THE ADAPTIVE FORMATION OF UREASE BY WASHED SUSPENSIONS OF PSEUDOMONAS AERUGINOSA

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During an investigation of nitrogen assimilation by a strain of *Pseudomonas aeruginosa* it was found that the nitrogen of ammonium sulfate was assimilated only during the course of oxidation of appropriate substrates (Berhneim and DeTurk, 1951). At this time it was also shown that this organism was able to form ammonia from urea, uric acid, xanthine, and hypoxanthine during the oxidation of substrates, but was unable to do so in the absence of such substrates. The formation of ammonia from urea and purines has been shown to depend upon the adaptive formation of urease, and the present observations are concerned with the conditions which affect the formation of this enzyme.

MATERIALS AND METHODS

A strain of *Pseudomonas aeruginosa* was grown for 24 hours in 100 ml of nutrient broth (Difco) in Erlenmeyer flasks. The cells were harvested, washed once with distilled water, and resuspended in 7 ml of Na-K-phosphate buffer, 0.05 M and pH 7.7. Oxygen consumption and carbon dioxide production were measured at 36 C in Warburg vessels. The gas phase was air. Each vessel contained 0.5 ml of a suspension of cells in a total volume of 2 ml. At the conclusion of experiments the entire flask contents or aliquots were taken for analyses. Uric acid was estimated by the method of Benedict and Franke (1922) on trichloracetic acid filtrates (3.3 per cent). Urea estimations were made on Somogyi filtrates by the method of Archibald (1945) or manometrically according to Umbreit, Burris, and Stauffer (1949). In the manometric estimation Arlington urease was used. Ammonia was estimated by the method of Speck (1949) on trichloracetic acid filtrates.

RESULTS

When washed cells were incubated with urea, it could be quantitatively recovered after 5 hours. When succinate was also present, it was oxidized, and the urea disappeared after a short latent period of 30 minutes. When urea was added after the succinate had been completely oxidized, it disappeared without a latent period. Under the latter circumstances no ammonia was assimilated, and the theoretical amounts of ammonia and carbon dioxide were obtained. Urea disappearance following the oxidation of 1.0 and 0.5 mg of succinic acid is shown in figure 1. It can be seen that the *rate* of urea disappearance is a function of the amount of

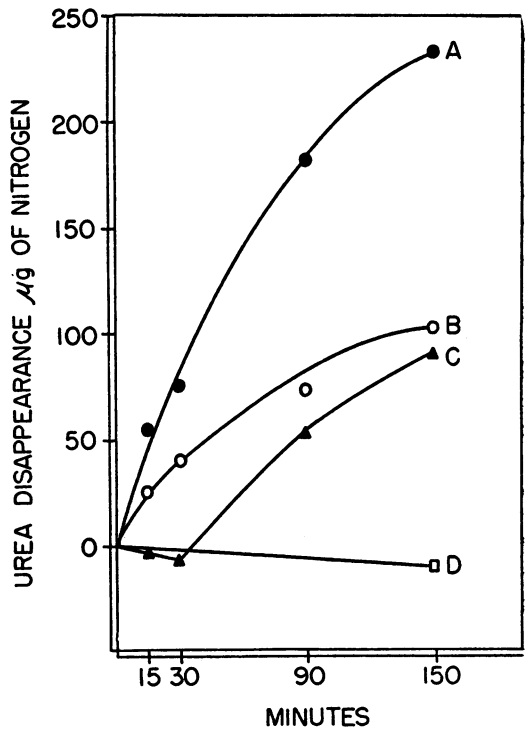

Figure 1. The effect of succinate oxidation on urea disappearance.

A. 0.5 mg of urea added after oxidation of 1.0 mg of succinic acid; B. 0.5 mg of urea added after oxidation of 0.5 mg of succinic acid; C. 0.5 mg of urea added together with 1.0 mg of succinic acid; D. 0.5 mg of urea.

succinate which has been previously oxidized, and this holds true over a range of 0.05 to 1.0 mg of succinic acid. The amount of succinate oxidized, however, does not determine the final *amount* of urea which can be degraded.

The urease activity could be demonstrated in broken cell suspensions. When suspensions of cells were incubated in Erlenmeyer flasks until succinate oxidation was complete, the urease activity remained after the cells were broken by sonic vibration. Broken cell suspensions similarly treated, but without previous succinate oxidation, showed no activity. Heating for 10 minutes at 100 C destroyed the activity, and the addition of heat-inactivated suspension to suspensions which had not oxidized succinate did not confer activity. When active broken cell suspensions were centrifuged for 2 hours at 80,730 × G, the activity remained entirely in the supernate. The enzyme was susceptible to heavy metals. $HgCl_2$ (9×10^{-6} M) inhibited 99 per cent, and Na_2HAsO_3 (1.5×10^{-4} M) inhibited 11 per cent KCN (0.5 mg), streptomycin (0.2 mg), terramycin (0.1 mg), and chloramphenicol (0.1 mg) did not inhibit. The enzyme is quite specific. No ammonia was derived from the following compounds: thiourea, formamide, oxamic acid, oxamide, guanidine, and aminoguanidine. Small amounts were sometimes derived from acetamide.

These observations indicate that the urea splitting ability is due to a soluble enzyme. The sensitivity to metals and the specificity suggest that it is urease. The experiments with broken cell suspensions indicate that in the absence of previous oxidation the lack of activity cannot be attributed to failure of urea to penetrate into the cell.

It seems most likely that the enzyme is adaptively formed and depends upon oxidation to supply the energy for its synthesis. The amount of oxidation would then determine the amount of enzyme formed. Dinitrophenol during the oxidation of succinate inhibited the production of enzyme as might be expected (table 1), and streptomycin, which also inhibits the formation of adaptive enzyme (Fitzgerald and Bernheim, 1948), inhibits the development of urease activity. NaF for unexplained reasons greatly increased the subsequent urease activity, when added during the period of oxidation, but had less effect when added later with urea. It did not affect the rate of succinate oxidation in any of the concentrations used.

TABLE 1

The effect of ammonium chloride, urea, dinitrophenol, and sodium fluoride on the development of urease activity

	μg Urea-N Disappearance		Time after Addition of Urea
	Additions with succinic acid	Additions after succinic acid	
0.5 mg succinic acid	40	—	60 minutes
+0.05 mg DNP	21	34	
+0.1 mg DNP	9	46	
+0.05 mg NaF	66	—	
+0.1 mg NaF	70	46	
+0.5 mg NaF	50	45	
1.0 mg succinic acid*	128	—	60 minutes
+10 μg NH_4Cl-N	102	134	
+20 μg NH_4Cl-N	73	134	
+30 μg NH_4Cl-N	32	136	
+40 μg NH_4Cl-N	4	139	
1 mg succinic acid	74		30 minutes
+20 μg urea	52		
+40 μg urea	36		
+60 μg urea	15		

Vessels contained washed cells and succinic acid as indicated. 0.5 mg of urea was added from side bulbs after the succinate was oxidized. After a further period as indicated, the reaction was stopped and urea estimated. The first column shows urea disappearance when NH_4Cl, urea, DNP, or NaF was incubated with the succinic acid. The second column shows urea disappearance when these were added together with urea at the end of succinate oxidation.

* All vessels in this experiment contained 1×10^{-4} M Na_2SO_4 to maintain maximum nitrogen assimilation.

Ammonia assimilation takes place only during the course of oxidation and probably depends upon oxidation for energy. Ammonium chloride, present during the oxidation of 1.0 mg of succinic acid, was assimilated, and the assimilation was associated with a suppression of urease formation (table 1). The suppression of enzyme formation by assimilation increased linearly with increasing amounts of NH_4Cl. With 1.0 mg of succinic acid and 40 μg of nitrogen as ammonium chloride, no urease activity de-

veloped. This amount of nitrogen, which completely suppressed enzyme synthesis, is the amount which can be assimilated by 1.0 mg of succinic acid. Small amounts of urea present with the succinic acid behaved in the same way as ammonia and reduced the development of urease activity. It would appear that the ammonia initially formed from urea is assimilated at the expense of further enzyme formation. Indeed when ammonia, urea, and succinate were all added at the beginning of the experiment, no urea disappeared, i.e., no urease was formed. The amount of ammonia assimilated, however, was the same in the presence of urea as in its absence. This indicates that ammonia assimilation takes precedence over enzyme formation.

The development of urease activity was not peculiar to succinate oxidation. Glucose, citrate, α-ketoglutarate, fumarate, oxalacetate, pyruvate, and acetate were also effective, though their effectiveness varied on a molar basis. Citrate and α-ketoglutarate were highly effective, whereas pyruvate and acetate were least effective. In general the substrates which were most effective in promoting urease activity were also most active in promoting nitrogen assimilation.

Organisms were grown in nutrient broth to which had been added 0.1 per cent of urea, 0.5 per cent of succinic acid, or both. Washed urea-grown cells showed no urease activity, and no urea disappeared from the medium during growth. Succinate-grown cells split urea equivalent to 117 μg of nitrogen in one hour, whereas cells grown in both succinate and urea split urea equivalent to only 13 μg of nitrogen. During growth, there was a small loss of urea from the succinate-urea medium amounting to 40 μg of nitrogen per ml. All types of cells, regardless of the growth medium, developed urease activity during the *in vitro* oxidation of succinic acid. The ammonia-nitrogen in the medium increased 34 to 36 μg per ml during growth in broth, urea, and succinate-urea media but remained at the initial value of 12 μg per ml during growth in succinate medium.

These experiments indicate that enzyme is formed in large amount only during growth in succinate. No enzyme is formed during growth in urea and a very lit le during growth in both succinate and urea. These findings suggest that in nutrient broth nitrogen requirements are met through the assimilation of ammonia from the deamination of amino acids, peptides, and other nitrogenous constituents. Under these circumstances urease would be formed only when oxidation was in excess of that required for nitrogen assimilation since ammonia is preferentially assimilated. Such conditions would exist in media enriched with succinate. In media containing both succinate and urea, enzyme formation is held at a minimum by the ammonia initially formed from urea.

When uric acid was added to washed cell suspensions, it was slowly oxidized at a rate of about 30 μL of oxygen per hour. This is approximately the rate of the endogenous respiration. Under these circumstances uric acid disappeared from the medium, but no ammonia was formed. When uric acid was added together with succinate or following the completion of succinate oxidation, it was oxidized at the same slow rate. Under these conditions, however, ammonia appeared in the medium. Broken cell suspensions were inactive. Table 2 shows the oxygen consumption and carbon dioxide evolution from 2.38 μM of uric acid. With washed cells, repeated experiments gave values of 1.1 to 1.2 moles of oxygen and 2.1 to 2.8 moles of carbon dioxide per mole of uric acid. When the uric acid was added after the complete oxidation of 1.0 mg of succinic acid, the total oxygen consumed was unchanged, but carbon dioxide production was increased to approximately 4 moles per mole of uric acid. These later values are ½ mole of oxygen and one mole of carbon dioxide less than that theoretically required if uric acid is metabolized to carbon dioxide, water, and

TABLE 2

Oxygen consumption and carbon dioxide production from uric acid

	O_2 Consumption		CO_2 Production	
	μL O_2	Moles O_2 per mole of uric acid	μL CO_2	Moles CO_2 per mole of uric acid
Cells + 2.38 μM uric acid	60	1.1	114	2.1
Cells + 2.38 μM uric acid added after oxidation of 1 mg succinic acid	60	1.1	199	3.7

TABLE 3

The production of urea and ammonia from uric acid by washed cells and cells which have oxidized succinic acid

	Urea Archibald Method		Urea Manometric Method		NH_3-N	
	μM per ml	Moles per mole of uric acid	μM per ml	Moles per mole of uric acid	μM per ml	Moles per mole of uric acid
A. Cells	0.03		0		0.45	
B. +1.628 μM uric acid per ml	3.6	2.2	2.7	1.7	0.05	0.3
C. +1.628 μM uric acid per ml, after oxidation of succinic acid	0.03	0.02	0	0	5.1	3.1

Cells from 300 ml of culture were divided among 3 flasks. 13.0 mg of succinic acid were added to flask C, and when oxidation ceased, uric acid was added to flasks B and C. Aliquots were immediately taken for estimations of initial urea and ammonia, and when uric acid oxidation ceased, final estimations were made. The small initial valves have been subtracted.

ammonia by way of allantoin, allantoic acid, urea, and glyoxylic acid. However, study of these intermediates showed that glyoxylic acid was not completely oxidized. It consumed only ½ mole of oxygen and evolved only one mole of carbon dioxide per mole of substrate. Accordingly the values for cells which have had a previous period of oxidation are in keeping with the metabolism of uric acid by this pathway. Campbell (1954) has recently shown that an unidentified species of *Pseudomonas* oxidizes allantoin *via* this pathway. In this organism, however, glyoxylic acid was completely oxidized to carbon dioxide and water. Urea was one of the end products.

Table 3 shows the results of analyses for ammonia and urea. It can be seen that control cells accumulated urea in approximately the theoretical amount of 2 moles per mole of uric acid and that no ammonia was formed. Cells which had oxidized succinate, however, accumulated ammonia instead of urea. In other experiments ammonia values more nearly approached the theoretical value of 4 moles per mole of uric acid, and it may be that there was a small amount of assimilation at times. The agreement between the colorimetric and urease methods for urea estimation leaves little doubt that the material which accumulates is urea rather than allantoic acid or another intermediate since the manometric method did not evolve carbon dioxide from allantoic acid, pyruvate, or casein hydrolyzate under the conditions of the assay. Allantoin and allantoic acid were oxidized even more slowly than uric acid. This may be due to inability to gain access to the cell. However, these intermediates did provide small but definite increases in ammonia when incubated with cells which had oxidized succinate. As in the case of urea, ammonia production from uric acid was diminished when NH_4Cl was present during the period of succinate oxidation, and the presence of small amounts of uric acid during the oxidation produced the same result.

DISCUSSION

These findings show that washed cells which have not oxidized substrate are incapable of attacking urea. When urea is added together with an oxidizable substrate, there is a latent period following which urea disappears and ammonia is produced. When the substrate is completely oxidized before the addition of urea, there is no latent period. This suggests the adaptive formation of urease in the absence of added urea. The enzyme is not associated with the particulate fraction of broken cell suspensions and appears to be urease as evidenced by its specificity and sensitivity to metals. It would appear that oxidation is necessary as a source of energy for the adaptation since urease is not an oxidative enzyme.

In the absence of previous oxidation, uric acid is slowly oxidized by washed cells, and 2 moles of urea are produced per mole of uric acid oxidized. The oxygen consumption and carbon dioxide evolution are compatible with an oxidative pathway involving allantoin, allantoic acid, and glyoxylic acid. The latter, however, is not oxidized to completion, and urea is one of the end products.

In cells which have oxidized a substrate before the addition of uric acid, no urea accumulates. Instead ammonia is formed, and 2 additional moles of CO_2 are evolved per mole of uric acid. It seems likely that small amounts of endogenous purines may give rise to sufficient urea to direct the formation of urease if an energy source is

provided. This would explain the formation of enzyme in the apparent absence of substrate.

It has been shown previously (Bernheim and DeTurk, 1953) that adaptation to benzoic acid in this organism is facilitated by previous oxidation of succinate as evidenced by a decrease in the latent period. The assimilation of ammonia during the oxidation further shortened the latent period, and when a few micrograms of benzoic acid were added to direct the adaptation, it was virtually abolished. It has also been shown (Halvorson and Spiegelman, 1952) that adaptation depletes the amino acid pool. It was felt that nitrogen assimilation facilitated the benzoate adaptation by augmenting the amino acid pool.

In the present experiments ammonia assimilation had the reverse effect and prevented the development of urease activity. This may be due to a specific interference by ammonia with the enzyme formation, or it may be due to competition for a limited supply of energy since both the nitrogen assimilation and adaptation are dependent on oxidation. Since the presence of ammonia completely suppresses urease activity, whereas the presence of urea does not affect assimilation, it appears that nitrogen is preferentially assimilated.

SUMMARY

Washed cells of a strain of *Pseudomonas aeruginosa* adaptively form urease during the oxidation of a variety of substrates in the absence of added urea.

Uric acid, hypoxanthine, and xanthine are slowly oxidized by washed cells. Oxygen consumption and CO_2 evolution suggest a pathway involving allantoin, allantoic acid, and glyoxylic acid, and urea accumulates in amounts equivalent to the uric acid added.

Cells which have previously oxidized an added substrate oxidize uric acid *via* the same pathway. These cells, however, accumulate ammonia in place of urea because urease has been formed as a result of the oxidation.

Ammonia is preferentially assimilated during the course of oxidation and may completely suppress urease formation.

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