The Biochemistry of the Nitrifying Organisms

2. THE FREE-ENERGY EFFICIENCY OF *NITROSOMONAS*

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The free-energy efficiencies of a number of autotrophic micro-organisms have been calculated by Baas-Becking & Parks (1927) as the ratio between the free energy consumed in carbon dioxide assimilation and the free energy released by the primary inorganic oxidation. These calculations were based on the results of determinations, published by various workers, of the amounts of organic carbon found in initially inorganic media in which various autotrophs had oxidized measured amounts of substrates. In the case of *Nitrosomonas*, which obtains all the free energy needed for carbon dioxide assimilation from the free energy released by the oxidation of ammonia to nitrite, the calculation runs as follows: The reduction of 1 g. of carbon from carbon dioxide to the oxidation level at which carbon is found in cellular constituents requires approximately 10 000 cal. of free energy. The oxidation of 1 g. of nitrogen from ammonia to nitrite releases about 4700 cal. of free energy. (The exact figure depends on the pH of the medium, concentrations of reactants, and other relevant factors.) The free energy efficiency of *Nitrosomonas*

is therefore $\frac{C_o}{N_n} \times \frac{10\,000}{4\,700} \times 100\%$, where $C_o =$ g.

carbon assimilated by the culture and $N_n =$ g. nitrite N formed by the culture. Baas-Becking & Parks (1927) used the value of 1/35, obtained by Winogradsky (1890b), for the ratio C_o/N_n and thus concluded that the free-energy efficiency of *Nitrosomonas* was about 6%. This value has been widely accepted (e.g. Stephenson, 1949), although Nelson (1931) showed that it could be considerably exceeded, since analyses of his cultures of *Nitrosomonas* revealed that C_o/N_n was approximately 1/15, corresponding to a free-energy efficiency of 14%. The results of Hes (1937) indicated that the free-energy efficiency of *Nitrosomonas* was somewhat higher in young cultures than in older ones, Winogradsky (1890a) had obtained similar indications in his preliminary investigations on carbon assimilation by *Nitrosomonas*, but he apparently regarded these preliminary results as unreliable. As Van Niel (1943) has pointed out, the whole subject of the free-energy efficiencies of the autotrophic micro-organisms might well be reinvestigated since the published data are too scanty to

permit a reliable interpretation. The present work is a study of the free-energy efficiency of *Nitrosomonas* as defined above and determined at various stages during the growth of cultures of the organisms.

MATERIALS AND METHODS

The organisms were cultured in the way already described (Lees, 1952). For some experiments the volume of the cultures was increased to 400 ml. and a larger culture vessel used, but this change in no way affected the results obtained.

Determination of organic carbon formed in a culture. When the amount of nitrite formed in a culture had attained a suitable value, the whole of the culture was centrifuged to separate the precipitate of inorganic carbonates to which the organisms adhere (Winogradsky, 1890a, b; Meiklejohn, 1950). The precipitate, with its adherent organisms, was washed at the centrifuge with distilled water until the washings were free of NH_3 , suspended in about 10 ml. of distilled water, and acidified with 10 N- H_2SO_4 to a pH of 2.0. The acid suspension was made up to a known volume and a measured sample transferred to a Van Slyke carbon tube (Van Slyke & Folch, 1940), together with 1 ml. conc. HCl to destroy any traces of carbonate. After the contents of the tube had been evaporated to dryness at 105°, the organic carbon in the residue was determined by the method of Van Slyke & Folch (1940). Blank determinations were made in a culture set up at the same time and in the same way as the 'test' culture but inoculated with a boiled inoculum.

Organic N determinations. From time to time the amount of organic N in a culture as well as organic C, was determined. For this determination, a sample of the suspension acidified with H_2SO_4 (see above) was digested according to a micro-Kjeldahl procedure and the final NH_3 determination carried out as already described (Lees, 1952). It was found that the ratio (organic C):(organic N) always fell between 3.5 and 4.0. The constancy of this ratio thus provided a check on the accuracy of the organic C determinations.

Nitrite N. This was determined as already described (Lees, 1952).

RESULTS

If the free-energy efficiency of *Nitrosomonas* is constant throughout growth of a culture $\frac{C}{N} = K$, where

$C = \mu\text{g. organic C formed/ml. culture}$, $N = \mu\text{g. NO}_2\text{-N formed/ml. culture}$, K is a constant, therefore $\log C = \log K + \log N$, and a plot of $\log C$ against $\log N$ should yield a straight line of unit slope with an intercept equal to $\log K$ when $\log N$ is zero.

The experimentally determined points for $\log C$ and $\log N$ are shown in Fig. 1. The regression line shown, which was obtained by the least squares method, fits the equation $\log C = 0.73 \log N - 0.35$, whence $2.2 C = N^{0.73}$. Thus $C/N = 1/(2.2 \times N^{0.27})$ and the free-energy efficiency (see Introduction) is $97/N^{0.27} \%$ or $77/C^{0.37} \%$. The free-energy efficiency of *Nitrosomonas* thus falls as the $\text{NO}_2\text{-N}$ and organic-carbon content of the culture increase. It should be noted that the free-energy efficiency so calculated is derived from the ratio of the total carbon assimilated to the total nitrogen oxidized; it may thus conveniently be thought of as an overall free-energy efficiency as contrasted with the instantaneous free-energy efficiency. A line calculated from the regression line and showing the fall of overall free-energy efficiency with increasing $\text{NO}_2\text{-N}$ concentration is included in Fig. 1. Experimental points obtained by other workers are plotted for comparison.

Fig. 1. The relation between organic carbon assimilated from carbon dioxide and nitrite nitrogen produced from ammonia in cultures of *Nitrosomonas*. Points determined by: (x), present authors; (O), Winogradsky, 1890a; (●), Winogradsky, 1890b; (⊗), Nelson, 1931; (●), Bömeke, 1951. Solid line, regression line calculated to present results. Dotted line, free-energy efficiency calculated from regression line.

DISCUSSION

The results show that the overall free-energy efficiency of *Nitrosomonas* fell as the nitrite concentration and organic carbon concentration of the culture rose. The instantaneous free-energy efficiency of the organisms at any particular time is given by the first differential, dC/dN , of the expression relating the nitrite-nitrogen concentration with the organic-carbon concentration (see Results). It may therefore be easily calculated from the equation to the regression line that the instantaneous free-energy efficiency, dC/dN , is 0.73 of the overall free-energy efficiency.

The figure shows that the results obtained by other workers agree well with our own, with the exception of the results of Bömeke (1951) which

indicate a rather lower amount of organic carbon in the cultures than might be expected from the amount of nitrite formed therein. Unfortunately the experimental basis of Bömeke's work was rather unsatisfactory. He did not actually measure the amount of nitrite formed in his cultures, but was content to assume that 80–90% of the ammonia he added to the medium before sterilization was converted to nitrite. Moreover, he allowed his cultures to remain without ammonia, and in contact with the nitrite they had produced, for 3–4 weeks before performing any carbon analyses on them. His organisms were thus kept for a period in a state where respiration, with consequent loss of carbon, could proceed but where carbon assimilation, which is dependent upon ammonia oxidation, could not. It is therefore possible that there was an appreciable overall loss of carbon from the cultures during this 3- to 4-week period. Since Bömeke's 'nitrite' figures may be too high, and his carbon figures may be too low, there are reasonable grounds for supposing that the points corresponding to his analyses in Fig. 1 could, with advantage, be displaced upwards and to the left. This would bring them more closely into line with our own results and the results obtained by other workers. The observed fall in free-energy efficiency of growing cultures of *Nitrosomonas* may be due to either or both of two causes. If, as seems probable (Kermack & Lees, 1952), respiratory and oxidative activities in growing cultures of *Nitrosomonas* increase more rapidly than does the assimilative activity, then the free-energy efficiency of the cultures would fall during growth. Secondly, the end product of *Nitrosomonas* metabolism is nitrite, an ion highly toxic to most living organisms. It seems most unlikely that in view of this toxicity the intracellular nitrite concentration of *Nitrosomonas* could ever be high, certainly not as high as 0.1 M, a concentration often reached in *Nitrosomonas* cultures. Engel (1941) has suggested that ammonia oxidation by *Nitrosomonas* takes place on the cell surface; if this were so it may well be that nitrite never enters the cell at all and that the intracellular nitrite concentration is kept small. But since nitrite is not only toxic, but is easily diffusible, one is driven in any case to postulate that *Nitrosomonas* possesses some 'nitrite-excreting' mechanism for maintaining a concentration gradient of nitrite between the interior of the cell and the culture medium. Such a mechanism could not operate without a supply of free energy, and since the only primary source of free energy available to the organisms is ammonia oxidation, the operation of a 'nitrite-excreting' mechanism would demand the oxidation of some extra quantity of ammonia, over and above the quantity required to maintain the normal respiratory and assimilative requirements of the cell. Moreover, the rate at

which the nitrite-excreting mechanism consumed energy would increase as the nitrite concentration increased in the medium surrounding the cell. Hence, the free-energy efficiency of the cultures would fall with increasing nitrite concentration in the medium, i.e. it would fall as the cultures grew.

We now intend to investigate, by means of the Warburg technique, the respiration of *Nitrosomonas* cultures at various stages in their growth; we also hope to investigate the effect of various concentrations of nitrite on the respiration of the cultures. These experiments should lead to a better understanding of the causes underlying the fall in the free-energy efficiency of *Nitrosomonas* cultures during growth.

SUMMARY

1. The free-energy efficiency of *Nitrosomonas* falls as the amount of carbon assimilated and the amount of ammonia oxidized increase; the free-energy efficiency thus falls during the growth of cultures of the organisms.

2. The low free-energy efficiencies reported by other workers for *Nitrosomonas* are consistent with the high nitrite concentrations at which they were determined.

3. Possible reasons for the observed fall in free-energy efficiency are discussed.

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The Ultraviolet Absorption Spectrum of Ribonuclease

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No detailed study of the absorption spectrum of ribonuclease appears to have been made. It is well known that this enzyme contains no tryptophan, while its cystine and phenylalanine contents (Brand, 1948) are such as to make a negligible contribution to its absorption spectrum in the region above 2700 Å. (see below). In addition, electrophoretic, ultracentrifugal and solubility studies all indicate homogeneity (Northrop, Kunitz & Herriott, 1948), although recent observations (Martin & Porter, 1951; Hirs, Stein & Moore, 1951) raise anew the question as to what is meant by a homogeneous protein preparation, at least in so far as ribonuclease is concerned.

The quantitative method introduced by Holiday (1936) for the spectrophotometric estimation of the tyrosine and tryptophan contents of proteins has been widely applied and has proved it is useful in a number of instances. There are, however, examples where serious divergences exist between the results obtained by this method, and chemical analyses. If,

as appears most likely (Beaven, Holiday & Jope, 1950), absorption due to peptide linkages is negligible in the region of the absorption maxima of the aromatic amino-acids, it would seem reasonable to expect that a study of the differences obtained by the two methods may lead to information regarding the nature of the changes undergone by the aromatic amino-acids upon incorporation into a protein molecule. This assumption is naturally dependent upon the correctness of chemical analyses, regarding which doubts still exist in some instances (Tristram, 1950).

Further interest is lent to a study of ribonuclease by the observation of Kunitz (1940) that it is digested in acid medium by pepsin, with resulting denaturation and loss of enzymic activity. Since pepsin is known to attack linkages between α -carboxylic groups of dicarboxylic amino-acids and the α -amino radical of an aromatic amino-acid (Bergmann & Fruton, 1941), observation of the spectrum both before and after digestion may be