Studies with a *Pseudomonad* able to Grow with Creatine as Main Source of Carbon and Nitrogen

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SUMMARY: An organism able to grow in a simple medium with creatine as the main C and N source was isolated from garden soil and subsequently identified as *Pseudomonas ovalis* Chester. Organisms harvested from a creatine-containing medium destroyed creatine with the uptake of O₂ and the formation of CO₂, NH₃ and urea. With the exception of urea the quantities of reactants fell short of those required by theory, and only part of the deficiency could be attributed to oxidative assimilation. Optimal conditions for the growth of active organisms and for the destruction of creatine were determined. In these conditions the organisms destroyed, in addition to creatine, only arginine and agmatine from a variety of compounds tested; compounds not attacked included creatinine and glycocyamine. Ability to oxidize creatine was partially lost during repeated washing and storage of the organisms, and was inhibited by *p*-chloromercuribenzoic acid and fatty acids.

Micro-organisms able to use creatine as a substrate for growth have been known for many years (den Dooren de Jong, 1926; Twort & Mellanby, 1912). The classical work of Dubos and Miller revealed further species adaptively capable of destroying creatine (as well as creatinine and other substrates) in washed suspension (Miller & Dubos, 1936; Dubos & Miller, 1937, 1938). The use of a washed suspension of one of these species allowed a discrimination to be made between creatinine and other Jaffe-positive material in human blood. More recently organisms which can similarly use for growth creatine or creatinine as sole source of carbon and nitrogen, and which can as suspensions of the organisms degrade these two compounds, have been isolated from a number of sources (Beard, 1943, 1944; Kopper & Beard, 1947; Roche, Girard, Lacombe & Mourgue, 1948).

In 1949 it became apparent to workers in this Department that the use of an enzyme system specifically capable of destroying creatine would materially aid in investigations on the excretion of this compound. Unfortunately, none of the organisms mentioned above possesses the necessary degree of specificity. All appear to destroy creatinine, and some also to degrade guanidine compounds which give a colour in the method (Ennor & Stocken, 1948) used for creatine estimation. It was decided, therefore, to attempt to isolate a micro-organism, washed suspensions of which could destroy creatine only. In this paper are reported some of the properties of a *pseudomonad* isolated by specific enrichment; a preliminary account of it has already been given (Nimmo-Smith, 1949).

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METHODS

Organism. The organism (whose isolation is described in Results) was maintained by monthly transfer on tryptic-casein agar slopes incubated for 24 hr. at 30°. It was provisionally identified as *Pseudomonas eisenbergii* (*P. non-liquefaciens*), but a more thorough examination by Dr S. T. Cowan leads him to think that it is a strain of the related species *P. ovalis* Chester. A culture has been deposited as no. 7914 in the National Collection of Type Cultures.

Medium. The basal medium used throughout contained: KH_2PO_4 , 1.9 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 9.3 g.; NaCl, 5 g.; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 10 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg.; $\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$, 10 mg.; yeast extract (Difco), 0.5 g.; distilled water to 1 l.; pH 7.0. To this basal medium was added the desired growth substrate (creatine, arginine, etc.) at 2.5–5.0 g./l.

Growth experiments. The above medium (or indicated modifications of it) was distributed (for 5 ml. final volume) in 50 ml. Erlenmeyer flasks. After autoclaving for 7 min. at 10 lb./sq.in. each flask was inoculated with 0.5 ml. of a 1/100 dilution of a 24 hr. culture in the basal medium + creatine. When a non-adapted inoculum was wanted about the same number of organisms from a tryptic casein agar slope culture were used. All incubations were at 30°. Inability to grow in any particular set of conditions was not concluded until after at least 72 hr. In later experiments, medium (final volume 4.0 ml.) was incubated in sloped $6 \times \frac{3}{4}$ in. test-tubes and growth was assessed with a photoelectric colorimeter (Evans Electroselenium Ltd.; EEL.) with a neutral density filter.

Preparation of suspensions of organisms. The medium (100 ml.) containing 0.5 g. creatine or other substances stated, was distributed in Roux bottles and autoclaved for 7 min. at 10 lb./sq.in. Each bottle was inoculated with 0.1 ml. of a culture of the organism in the same medium and incubated in the horizontal position at 30°. After 20–24 hr. the organisms were harvested on the centrifuge, washed with 0.02 M-phosphate buffer pH 7 (50 ml.) and finally suspended in 5 ml. buffer or water to give a concentration of *c.* 5 mg. dry wt. organisms/ml. The relationship between dry weight and instrument reading of either the EEL. or the Hilger Spekker photoelectric colorimeter was established. A reading on the EEL. instrument of 23.5 corresponded to a suspension of 0.5 mg. dry wt. organisms/ml. Suspensions prepared in this way were often used at once, but could be stored at +2° for at least a week in semi-anaerobic conditions with only slight loss of activity.

Activity of suspensions. Two main methods were used to explore the specificity of the harvested organisms and to characterize the process by which creatine was destroyed.

(1) One of the objects of this work was to isolate an organism which could destroy creatine but not other compounds which give a colour with diacetyl and α -naphthol in alkaline medium (Voges & Proskauer, 1898; Barritt, 1936). In all the earlier experiments, therefore, organisms were shaken with a known concentration of each compound to be tested, and the amount removed

determined colorimetrically. Once they had been established, the conditions optimal for creatine removal were used in the specificity tests. Thus a volume of 4.0 ml., containing 0.5–2.0 mg. dry wt. organisms, 1.0 mg. creatine or an equivalent weight of one of the other substrates, and buffered at pH 7.8 with 0.05 M-phosphate, was shaken for 30–120 min. at 30°. The reaction was stopped by adding 6.0 ml. of a solution of *p*-chloromercuribenzoic acid (0.67 g./l.).

The organisms were removed by centrifuging and the amount of substrate which remained in the supernatant was estimated by the method of Eggleton, Elsdon & Gough (1943), as modified by Ennor & Stocken (1948). For each compound tested the rate of colour-production and the relationship between concentration and colour intensity were established. The colour produced by creatine and by dimethylguanidine was measured 20 min. after the addition of reagent; the colour produced by all other compounds was read at 40 min. Creatinine was estimated by the Jaffe reaction.

(2) In the second method the conventional Warburg manometric technique was used, both to confirm some of the findings in the specificity tests and also to study more closely the kinetics and products of creatine catabolism. Usually, 1.0 ml. suspension (equivalent to *c.* 5 mg. dry wt. organisms) and 1.0 ml. 0.2 M-phosphate buffer, pH 7.8, were placed in the main compartment of the flask and 0.5 ml. 0.033 M-creatine tipped in from the side-bulb after equilibration at 30°. When ammonia was to be estimated the reaction mixture was buffered at pH 7.0. The reaction was usually stopped by heating the contents of the flasks for 5 min. in a boiling water-bath; after centrifuging the supernatant fluid was used for analysis.

Creatine was estimated as above. It was found (D. D. Woods, unpublished observations) that ammonia could be estimated in the presence of urea by distilling in the Markham (1942) apparatus after the addition of 0.35 M-borate buffer of pH 8.5. Urea was determined by the difference in ammonia content of the solution before and after incubation with urease.

Chemicals. Some of the compounds listed in Table 5 were gifts generously made. We are indebted to Imperial Chemical Industries (through the courtesy of Dr F. L. Rose) for compounds 2 to 8, to Dr H. King for compounds 9 to 12 and 15 to 17, and to Dr P. C. Spensley for compound 13. We are grateful also to Dr L. A. Stocken for a generous supply of *p*-chloromercuribenzoic acid. The other materials used were of commercial origin and, with two exceptions, were not further purified. Creatine was recrystallized according to Hunter (1928), and the sample of decamethylenediguanidine was converted from the carbonate to the hydrochloride.

RESULTS

Isolation of the organisms

The medium originally used was similar to that described above, but without yeast extract and containing 0.5 % (w/v) creatine; it was buffered to pH 6, 7 or 8. Samples (25 ml.) in 250 ml. Erlenmeyer flasks were inoculated with *c.* 1 g. garden soil from several sources, and incubated at 18, 25, 30 and 37°. After

incubation for 3 days diffuse microbial growth was seen in those flasks which had been inoculated from ground recently used as a chicken-run. Growth was heaviest at 25 and 30°, but seemed to be unaffected by the pH range covered. Morphological appearance and the production of a greenish yellow pigment with an electric-blue fluorescence in ultraviolet light suggested that it was the same organism growing in each flask. The culture obtained at 30° and pH 7 was subcultured five times in these conditions, with 2 days of incubation on each occasion. The fifth subculture was plated on the creatine-containing medium solidified with 1·5 % (w/v) agar, and single colonies isolated. Tested on a variety of media the organism behaved as though in pure culture and was considered to be in a state suitable for further investigation.

As stated above, the organism is probably a strain of *Pseudomonas ovalis* Chester. It was isolated only from soil taken from this one situation; even after 14 days of incubation flasks inoculated with soil from other sources showed no visible growth.

Fig. 1. Effect of yeast extract and of casein hydrolysate on growth of *Pseudomonas ovalis*. Basal medium supplemented with 5 mg./ml. creatine (A), creatine + 0.5 mg./ml. Difco yeast extract (B), creatine + 0.1 mg./ml. acid-hydrolysed casein (C), yeast extract alone (D) or acid-hydrolysed casein alone (E). Incubation at 30° in sloped test-tubes.

Some growth properties of the organism

The organism grew very well on all the common laboratory media tested, but only under aerobic conditions.

Effect of yeast extract. In the simple medium with creatine as only added organic constituent growth was less rapid than in more complex media. Inclusion of 0.05 % (w/v) yeast extract decreased the lag period and somewhat increased the rate and extent of growth (Fig. 1). Yeast extract was normally

included in the medium used for growth tests and always when the cultures were to prepare suspensions of organisms.

An attempt was made to discover what component of the yeast extract was responsible for its stimulatory effect. Several vitamins were tested, singly or in groups, at various concentrations. Neither biotin, pyridoxin, pyridoxal, nicotinic acid, pantothenic acid, riboflavin, thiamine nor *p*-aminobenzoic acid improved growth. Acid-hydrolysed casein caused a similar marked decrease in the lag period (Fig. 1) which could not be traced to the effect of any particular amino acid. The single addition of almost any amino acid has some effect; glutamic acid (and glutamine), aspartic acid, histidine, proline, and hydroxyproline were the most active. Both yeast extract and acid-hydrolysed casein also improved growth on the basal medium containing glucose and ammonium salts (quantities as in Table 1) in place of creatine. In the presence of yeast extract the further addition of either glucose or of ammonium salt to the creatine medium led to a considerable increase in total growth (Table 4).

pH range. Growth of the organism was remarkably independent of hydrogen-ion concentration, and was almost equally good between pH values of 5.4 and 9.0 with an ill-defined optimum in the region of pH 8; pH 7.0 (which was also about the optimum for creatine destruction) was chosen for all subsequent work.

The importance of other ions was also investigated briefly. A fairly high concentration of NaCl was found to be obligatory; below 0.1 % (w/v) growth was suboptimal, and above 2 % the salt was inhibitory. Traces of Ca, Mg, Mn and Fe were essential.

Temperature. A temperature of 30° was near the optimum for growth. Incubation at 25° resulted in a slight decrease; raising the temperature to 37° decreased growth at 24 hr. to about one-half of that at 30°.

Aeration. Roux bottles incubated horizontally provided the largest crops. Growth after 24 hr. was decreased considerably when the bottles were kept in the vertical position, or even when they were sloped so as to present half the normal surface area to the atmosphere.

An attempt was made to increase the crop by cultivating in medium continuously agitated by a magnetic stirrer. Although this procedure must have resulted in increased aeration, growth after 24 hr. was only one-third of that in undisturbed Roux bottles. A similar decrease was observed in Roux bottles which were shaken by hand every few hours. To test whether a raised oxygen tension might be inhibitory, a Roux bottle was filled with oxygen after inoculation; growth at 24 hr., however, was as good as in a bottle exposed to air in the usual way.

Substrates for growth. In testing the ability of the organism to use a variety of single substances for growth these were added to the basal medium without yeast extract to give a concentration of 0.3 % (w/v); when pairs of substances were tested each was present at 0.25 %. Failure to grow was recorded when no visible growth occurred after 3 to 4 days. A number of nitrogenous organic compounds were tested for their ability to support growth (Table 1); non-nitrogenous organic compounds were tested in the presence of ammonium

salts. Of most interest was the limited ability to grow on guanidine derivatives; of those tested only creatine, arginine and the amine of arginine, agmatine, supported full growth. There was a limited ability to use guanidine itself, but none to use creatinine or glycoeyamine. Urea was used as a nitrogen source (in the presence of glucose as a carbon source), and sarcosine as source of both nitrogen and carbon.

Table 1. *Substances tested for their ability to support growth of Pseudomonas ovalis Chester as source of C and N*

Single compounds at 0.3 % (w/v) in the basal medium; NH_4^+ salt and carbon source at 0.25 % each. 0 = no growth up to 5 days; + + + + = abundant growth, etc.

(a) Guanidine derivatives			
Creatine	+ + + +	Methylguanidine	0
Creatinine	0	Dimethylguanidine	0
Glycoeyamine	0	Ethylguanidine	0
Arginine	+ + +	Tetramethylenediguanidine	0
Agmatine	+ + + +	Decamethylenediguanidine	0
Guanidine	+		
(b) Possible primary breakdown products of creatine			
Sarcosine	+ + + +	Urea + glycine	0
Glycine	0	Urea + glucose	+ + + +
Urea	0		
(c) Amino acids and miscellaneous compounds			
Alanine	$\pm$	Asparagine	+
Serine	$\pm$	NH_4 + glucose	+ + + +
Aspartate	0	NH_4 + acetate	++
Glutamate	0	NH_4 + citrate	+
Methionine	0	Choline	+ + +
Tryptophan	0	Indole	0

Action of suspensions on creatine

When suspensions of organisms harvested from the creatine medium were incubated aerobically with creatine, the substrate disappeared at a uniform rate until all had been removed (Fig. 2). Organisms destroyed their own (dry) weight in 1–2 hr.

The disappearance of creatine was accompanied by uptake of O_2 and by formation of CO_2 , NH_3 and urea. In the absence of any added substrate suspensions of organisms had a Q_{O_2} of 20–30; this endogenous uptake was always subtracted from the values observed in the presence of substrate to give the recorded values. In the presence of creatine the corrected Q_{O_2} was about 100 and O_2 uptake continued at a regular rate during the removal of creatine. At, or slightly after, the time when the creatine had completely disappeared the rate of O_2 consumption abruptly fell to a much lower level. Yet even when incubation was continued for many hours after the 'break' in the curve, the rate of O_2 consumption never fell quite to the value without substrate (Fig. 2).

At the time of the 'break' in the O_2 uptake curve about 1.4 mole O_2 /mole creatine (limits 1.3–1.5 mole) were taken up. Production of CO_2 followed a

course very similar to that of O_2 uptake (Fig. 2). At the 'break' about 1.5 mole CO_2 /mole creatine were formed.

Fig. 2. Uptake of O_2 and evolution of CO_2 in relation to creatine disappearance. Organisms (equiv. to 5 mg. dry wt.) in 0.08 M-phosphate buffer (pH 7.8) with 16.7μ mole creatine; total volume 2.5 ml. Incubated at 30° in air.

Fig. 3. Creatine removal (x), O_2 uptake (●), urea (○) and NH_3 (▲) formation by suspensions of *Pseudomonas ovalis*. Organisms 6 mg. (dry wt.); other additions as in Fig. 2.

About 0.7 mole NH_3 (limits 0.65–0.75) was formed (Fig. 3). On two occasions ammonia was estimated as volatile base in the Markham apparatus and by its colour production with Nessler's reagent. At the concentrations used

methylamine, a possible product of creatine breakdown, behaved as ammonia on steam distillation but gave no colour with Nessler's reagent. Since the values given by the two methods were in excellent agreement it was concluded that no methylamine was being produced. At all stages of the reaction 1 mole urea/mole creatine was formed (Fig. 3).

Amino acids were sought by paper partition chromatography, fatty acids by distillation with phosphoric acid in the Markham apparatus, and formaldehyde by its colour reaction with chromotropic acid. None of these compounds was detected in the reaction mixture.

Oxidative assimilation. The degradation of creatine to CO_2 , ammonia and urea required in theory the appearance of 3 mole CO_2 and one each of the other two products, together with the uptake of 3 mole O_2 . The considerable discrepancies between the observed values (with the exception of urea) and the theoretical suggested either that there were undetected products or that some of the creatine might be undergoing oxidative assimilation (Clifton, 1946). To test the latter possibility organisms were incubated with creatine in the presence of sodium azide or 2:4-dinitrophenol. Different batches of organisms varied considerably in their sensitivity to azide and the optimal concentration varied by as much as a factor of three from one suspension to another. In the presence of that concentration of azide (*c.* 10^{-3}M) which had the most marked effect the O_2 consumption at the 'break' was increased only by a factor of about 1.2 (Table 2); there was a similar proportionate increase in CO_2 and NH_3 production. It is clear that it is not possible to account for the missing creatine by oxidative assimilation unless this is considerably greater than it was possible to demonstrate.

Table 2. *Effect of azide on the oxidation of creatine by Pseudomonas ovalis Chester*

Organisms (equiv. to 4 mg. dry wt.) were incubated in 0.067 M-phosphate buffer (pH 7) with $16.7\mu\text{mole}$ creatine in presence or absence of sodium azide ($2\mu\text{mole}$); total volume 3 ml. The reaction was stopped after 100 min.; the 'break' in O_2 uptake occurred after 60 min. in absence of azide and after 90 min. in its presence. All creatine had been removed in both cases.

	Moles/mole creatine removed		
	Azide absent	Azide present	Theory*
O_2 taken up	1.55	1.95	3.0
CO_2 formed	1.74	2.01	3.0
NH_3 formed	0.70	0.81	1.0
Urea formed	0.99	1.01	1.0

* For complete oxidation according to

Several attempts were made to confirm this 'uncoupling' effect with dinitrophenol. On only one occasion was the O_2 uptake increased (to 2.0 mole); at all other times dinitrophenol was without effect.

Kinetics of creatine destruction

Concentration of organisms. Below a concentration of *c.* 3 mg. dry wt. organisms/ml. the rate of creatine removal and O₂ uptake was directly proportional to the concentration of organisms. Above this concentration O₂ consumption increased only slowly and was never more than 1000 μ l./hr. At high suspension densities the factor limiting rate of O₂ uptake appeared to be the rate at which O₂ diffused into the reaction mixture or into the organisms. When the flasks were filled with O₂ instead of with air a far greater rate of uptake was attained (Fig. 4).

Fig. 4

Fig. 5

Fig. 4. Effect of O₂ tension on activity of suspensions of *Pseudomonas ovalis*. Organisms (different concentrations) with other additions as in Fig. 2. Shaken at 30° in air (●) or O₂ (○). Not corrected for endogenous O₂ uptake.

Fig. 5. Influence of initial creatine concentration upon rate of creatine removal by *Pseudomonas ovalis*. Different amounts of creatine were incubated with suspensions of organisms equivalent to 1.76 mg. dry wt. in 4 ml. 0.05 M-phosphate buffer (pH 7) for 50 min. at 30°.

Creatine concentration. Oxygen uptake reached a maximum rate when the creatine concentration was about 10^{-2} M. The rate of creatine disappearance, however, continued to increase up to at least 4.7×10^{-2} M, the highest concentration tested. Over almost a 100-fold range of concentration there was a linear relationship between the logarithm of the initial creatine concentration and the logarithm of its rate of destruction (Fig. 5).

pH range. The optimum pH for destruction of creatine in 0.05 M-phosphate buffer was in the region of 8.0. There was no sharp peak in the pH-activity curve (Fig. 6). In spite of the slightly decreased activity in the presence of 0.05 M-borate a much better-defined optimum than with phosphate was found at pH 8.25 (Fig. 6). Where comparison could be made the rate of creatine

removal was even less in the presence of veronal; for instance at pH 7·8 the rate was one-third of the rate in phosphate. As the pH value was increased to the end of the veronal range (about pH 9·4) the rate rose steadily to 70 % of that at the phosphate optimum.

Fig. 6

Fig. 7

Fig. 6. Effect of pH and of nature of buffer on creatine destruction of *Pseudomonas ovalis*. Four ml. of 0·05 M-phosphate (●), borate (○), or veronal (×) buffer containing the equivalent of 1·08 mg. dry wt. organisms and 1·0 mg. creatine; incubated at 30° for 45 min.

Fig. 7. Inhibition by *p*-chloromercuribenzoate of creatine removal by *Pseudomonas ovalis*. Organisms (equivalent to 1·35 mg. dry wt.) in 4 ml. 0·05 M-phosphate buffer (pH 7) with 1·0 mg. creatine; incubated 1 hr. at 30°.

Inhibitors. *p*-Chloromercuribenzoic acid (as the sodium salt) completely inhibited destruction of creatine. With a suspension equivalent to 0·2 mg. dry wt. organisms/ml. 50 % inhibition was induced by $6·6 \times 10^{-5}$ M-*p*-chloromercuribenzoate (Fig. 7). In a further experiment, with a suspension of organisms ten times more concentrated, O₂ uptake was inhibited 50 % by a $4·5 \times 10^{-5}$ M-*p*-chloromercuribenzoate.

During investigation of the effect of pH value upon the reaction it was discovered that acetate caused a powerful inhibition (Fig. 8). Other fatty acids were also inhibitory, the order of decreasing activity being acetate, propionate, butyrate, formate; these tests were made at pH 6. Creatine removal was inhibited 50 % by acetate at 5×10^{-3} M, propionate at 7×10^{-3} M and butyrate at 2×10^{-2} M. Formate was much less effective; at 5×10^{-2} M (the highest concentration tested) it produced only 25 % inhibition.

Inhibition by acetate was completely independent of creatine concentration. A concentration of acetate expected to cause about 50 % inhibition was added to a series of flasks, in which the creatine concentration was varied over a 64-fold range. The degree of inhibition varied only between 50 and 65 %.

Loss of activity on storage and washing. Harvested organisms lost some activity either during storage at 0° (especially when exposed to air) or on further washing with phosphate buffer (Table 3). Activity was partly or wholly restored by Difco yeast extract (1 mg./ml.) or by DL-cystine (125 mg./ml.).

Table 3. *Loss of creatine-destroying activity induced by washing Pseudomonas ovalis with phosphate buffer, and stimulation by yeast extract*

Organisms (equivalent to 0.55 mg. dry wt.) were incubated for 1 hr. at 30° in a volume of 4 ml. at pH 7.8 with 1000 µg. creatine, with or without yeast extract (1 mg./ml.). Organisms from normal suspensions (once washed) were centrifuged and washed repeatedly with 0.02M-phosphate buffer pH 7; samples were taken after each washing.

Number of extra washes	Suspension	
	No yeast extract	With yeast extract
	Creatine removed (µg.)	
0	473	552
1	308	496
2	200	400
3	185	420

Adaptive nature of creatine oxidation

Only organisms grown with creatine as main carbon and nitrogen source possessed full ability immediately to remove creatine. Organisms grown with either arginine or with glucose+ammonia removed no creatine during an incubation period (1 hr.) when creatine-grown organisms had removed the whole of the substrate. When incubation was continued beyond 1 hr. organisms grown in the absence of creatine began slowly to oxidize this compound and continued at an increasing rate (Fig. 9).

As described earlier, the addition of either glucose, or of ammonium salt, or of both, considerably increased the total amount of growth in the creatine medium. Organisms grown in this way, however, showed a loss of intrinsic activity which more than offset the increased growth (Table 4). Organisms were also grown on the usual creatine medium solidified with 3% (w/v) agar; growth was then about 70% better than in the liquid medium, but activity/amount of organism was decreased almost proportionately.

Specificity of action of suspensions

Organisms were grown on media containing creatine, arginine, or glucose+ammonium salt and their activity towards a number of guanidine derivatives and related compounds was tested. Some of the results of such experiments, in which the criterion of activity was the disappearance of substrate, are summarized in Table 5. Tests on the specificity of creatine-grown organisms were extended to include several further compounds which develop a colour with diacetyl and α -naphthol in alkaline solution. None of the compounds tested was attacked (Table 6). In addition, manometric experiments showed that organisms grown in a creatine medium were unable to oxidize creatine

Table 4. *Effect upon growth of Pseudomonas ovalis and upon creatine-oxidising activity of supplementing the creatine medium with ammonium salts and glucose*

Cultures of *P. ovalis* were incubated in Roux bottles at 30° for 24 hr.; the normal creatine-containing medium had the supplements stated. Organisms (equivalent to 8–16 mg. dry wt.) incubated in 0.08 M-phosphate buffer (pH 7.8) with 16.7 μ mole creatine. Endogenous O₂ uptake not subtracted.

Supplement to growth medium	Growth (mg. dry wt./100 ml.)	Q _{O₂} with creatine
(1) Nil	38	124
(2) (NH ₄) ₂ SO ₄ , 0.25 % (w/v) + NH ₄ Cl, 0.25 % (w/v)	51	86
(3) Glucose, 0.5 % (w/v)	69	50
(4) (2) + (3)	83	33

Table 5. *Influence of the growth substrate upon the relative ability of suspensions of Pseudomonas ovalis to remove various substrates*

Suspensions of organisms (equivalent to 1.0–1.3 mg. dry wt.) harvested from the basal medium with indicated supplements and incubated for 1 hr. with 1.0 mg. creatine or an equivalent weight of the other substrates.

Substrate	Relative rate* of removal of substrate by organisms grown on		
	Creatine	Arginine	NH ₄ ⁺ + glucose
Creatine	100	0	0
Arginine	10	100	0
Agmatine	30	50	0
Glycoeyamine	0	0	0
Creatinine	0	0	0

* Rate of removal of creatine by creatine-grown organisms = 100.

Table 6. *Guanidino compounds not attacked by creatine-grown Pseudomonas ovalis*

(a) Monoguanidino derivatives

No.	R =
1	—H
2	—CH ₃
3	—CH ₂ —CH ₃
4	—(CH ₂) ₂ —CH ₃
5	—CH=(CH ₃) ₂
6	—(CH ₂) ₃ —CH ₃
7	—CH ₂ —CH=(CH ₃) ₂
8	—(CH ₂) ₂ —N=(C ₂ H ₅) ₂
9	—(CH ₂) ₁₁ —CH ₃
10	—(CH ₂) ₁₃ —CH ₃
11	—(CH ₂) ₁₅ —CH ₃
12	—(CH ₂) ₁₇ —CH ₃
13	—2-benziminazole

(b) Diguanidino derivatives

No.	n =
14	4 (arcaine)
15	5
16	6
17	8
18	10 (synthalin)
19	12 (synthalin B)

phosphate, dimethylguanidine, hydantoic acid or urea oxalate. Absence of O_2 uptake confirmed their inability to oxidize guanidine, methylguanidine or creatinine.

When creatine-grown organisms were incubated in the usual way with glycoeyamine, O_2 consumption was about 50 % higher than the endogenous value; i.e. there was a Q_{O_2} of about 10 apparently due to the oxidation of glycoeyamine. On the other hand, colorimetric estimation showed no loss of glycoeyamine, and even after 5 hr. of incubation, no urea or ammonia was detected

Fig. 8

Fig. 9

Fig. 8. Inhibition by fatty acids of *Pseudomonas ovalis*. Organisms (equivalent to 1.17 mg. dry wt.) incubated as in Fig. 7 except that buffer was pH 6. Acids brought to pH 6 before addition.

Fig. 9. Effect of growth substrate upon oxidation of creatine by suspensions of *Pseudomonas ovalis*. Organisms (equivalent to 4.5 mg. dry wt.) grown on medium containing creatine (A) or glucose and ammonium salts (B) and incubated in 0.08 M-phosphate buffer (pH 7.8) with 16.7 μmole creatine.

in the reaction mixture. It was thought just possible that glycoeyamine was being converted to guanidine (by oxidation of the acetic acid side-chain) or to some other guanidino compound giving a similar colour reaction; yet paper chromatography gave no evidence of this. It was concluded that such suspensions of organisms were unable to oxidize glycoeyamine.

DISCUSSION

The micro-organism isolated in the present work appears to produce an adaptive system of enzymes which enable it to grow with creatine as main source of carbon and nitrogen. It differs from other organisms having this property in its high degree of specificity. In particular it does not attack creatinine; it is unable to grow on a medium containing creatinine as main organic constituent,

and washed organisms do not destroy creatinine. The only other guanidine derivatives which are attacked by creatine-grown organisms are arginine and agmatine, which are destroyed relatively slowly. The mechanisms of degradation of these two compounds are probably different from that of creatine, since organisms grown on an arginine medium are unable to metabolize creatine. This degree of specificity provides the basis for a method for the selective destruction of creatine in a mixture which also contains creatinine or other guanidine derivatives. It has been exploited by Ennor & Stocken (1953) in determining the urinary excretion of creatine.

Of the products of the reaction in which creatine was destroyed only urea was found in theoretical yield. The formation of 1 mole urea from 1 mole guanidine derivative appears to be a rather constant finding in other cases where this point has been investigated (Dubos & Miller, 1937; Kopper & Beard, 1947; Krebs & Eggleston, 1939; Roche *et al.* 1948). An exception to this was found with arginine; Hills (1940) showed that certain Gram-positive cocci contain an enzyme, arginine dihydrolase, which hydrolyses arginine directly to ornithine, NH_3 and CO_2 without the intermediate formation of urea. Roche, Lacombe & Girard (1950) studied the action on arginine of growing cultures of our strain of *Pseudomonas ovalis* Chester, and concluded that it also produces an arginine dihydrolase.

Further work on the reactions involved in the degradation of creatine by this organism is described in the following paper (Appleyard & Woods, 1956).

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