolysis. The inhibition is competitive.

6. Phosphorolysis is greatly accelerated by the addition of washed suspensions of *Esch. coli*, which contain ornithine decarboxylase. Hydroxylamine, which inhibits ornithine decarboxylase does not fully abolish the accelerating effect of *Esch. coli*. It is concluded that a factor in addition to ornithine decarboxylase promotes phosphorolysis. Its nature is unknown.

7. Carbamyl and acetyl derivatives of amino acids do not affect the rate of arsenolysis but some compounds of this group (carbamylglutamate, carbamylglutamine, carbamylalanine and acetylglutamate) accelerate the rate of phosphorolysis in the presence of ornithine decarboxylase or the factor of *Esch. coli*.

8. Among animal tissues liver was the only one in which arsenolysis of citrulline was found. Avian liver was inactive. It is assumed that the enzymes responsible for arsenolysis are components of the system which synthesizes citrulline in the living cell.

9. A mechanism of arsenolysis and phosphorolysis is proposed. It involves the formation of a phosphorylated or arsenolated enzyme.

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