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ON THE EFECT OF SOME CARBONYL COMPOUNDS AND THEIR OXIMES UPON THE NITRATE REDUCTASE*

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On the mechanism of the nitrate reductase action, it might be recognized that the hydrogen atom which is detached from certain coexisting substances, hydrogen donators, by the relevant dehydrogenases, is provided to the nitrate via intermediates such as dinucleotides and methylene blue, as postulated by Yamagata (1938) and Egami and Sato (1948). From this point of view, it is conceivable that the fall of activity of the nitrate reductase of silkworm tissue after extraction with water or buffer is partly due to the removal of the hydrogen donators; and that when one of such substances is added, the activity will be revived at least to some extent. Thus the substances with hydrogen donator specificity and dehydrogenases concerned can be determined. On the basis of this presumption the activating ability of malic acid, succinic acid, malonic acid, fumaric acid, glutamic acid, asparagine, fructose and sucrose on the worm tissue enzyme was confirmed (Omura, 1954b). As for mice (Omura, 1954c), citric acid, succinic acid, malic acid, lactic acid, galactose, rhamnose and mannose were available for recovery. In the case of cattle liver reductase (Omura, 1954c) the acceleration of reduction by succinic acid, rhamnose, galactose, mannose, glycine, alaine, asparagine, acetaldehyde and ethylalcohol was similarly observed. As can be seen from these results, however, these facts are specific to the enzyme materials. For instance, malic acid, which had the hydrogen donator effect for the worm, had an inhibitory reaction on the nitrate reductase of mice. Therefore, attention must be paid

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to these facts in examining the influences of the substances on the enzyme.

EXPERIMENT

Although it is supposed that nitrate reductase may play an essential part in the nitrogen assimilation of plant and micro-organisms, this is doubtful in the case of animals. Concerning its biological significance, however, Egami (1950) observed the relationship between this reductase action and oxygen consumption by using a bacterial enzyme and postulated the idea of "nitrate respiration." This hypothesis may be applicable to the animal. As is well known, carbonyl compounds such as keto acid and aldehyde occupy the key point in respiration and other biological metabolisms. Following the preceding studies, therefore, the influence of such compounds on the nitrate reductase was tested. Carbonyl compounds used were acetone, pyruvic acid, α-ketoglutaric acid and acetaldehyde as representatives respectively of ketone, mono- and di-keto acid, and aldehyde which are of biological importance. In our institute, on the other hand, investigations on the oxime metabolism such as oximase (Yamafuji, Kawakami and Shinohara, 1952; Yamafuji and Omura, 1952; Yamafuji and Yoshihara, 1954), transoximation (Yamafuji and Akita, 1952) and its enzyme (Yamafuji, Omura and Miura, 1953), and virus provoking action of oximes (Yamafuji and Omura, 1950a, b), have been in progress for a number of years. In connection with this subject, the effect of oximes upon the nitrate reductase also was studied. The oximes used were the oximes of the carbonyl compounds cited above and synthesized in our laboratory. Among those oximes, it is probable that the oximes of pyruvic acid, α-ketoglutaric acid and acetaldehyde are found in biological materials.

Results for the cattle liver enzyme have been reported already (Omura, 1954c). Similar researches were performed using other enzymes, because the influences of the acids or sugars varied with enzyme materials as stated above.

The method of estimation of the nitrate reductase was the same, on the whole, as that in the foregoing papers. Mice viscera (liver, spleen, kidney and heart) were taken immediate after decapitation, washed repeatedly in sterilized water, ground thorough-

ly with small amounts of quartz sand in a mortar and suspended in M/15 phosphate buffer, pH 6.0, to make a 10% (w/v) suspension. After centrifugation, the residue was resuspended in the same solvent of the same volume and 5 cc of this preparation were used for each experiment. Fowl liver enzyme also was prepared in a similar manner. The concentration of nitrate in the reaction mixture was 10^{-2}M, and reaction was allowed to proceed for 2 hours at 40°C under anaerobic conditions in a Thunberg tube, as usual. The amounts of carbonyl compounds and their oximes tested were twice that of the nitrate. Of course, these compounds were neutralized before being used. The measurement of nitrite produced was carried out colourimetrically in the usual way after clarification of the reaction mixture. The results are shown in Table 1 with that of the cattle liver for the sake of comparison. In the case of the cattle liver, however, five times the amount of nitrate was added in order to overcome the hindrance by native substrates, for the liver extract which was used as the enzyme solution, contained both the reductase and the substrates.

Table 1. The effects of some carbonyl compounds and their oximes upon the nitrate reductase of animals.

		None	Acetone	Acetone-oxime	Pyruvic acid	Pyruvic acid-oxime	α-Keto glutaric acid	α-Keto glutaric acid-oxime	Acet-aldehyde	Acet-aldehyde-oxime
Cattle	$NO_2/\mu M$	9.6	9.1	14.4	3.0	6.0	13.7	5.5	62.3	6.5
	Ratio	100.0	94.8	150.0	31.3	62.5	142.7	55.6	648.6	67.6
Mice	$NO_2/\mu M$	24.2	25.6	12.6	18.6	0.8			26.7	1.5
	Ratio	100.0	105.7	52.1	76.9	3.3			110.3	6.2
Fowl	$NO_2/\mu M$	8.1	9.7	9.7	7.0	4.2	8.2		5.1	4.8
	Ratio	100.0	107.4	107.4	86.4	51.9	101.2		63.0	59.3

On the silkworm reductase the experimental conditions were slightly different. The larvae of strain P-22×P-21 were sacrified on the 5th day of 5th instar. The enzyme solution was prepared from the residue of tissue which had been extracted twice with phosphate buffer. Moreover, the reaction was carried out with glutamic acid which had the highest reactivating ability among

the substances tested. Under these circumstances similar tests were performed. The concentration of compounds tested also was 10^{-2}M, identical with that of nitrate and glutamic acid. The results obtained are presented in Table 2. Reactivation by glutamic acid showed the increase of relative activity from 66.9 to 100.0.

From the figures shown in both tables, it is evident that the carbonyl compounds also exerted, as expected, a peculiar effect on the enzymes according to the materials used. As regards the cattle liver enzyme, acetaldehyde had very strong activating capacity as demonstrated by Bernheim and Dixon (1928) who claimed the conference of aldehyde oxidase, a result which is contrary to the observation of Egami et al (1950) who showed an inhibitory action. On the contrary, however, this aldehyde interfered with the action of mice enzyme and had almost no effect on the fowl liver and silkworm tissues. Pyruvic acid, most popular intermediate in biological metabolisms, on the other hand, caused

Table 2. The effects of some carbonyl compounds and their oximes upon the nitrate reductase of the silkworm tissue.

H donator added	None	Glutamic acid						
Carbonyl comp. or oximes	None	None	Acetone	Acetone-oxime	Pyruvic acid	Pyruvic acid-oxime	Acet-aldehyde	Acet-aldehyde-oxime
NO_2, μM	52.4	76.8	69.2	32.0	130.0	7.4	82.0	10.0
Ratio	66.9	100.0	90.1	41.7	169.3	9.6	106.8	13.0

the depression of nitrate reduction of the former three enzymes while activation of worm reductase was brought about. Against the above, α-ketoglutaric acid provoked the increase of activity of cattle liver and did not influence the fowl, whereas no changes were observed when acetone was added. It is worth noticing, however, that their oximes, having the probability of being in the biological materials, with the exception of acetoxime, restrained the action of reductase though the degree of the retardation varied with enzymic materials and oximes.

It is our opinion that the action of oximase, oxime hydrogenating enzyme found in silkworm tissue primarily, may act on the following equation:

$$R\text{-}\underset{\underset{NOH}{\|}}{C}\text{-}COOH + 2H_2 \longrightarrow R\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH + H_2O$$

This equation coincides with that of the nitrate reductase:

$$HNO_3 + H_2 \longrightarrow HNO_2 + H_2O$$

In other words, oximes also may act as hydrogen acceptor in place of nitrates. Therefore, it is considered that oximes coexisting with nitrates in the reaction mixtures received competitively hydrogen atoms carried from hydrogen donators by the dehydrogenase and nitrate reductase. This means the nitrate reductase can be checked. Regarding the results with silkworms, shown in Table 2, if the native hydrogen donators contained in the enzyme solution provide their hydrogen preferentially, it amounts to 52.4 μM/L. Through the competition of pyruvic acid- and acetaldehyde-oxime with nitrate, 69.4μM and 66.8μM were taken by these oximes respectively. These amounts were over 52.4μM. Therefore, it is clear that oximes must get hydrogen from glutamic acid. As to the acetoxime (44.8μM) such evaluation could not be made, but glutamic acid is the most effective hydrogen donator we know, so that it must be applicable similarly.

If the above argument is the case, the oximes in the reaction mixture should be diminished under those circumstances. Now the same experiments were set up using the same enzyme as that of control in Table 2 with exception that 10^{-4}M pyruvic acid oxime was employed instead of nitrate.

As expected, the decrease of oximes was observed as shown in Table 3.

Table 3. Decrease of oxime by worm enzyme.

Reaction time, hrs.	2	4
Decrease of oxime, %	24.0	62.7

Hitherto we used the long reaction time, as the activity of oximase was too weak to estimate during a short period. Now the possibility arose, of proceeding with the measurement of oximase activity anaerobically using the Thunberg technique. On the contrary, no satisfactory decrease of oximes was seen with

other animals in which the conclusive activity of oximase was scarsely detected even after long periods of reaction, though the strong hindering action of oximes upon the nitrate reductase was confirmed. It is supposed, therefore, that oximes might influence the enzyme itself directly.

Then the effect of the variation of concentration of oxime on the reductase was pursued. In the study of Table 4, mice liver homogenate and acetaldehyde oxime were used.

Table 4. The effect of variation of concentration of acetaldehyde oxime upon the nitrate reductase of the mice liver.

Conc. of oxime, 10^{-2}M	0	0.5	0.1	0.05	0.01
NO_2, μM	22.8	11.7	20.8	21.5	23.8
Ratio	100.0	51.0	91.2	94.2	104.5

As far as 10^{-3}M of oxime, tenth the amounts of nitrates, the inhibition was observed. Of course the rate of retardation of reaction by oxime was decreased with the fall of the concentration of oxime. Sato and Egami (1949) established that the nitrate reductase of *Bacterium coli* is haemin protein. It is well known that catalase, similar haemin protein, is very sensitive to hydroxylamine, hydrolysing product of oximes. Akita (1951) in our institute observed that 4×10^{-4}N acetoxime depressed the catalase activity of yeast only to about 75% of the original, while almost all activity of the same catalase, about 96%, was interferred with by hydroxylamine of the same concentration. Moreover, Kaplan (1953) reported the inhibition by this amine upon crystalline alcohol dehydrogenase. As for the nitrate reductase, we have no information on the inhibition by such a substance. At the beginning of a series of the author's investigations, however, the following observation was made (Yamafuji, Omura and Sakamoto, 1952; Omura, 1954a). When the suspension of whole bodies of silkworms fed with hydroxylamine was used as enzyme, no nitrate reduction was detected. On the other hand, the nitrate reductase of the alimentarytract-free tissue of the same caterpillars was activated. At that time, I thought that this might be ascribed to the hydroxylamine remaining in the body, although the enzyme

was activated. But more detailed study in vitro on this problem has not been pursued.

Therefore the depression of the reductase activity by oxime might be regarded with suspicion, as the inhibition may be due to the action of hydroxylamine, not to the oxime itself. In fact, it is known that oximes can dissociate in solution even though very slightly. Then the effect of hydroxylamine upon the same enzyme was tested. Conditions were, of course, the same as that of the former experiments.

Table 5. The effect of hydroxylamine upon the nitrate reductase.

Conc. of NH_2OH, $10^{-2}M$	0	0.2	1.0	0.5	0.1	0.05
NO_2, μM	37.5	10.8	15.2	25.2	35.1	36.9
Ratio	100.0	28.8	43.2	67.2	93.6	98.4

It has been proved that hydroxylamine, too, had the inhibiting ability. However, the comparison of these data with those which were obtained in the experiments with acetaldehyde oxime revealed that both oxime and amine have almost the same degree of inhibition ability on the nitrate reductase of mice liver. The degree of dissociation of oximes, however, is very small and outside of the considerations in this case, as is known from experiences so far on the investigations on oximes. Therefore, in the case of oxime too, if it is argued that hydroxylamine dissociated from oxime brings about the hindrance of the reaction, so the rate of decrease of activity by oxime must be far smaller than that of the amine. Eventually, it will be pointed out that the cause of inhibition was due to the oxime itself not to the hydroxylamine produced from oxime.

SUMMARY

Acetaldehyde interfered with the activity of mice nitrate reductase and did not have any influence upon that of fowl and silkworm tissue. On the other hand, pyruvic acid inhibited the action of reductase of mice and fowl, whereas the activation of worm enzyme was brought about. Against the above, acetone had almost no effect. But their oximes caused the inhibition in

all cases, although the degree of the depression of the activity varied with the kinds of oximes and enzymic materials.

This inhibition is partly due to the competition of oximes with nitrates for hydrogen atoms, and partly to the direct effect of oximes upon the enzyme itself. In concentrations as far as 10^{-3}M acetaldehyde oxime, the inhibition was observed on the mice reductase. Hydroxylamine, too, had about the same degree of influence as that of acetaldehyde oxime.

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