Studies on Nitrate Reductase. VII. Reinvestigation on the Identity of the Enzyme with Cytochrome *b*[1]

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Previous studies in this laboratory,[3~6] together with informations from other laboratories,[7~10] have clarified that nitrate reductase is the enzyme functioning as the terminal component of the catalytic system, by which hydrogen atoms (or electrons) are transported from metabolites such as succinate, formate and alanine to the final acceptor

(1) The results and discussions described in this paper were, for the most part, already presented at the 3rd Symposium on Enzyme Chemistry, October 1950, TOKYO. Its abstract has been published in Japanese (*Symp. Enzyme Chem.*, **6**, 40 (1951))

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(3) F. Egami and R. Sato, *J. Chem. Soc. Japan*, **68**, 39 (1947).

(4) F. Egami and R. Sato, *J. Chem. Soc. Japan*, **69**, 160 (1948).

(5) R. Sato and F. Egami, This Bulletin, **22**, 137 (1949).

(6) R. Sato, *Scienca Revuo*, **2**, 122 (1950).

(7) S. Yamagata, *Acta Phytochimica*, **10**, 283 (1938).

(8) S. Yamagata, *Acta Phytochimica*, **11**, 145 (1939).

(9) E. Aubel and F Egami, *Compt. rend. acad. sci.*, **202**, 675 (1936).

(10) E. Aubel and F. Egami, *Bull. soc. chim. biol.*, **18**, 301 (1936).

nitrate. The role of this enzyme in nitrate reduction is therefore quite analogous to that of the terminal heme enzyme in respiratory mechanisms. Corresponding to this functional similarity, one of the present authors (Sato) and Egami[5] suggested in an earlier report of this series that nitrate reductase of *Escherichia coli* may be an iron enzyme and is probably identical with cytochrome *b* of *E. coli* type.[11] This view appeared to be able to account for almost all the results obtained up to that time. As to this identity, however, some doubts were presented even at that time, since there was no evidence to indicate that the cytochrome in question does combine with cyanide, a well known inhibitor of nitrate reductase. On the contrary, Pappenheimer and Hendee[12] have reported that cytochrome *b* of *Corynebacterium diphtheriae*, apparently identical with that of *E. coli*, is insensitive to cyanide in its aerobic function. Sato (one of the present authors) and Egami[5] themselves also failed to observe any change in the absorption bands of the cytochrome *b* contained in a cell-free preparation of nitrate reductase from *E. coli* when cyanide was added to this preparation. In order to correlate this observation with the above view, they had to assume a rather incredible fact: cyanide does combine with the cytochrome *b* and so inhibit the activity of nitrate reductase, but this combination causes no alteration of the absorption spectra of the cytochrome (at least α- and β-bands of the reduced cytochrome). It has been since considered desirable to study the problem more extensively and to settle the contradictory points.

In our attempts to solve the problem, it was discovered that thiourea inhibits nitrate reductase considerably. This reagent differed from the other known inhibitors of nitrate reductase such as cyanide, azide and carbon monoxide in exerting only a slight inhibition upon the oxygen uptake by *E. coli*. The fact that such an inhibitor as thiourea does exist seemed to provide another support for the invalidity of the suggested identity, because it was clear that the very cytochrome *b* is required in this thiourea-insensitive respiration.

Further investigations were then carried out using thiourea as a tool in order to obtain more advanced informations on the nature of nitrate reductase. The results of these investigations as well as consideration of the other data have shown that the previously proposed hypothesis is incorrect. Instead, it has been now clarified that cytochrome *b* of *E. coli* type is not identical with nitrate reductase itself, though it is closely related to the nitrate-reducing activity of this organism. The "nitrate reductase" which we previously so called is in fact a complex system consisting of the cytochrome *b* and a cyanide- and thiourea-sensitive factor, nitrate reductase itself.

Despite the revision of the view, it seems still tenable that nitrate reductase is an iron enzyme, because the carbon monoxide inhibited-activity of the enzyme can be restored by light.

Experimental

Materials and Methods

The resting cell suspension of *E. coli* grown on a nitrate-containing peptone-bouillon-agar medium was prepared as described in the preceeding paper.[13] The cell-free preparation of nitrate reductase employed in this investigation was obtained from *E. coli* by ultrasonic destruction of the cells and ammonium sulfate precipitation as previously described.[5] In the present study, however, the preparation was brought to dryness by lyophilization after 10 hours dialysis against a stream of water and stored at 0° in a vacuum desiccator. The presence of cytochrome *b* of *E. coil* type in this preparation was readily demonstrable by a hand spectroscope. It also contained a powerful activity of formic dehydrogenase as measured by the methylene blue technique.

Nitrate reduction was carried out anaerobically using Thunberg tubes. The rate of the reduction was measured by determining the amount of nitrite formed in the reaction mixtures by a colorimetric method as used in the previous studies.[13] The activity of dehydrogenases was followed by the ordinary Thunberg method. Manometric experiments were conducted with the use of a Warburg manometer. Spectroscopic observations with the resting suspension were made as follows. A Thunberg tube containing the samples was illuminated by a projector from a distance of about 5 cm. and the spectrum was observed by a hand spectroscope. A Beckman spectrophotometer was employed in spectroscopic investigations with the cell-free preparation.

Further details of the experimental conditions and methods are given in the tables and figures.

Results

Effect of Thiourea on Nitrate Reductase, Dehydrogenase and Oxygen Uptake of E. coli.— Fig. 1 shows the effect of thiourea on nitrate reduction by the cell-free preparation of nitrate

(11) This type of cytochrom *b* is also called cytochrome b_1, according to D. Keilin and C. H. Harpley (*Biochem. J.*, **35**, 688 (1941)).

(12) A. M. Pappenheimer Jr. and E. D. Hendee, *J. Biol. Chem.*, **177**, 701 (1947).

(13) R. Sato, M. Ebata and F. Egami, *This Bulletin*, **25**, 56 (1952).

reductase using sodium formate and methylene blue as hydrogen donator and intermediary carrier respectively.[3] It can be seen that the reduction was completely inhibited by about 0.1 M. of thiourea; half maximum inhibition was attained by $10^{-2.9}$ M. of the inhibitor. Under these conditions, however, the reduction occurs by a co-operative action of formic dehydrogenase and nitrate reductase; the former reducing methylene blue and the latter transfering electrons from the reduced dye to nitrate.[3,7] It was therefore necessary to decide which of the two enzymes concerned was actually inhibited by thiourea. This problem was pursued by studying the effect of the inhibitor on formic dehydrogenase using Thunberg technique. The results of these experiments, together with those on succinic dehydrogenase, are recorded in Table 1. As is shown in the table, the activity of formic dehydrogenase as well as that of the succinic enzyme was not inhibited by thiourea even at a concentration of 0.2 M. It is accordingly evident that nitrate reductase was the actual site of the thiourea inhibition.

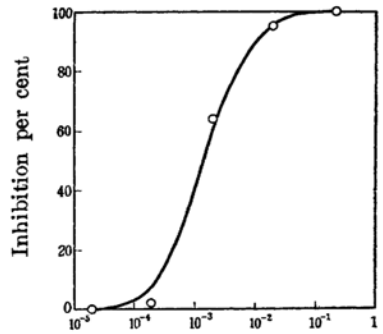


Fig. 1.—Inhibition of nitrate reduction by varying concentrations of thiourea. Each Thunberg tube contained 1 mg. of the cell-free enzyme preparation, 0.004 M. KNO_3, 0.02 M. sodium formate, 10^{-5} M. methylene blue, 0.1 M. phosphate buffer (pH 7.4), and varying concentrations of thiourea in a final volume of 5 ml. Temperature: 35°. Reaction time: 50 minutes. Control experiment without thiourea produced 165 μ M./l. nitrite under these conditions.

Table 1

Effect of Thiourea on Formic and Succinic Dehydrogenases of *E. coli*.

Each Thunberg tube contained 12 mg. of the resting cells, 0.1 M. phosphate buffer (pH 7.6), 0.002 M. substrate, and 1 : 25000 methylene blue in a final volume of 5 ml. Temperature: 36°. Figures indicate the decolorization time of the dye (in minutes).

Substrate	Without thiourea	With thiourea		
		0.2 M	0.1 M	0.01 M
Formate	15	—	16	16
〃	14	10	—	—
Succinate	8	—	—	5
〃	5	9	5	—

The effect of thiourea on the oxygen uptake by *E. coli* using formate (Fig. 2) and succinate (Fig. 3) as substrate was then studied. As shown in

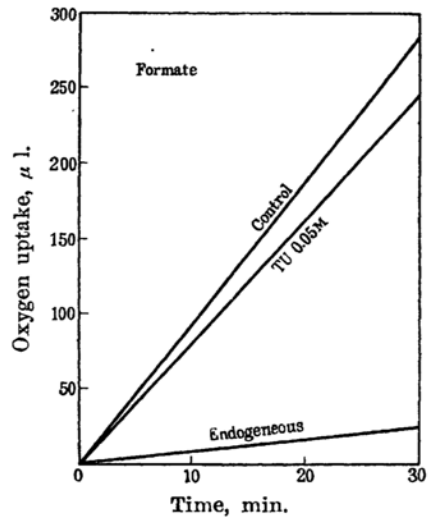


Fig. 2.—Effect of thiourea on respiration of *E. coli* (formate as substrate). Each vessel contained 2.5 mg. of the resting cells, 0.017 M phosphate buffer (pH 7.4), 0.017 M. sodium formate with or without thiourea (0.05 M.) in a final volume of 3 ml. Endogeneous respiration was measured without the addition of formate. Temperature: 36°. TU = thiourea.

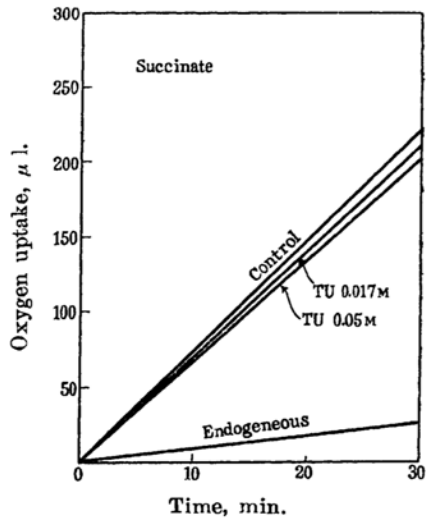


Fig. 3.—Effect of thiourea on respiration of *E. coli* (succinate as substrate). The same conditions as Fig. 2 except sodium succinate was used as substrate instead of formate.

Table 2

Qualitative Spectroscopic Observations using Resting Cell Suspension of *E. coli*

In the main compartment of a Thunberg tube the reactants in Column A were placed, and in the hollowed stopper those in Column D. After evacuation, the tube was incubated at 37° for about 20 minutes. The absorption bands of the ferrocytochrome *b* (at 560 mμ and 530 mμ) and the color of methylene blue (Mb) were observed and recorded in Column B. Then air was introduced into the mixture by removing the stopper and shaking vigorously. Changes observed after this procedure were recorded in Column C. The stopper was again plugged and the tube was incubated as before. Then the contents of the two parts of the tube were mixed and changes which occurrod as a result of this mixing were observed and recorded in Column E. All observations were conducted at room temperature. The *E. coli* suspension contained 7.5 mg. of the cells per ml. (in 0.2 M phosphate buffer of *p*H 7.6). ⧺, +, and ± indicate the intensity of the bands or the color. − shows the absence of the bands or the color.

	A (Main compartment)						B		C		D (Hollowed stopper)		E	
	E. coli suspension	Na succinate (0.1 M.)	Na formate (0.1 M)	KCN (0.1M)	Thiourea (1 M.)	Mb (0.02 %)	Bands of cytochrome	Color of Mb	Bands of cytochrome	Color of Mb	KNO_3 (0.5M)	Mb (0.02 %)	Bands of cytochrome	Color of Mb
1	2.0ml	0.5					⧺		−		0.5		±	
2	2.0		0.5				⧺		−		0.5		±	
3	2.0	0.5		0.5			⧺		⧺		0.5		⧺	
4	2.0		0.5	0.5			⧺		⧺		0.5		⧺	
5	2.0	0.5			0.5		⧺		−		0.5		+	
6	2.0		0.5		0.5		⧺		−		0.5		+	
7	2.0	0.5				0.5	⧺	−	−	⧺	0.5		±	⧺
8	2.0	0.5		0.5		0.5	⧺	−	⧺	+	0.5		⧺	−
9	2.0	0.5			0.5	0.5	⧺	−	−	⧺	0.5		⧺	±
10	2.0	0.5					⧺		−			0.5	−	⧺
11	2.0	0.5		0.5			⧺		⧺			0.5	−	⧺
12	2.0	0.5			0.5		⧺		−			0.5	−	⧺

Fig. 2 and Fig. 3, thiourea caused only a slight inhibition on the respiration of formate and that of succinate; only 12 per cent inhibition was seen in the respiration of formate by 0.05 M. of thiorea, a concentration sufficient to inhibit nitrate reductase almost completely. De Ritis and Scalfi[14] have also reported that thiourea does not appreciably influence the respiration of bacteria.

Spectroscopic Observations using Resting Suspension of E. coli.—One of the facts on which Sato and Egami[5] depended in suggesting the identity of nitrate reductase with cytochrome *b* of *E. coli* type was their spectroscopic observation that the reduced cytochrome *b* was rapidly oxidzed by the addition of nitrate. It seemed, therefore, of interest to use thiourea as an inhibitor in such spectroscopic experiments. Thus, we set ourself first to a qualitative investigation with the use of the resting cell suspension of *E. coli*. A hand spectroscope was employed for this purpose.

The results of this investigation are summarized in Table 2. On anaerobic incubation of the suspension with formate or succinate, the cytochrome *b* was converted to the reduced (or ferro-) form as indicated by the appearance of the absorption bands of the ferrocytochrome *b* (α-band at 560 mμ and β-band at 530 mμ). Thiourea and cyanide did not prevent this reduction. When air was introduced into the mixture, the bands disappeared at a rapid rate and the spectrum became that of the oxidized (or ferri-) cytochrome *b*. This aerobic oxidation of the ferrocytochrome *b* was completely inhibited by cyanide, but thiourea failed to inhibit it. In confirmation of the previous data,[5] the bands of the reduced cytochrome also greatly decreased their intensity by the addition of potassium nitrate. This oxidation by nitrate was also found to be sensitive to cyanide just like the aerobic process. A remarkable difference was, however, recognized between the two oxidation processes; in the former case, nitrate as oxidant, the disappearance of the reduced cytochrome bands was greatly inhibited by the presence of thiourea, in contrast to what was seen in the aerobic oxidation.

Incubation of the suspension with succinate or formate in the presence of methylene blue under anaerobic conditions resulted in, as is well known, the decolorization of the dye; the addition of nitrate into the decolorized mixture caused the immediate recolorization of the leuco methylene blue as previously reported.[3,7] This oxidation of the leuco dye by means of nitrate was found to be also inhibited by thiourea as well as by cyanide. Spectroscopically, it was confirmed that this recolorization of methylene blue was always accompanied by the disappearance of the bands of the reduced cytochrome *b*.

(14) F. De Ritis and L. Scalfi, *Boll. soc. ital biol sper.*, **22**, 703 (1946); *Chem. Abst.*, **41**, 3167 (1947).

Moreover it was found that the reduced cytochrome bands also disappeared when methylene blue was added to the mixture. But this disappearance was not inhibited by either cyanide or thiourea.

Finally, the effect of thiourea on the absorption spectra of the cytochrome *b* was examined. No change, however, could be detected in the spectra of either the oxidized or the reduced cytochrome even when 0.2 M. of thiourea was added.

Spectroscopic Experiments using the Cell-free Preparation.—A quantitative investigation by the use of a Beckman spectrophotometer was then undertaken with the object of ascertaining the findings of the above qualitative observations using a hand spectroscope. The cell-free preparation of nitrate reductase was adopted for this purpose.

Due to the turbidity and the relatively minute concentration of the cytochrome *b* contained in the preparation, the spectra obtained were rather indistinct. Nevertheless, it was not so difficult to identify the absorption maxima due to the reduced cytochrome *b*. Fig. 4 illustrates the spectra of the preparation at both the oxidized and reduced states measured at the wave length region ranging from 525 mμ to 575 mμ. The reduced state was realized by two methods; by the addition of sodium hydrosulfite and by incubation of the preparation with formate under an anaerobic condition (using liquid paraffin as a seal). In the latter procedure the incubation was carried out without any added intermediary hydrogen carrier such as methylene blue, but the cytochrome *b* was reduced to about 80 per cent of completion after 30 minutes (the reduction by hydrosulfite being assumed complete). This enzymatic method was used in the later experiments in preference to the hydrosulfite method; the reason of which was described in the previous paper.[5] It is clearly shown in Fig. 4 that the reduced spectra have two peaks at 560 mμ and 530 mμ corresponding to the α- and β-bands of the ferrocytochrome *b* respectively. On the other hand, no absoption maxima could be detected in the oxidized spectrum at least in this wave length region. The increasing optical density toward the shorter wave length region is not largely due to true absorption; the most part of this apparent absorption is a mere result of the scattering of light caused by the turbidity. (It is known that light scattering intensifies itself as the wave length becomes shorter). Net absorption at 560 mμ caused by the ferrocytochrome *b*, therefore, may

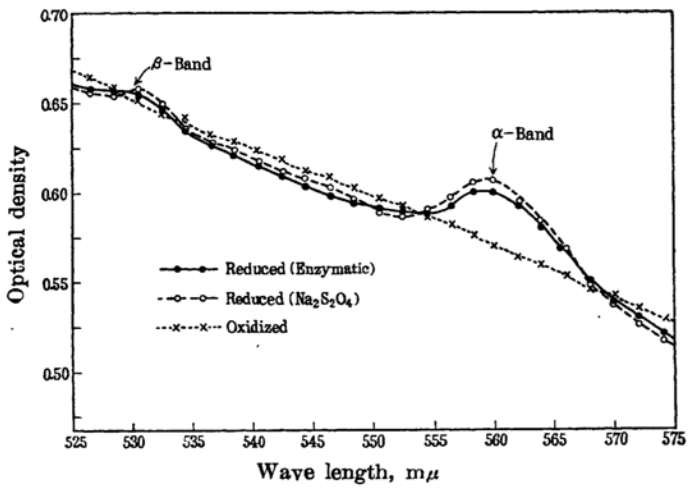


Fig. 4.—Absorption spectra of cell-free preparation of nitrate reductase. Each cuvette of the Beckman spectrophotometer (light path 1 cm.) contained 200 mg. of the cell-free preparation in a final volume of 4 ml. (phosphate buffer, 0.05 M, *p*H 7.4). Enzymatic reduction was carried out by incubating with 0.05 M sodium formate for 30 minutes at 37°. Liquid paraffin (1.5 cm. thick) was placed on the mixture to keep the anaerobic condition. Nonenzymatic reduction was carried out by adding a small amount of solid $Na_2S_2O_4$ and then placing liquid paraffin.

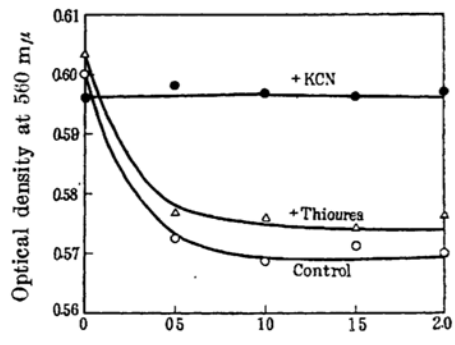


Fig. 5. Effect of KCN and thiourea on aerobic oxidation of ferrocytochrome *b*. The cell-free preparation of nitrate reductase was dissolved in 0.05 M. (*p*H 7.4) phosphate buffer containing 0.05 M. sodium formate (200 mg. of the preparation per 3 ml.). 3 ml. of this solution was pippetted into a cuvette of the Beckman spectrophotometer and liquid paraffin (1.5 cm. thick) was placed on it. The cuvette was then incubated for 30 minutes at 37°. Then each 1 ml. of either distilled water (control), or 0.04 M. KCN (+KCN), or 0.2 M. thiourea (+Thiourea) was added and the optical density at 560 mμ was measured. Then the liquid paraffin was removed by pipetting out as quickly as possible and air was bubbled into the mixture by a small syringe. The changes of the optical density was followed at each 30 seconds interval. Measurements were conducted at room temperature.

be estimated as about 0.03—0.04 in optical density unit.

By following the decrease of optical density at 560 mμ, the oxidation of the ferrocytochrome *b* by atmospheric oxygen (Fig. 5) and by potassium nitrate (Fig. 6) together with the effect of cyanide and thiourea on these processes were then studied. As shown in Fig. 5 and Fig. 6, it was confirmed that the reduced cytochrome *b* undergoes rapid oxidation by either introduction of air or by addition of nitrate. In both the cases the oxidation proceeded to completion within 30 seconds. In confirmation of the qualitative observations, it was further found that cyanide prevented both

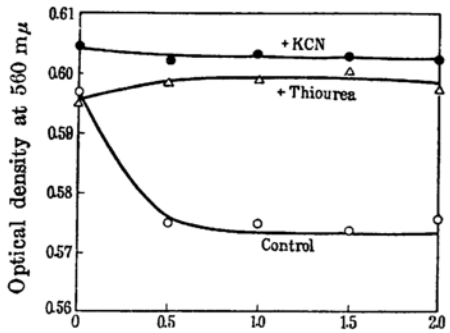


Fig. 6.—Effect of KCN and thiourea on oxidation of ferrocytochrome *b* by nitrate. Conditions and methods were the same as Fig. 5, except the following. Instead of removing the liquid paraffin and bubbling air, small amounts of solid potassium nitrate was added at zero time.

of the processes completely, but thiourea was inhibitory only for the oxidation by nitrate. No appreciable retardation of the aerobic oxidation was observed by the presence of thiourea (0.05 M.).

Attempts were also made to test the possibility that methylene blue may oxidize the ferrocytochrome suggested by the qualitative observation. But high concentration of methylene blue (0.001 per cent) used in these experiments made it impossible to conduct desirable measurements due to the intense general absorption of the dye. Even at lower concentrations of the dye no definite results were obtained.

Discussion

Nitrate reductase can be defined as the terminal enzyme of hydrogen (or electron) transporting pathways from suitable hydrogen (or electron) donators to nitrate; *viz.*, it is the enzyme which reacts directly with nitrate. If this enzyme is actually identical with cytochrome *b* of *E. coli* type as previously suggested,[5] the only possible scheme accounting for both the nitrate reduction and the aerobic respiration by *E. coli* should be as follows:

H-donators—dehydrogenases—····

····—cytochrome *b* (nitrate reductase) → oxidase?—O_2
→ NO_3^-

↑

KCN, CO, Thiourea

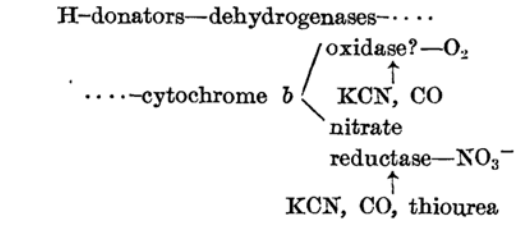
Here, the arrow indicates the site of action of the inhibitors. The intermediary components between dehydrogenases and the cytochrome *b* are not shown in this scheme for several reasons; (1) no definite information is as yet available, (2) this part of the scheme may be different according to the hydrogen donators, and (3) they are not important for the later discussions. The oxidase inserted between the cytochrome *b* and molecular oxygen will be discussed later.

The results reported in the present study, however, suggest that the above scheme as such is unlikely. First of all, this scheme can not provide any satisfactory explanations for the finding that thiourea does not appreciably influence the aerobic respiration of *E. coli*, in contrast to its fairly marked inhibitory action upon the nitrate reduction. Spectroscopic evidence also indicates that thiourea is not capable of preventing the oxidation of the ferrocytochrome *b* by atmospheric oxygen, while the disappearance of the reduced cytochrome bands by nitrate is completely inhibited by the same reagent. Furthermore, as already mentioned the above scheme is not consistent with the observations that no changes occur in the cytochrome bands by the addition of cyanide. The view claimed by Pappenheimer and Hendee[12] that diphtherial cytochrome *b* is not inhibited by cyanide further makes a protest against the scheme.

A satisfactory settlement of the matter, however, suggests itself on the basis of the very results obtained in the present study. The marked inhibition exerted by thiourea upon the nitrate reduction and the failure of the same reagent to inhibit the respiration necessarily lead to the assumption that a certain thiourea-sensitive factor may be involved in the nitrate-reducing mechanism; whereas no such factor may be required in the respiratory system. The role of this factor is suggested by the spectroscopic results to consist in the catalysis of the oxidation of the ferrocytochrome *b* by nitrate (but not by molecular oxygen). We should therefore regard this factor, according to the definition, as nothing but nitrate reductase itself. The contradiction concerning the cyanide inhibition of the nitrate reduction

mentioned above would also be able to be settled by taking this factor into consideration, because it seems more reasonable to attribute the cyanide inhibition to this new factor (nitrate reductase) rather than to the cytochrome *b*.

On the basis of the above considerations, a more acceptable scheme can be drawn for the processes in question instead of the former one.



This revised scheme is in agreement with all the results reported in this paper as well as with those in the foregoing studies. The reason why thiourea exerts marked inhibition upon the nitrate reduction without seriously affecting the aerobic respiration will be readily explained by this scheme. (The slight inhibition of the respiration by thiourea may be due to an entirely different mechanism from that for the nitrate reduction.) This scheme can also interpret the spectroscopic findings with ease. Furthermore, the cytochrome *b* is shown in this scheme not to be inhibited by either cyanide or carbon monoxide in accordance with the generally held concept that the cytochrome does not combine with these reagents. Thiourea should also be regarded as being inert to the cytochrome because of the inability of this substance to inhibit the respiration which requires the cytochrome as an essential catalyst.

A brief account of the aerobic pathway presented in the scheme seems desirable. Although there remains little doubt about the participation of the cytochrome *b* in the respiration of *E. coli*, further details of the process are as yet obscure. Pappenheimer and Hendee[12] have obtained evidence indicating that diphtherial cytochrome *b* is autoxidizable and the greater part of the oxygen uptake by a cell-free diphtherial extract can be accounted for in terms of this autoxidation. It is not clear, however, whether the same respiratory mechanism is functioning also in *E. coli*. From our spectroscopic observations it appears preferable to consider that the autoxidation of the cytochrome, if any, does not greatly contribute to the respiration of *E. coli*, because the aerbic oxidation of the reduced cytochrome *b* was strongly inhibited by cyanide. This fact in turn suggests that a cyanide-sensitive oxidase is necessary as a link between the cytochrome and oxygen. Keilin and Harpley[11] have already suggested that an iron enzyme, designated cytochrome a_2, functions as an oxidase of the cytochrome *b* in *E. coli*. Moreover, it has been found spectroscopically that this cytochrome combines with cyanide and carbon monoxide. The presence of a similar oxidase in Type I Pneumococcus and some other bacteria which lack cytochrome *c* and cytochrome oxidase has been reported by Sevag and Shelburne,[15] who found that the respiration of these bacteria is sensitive to both cyanide and carbon monoxide and attributed the inhibitions to this oxidase. It seems therefore by no means unreasonable to assume such an oxidase as the terminal enzyme in the aerobic mechanism of a group of bacteria characterized by the lack of cytochrome *c*-cytochrome oxidase system. The absence of this system in *E. coli* has been confirmed by the negative indophenol oxidase reaction of the organism.[16]

The role of methylene blue as an intermediary hydrogen carrier between dehydrogenase systems and nitrate reductase in the reconstructed system[3] may require an explanation. Previously, assuming the identity of the cytochrome *b* with nitrate reductase, the dye was simply considered as an artificial link between dehydrogenase systems and the cytochrome. Our spectroscopic observations, however, have provided indications, though qualitative and inconclusive, that the dye can serve also as an oxidant of the ferrocytochrome *b*. In addition, it has been confirmed that the reoxidation of the leuco dye by nitrate is sensitive to both cyanide and thiourea. These facts lead to the view that methylene blue might mediate between the cytochrome and nitrate reductase in this simple reconstructed system.

Throughout the above arguments the selective inhibition by thiourea on the nitrate reduction has been taken as the most conclusive evidence in favour of the nonidentity of nitrate reductase with the cytochrome *b*. A selective toxicity[17] of this compound on nitrate metabolism has been also reported by Fleury,[18] who found that thiourea exerts a strong fungistatic action on *Aspergillus niger* in media with nitrate as the sole source of nitrogen, while the toxicity is much less (or absent) when ammonium salts or organic nitrogen

(15) M. G. Sevag and M. Shelburne, *J. Gen. Physiol.*, **26**, 1 (1942).

(16) R. Sato, Unpublished data.

(17) W. W. Wainio *et al.* have reported (*J. Biol. Chem.*, **192**, 349 (1951)) that thiourea, even at a concentration of 10^{-2} M., does not exert any inhibition on cytochrome oxidase activity of a heart muscle preparation.

(18) C. Fleury, *Bull. soc. suisse botan.*, **58**, 462 (1948); *Chem. Abst.*, **43**, 3880 (1949).

sources are available. From these findings Fleury has concluded that thiourea inhibits the nitrate-reducing enzyme system of the mold. The precise mechanisms involved in these specific inhibitions, however, are left to be clarified. Presumably, the capacity of thiourea to form complexes with various metal ions might play a part in these phenomena in some manners. At any rate, further research about this point appears to be of promise for the elucidation of the nature of nitrate reductase.

As a result of the denial of the identity previously suggested between nitrate reductase and cytochrome *b* of *E. coli* type, the necessity has come to the fore again to consider the chemical nature of this enzyme. Neverthless, it seems still valid that the activity of nitrate reductase of *E. coli* is inseparably associated with the presence of iron in the molecule. The most clear-cut support for this view comes from the results of the previous study[5] showing the carbon monoxide inhibition of this enzyme and its reversal by light. Recently it has been further confirmed in this laboratory[19] that a small but definite inhibition by carbon monoxide is also seen in the nitrate reduction by *Bacillus subtilis* and *Acetobacter xylinum*; light reversed the inhibition also in these cases, This suggests that a similar iron enzyme may be responsible for the reduction also in these organisms. Its detailed features, however, are not yet clear.

The iron-protein nature of nitrate reductase receives further indirect support from an investigation dealing with the effect of iron-deficiency on the nitrate-reducing activity of *E. coli*.[20] It has been shown that the organism grown on iron-deficient media has a considerably low activity of nitrate reductase as compared to the cells supplied with a sufficient amount of iron. The activity of succinic dehydrogenase was not influenced by the iron content of the media. Similar results has also been obtained with *C. diphtheriae*.[21] In this connection, observations by Granick and Gilder[22] are worthy of special attention. Studying the growth-promoting activity of various porphyrin derivatives on strains of *Hemophilus influenzae*, they found that several iron porphyrin compounds such as iron meso- and iron hematoporphyrin can substitute iron protoporphyrin as an essential growth factor for this organism. But the organism grown on these unnatural growth factors was found to be completely devoid of the nitrate-reducing activity. On the basis of these observations they have concluded that iron protoporphyrin is required either for the formation of the function of the nitrate-reducing mechanism, the vinyl side chains of protoporphyrin being assumed to be essential.

There are, however, no definite indications in support of the possibility that nitrate reductase is actually a heme protein enzyme, and the clarification of the chemical nature of this enzyme is largely left for the future.

Summary

1. It has been found that thiourea has a marked inhibitory effect on the nitrate reduction by *E. coli*, but dehydrogenase activity and the aerobic respiration of the same organism are not considerably influenced by this compound.

2. Spectroscopically it has been further clarified that thiourea completely prevents the anaerobic oxidation of the ferrocytochrome *b* by means of nitrate, whereas the same reagent can not inhibit the aerobic oxidation of the cytochrome. Cyanide prevents both of the oxidation processes.

3. On the basis of these findings the previously suggested identity of nitrate reductase with cytochrome *b* of *E. coli* type has been criticized and it has been concluded that the cytochrome *b* is not identical with nitrate reductase itself, though it is closely associated with the nitrate-reducing mechanism of *E. coli*.

4. A scheme has been proposed which can account for both the nitrate-reducing and the respiratory mechanisms of *E. coli*.

5. The chemical nature of nitrate reductase has been discussed and the iron-protein nature of this enzyme has been again emphasized.

Addendum I: In a review quoting a previous paper[5] of this series, Wurmser[23] wrote that in the nitrate reduction nitrogen replaces oxygen for the Slater's respiratory scheme. In spite of the validity of this statement, it should be considered that nitrate, but not nitrogen, replaces it.

Addendum II: After this paper had been submitted to the editors, we were informed of the criticism by G. N. Cohen[24] against our earlier hypothesis on the identity of nitrate reductase with the cytochrome *b*. The nitrate-reducing mechanism of strict anaerobes and its differences from that of *E. coli* have been

(19) I. Yamashina, Unpubished data.
(20) M. Niwa, Unpublished data.
(21) R. Sato and M. Yoneda, Unpublished data.
(22) S. Granick and H. Gilder, *J. Gen. Physiol.*, **30**, 1 (1946).
(23) R. Wurmser, *Ann. Rev. Biochem.*, **20**, 8 (1951).
(24) G. N. Cohen, *Ann. Rev. Microbiol.*, **5**, (1951).

investigated by Mr. Tadao Katsura at the Institute of Infectious Diseases of Tokyo University in connection with us. This material will be published shortly.

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