学位申請論文

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主論文 第一部

STUDIES ON THE EFFECT OF DITHIOCARBAMATE ON THE RESPIRATION OF SEA-URCHIN SPERMS. I. THE AUGMENTATIVE EFFECT ON THE RESPIRATION.

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INTRODUCTION

It is well known that the dithiocarbamate, one of the metal chelating agents, forms complexes with some metals such as copper, zinc or iron.

Regarding the effect of the agent on the embryonic respiration, BODINE and FITZGERALD(1948) reported that the respiration of the grasshopper embryo was augmented by dithiocarbamate and that the agent formed a complex with copper in the iso-amyl alcohol extract of the embryo homogenate. More recently, MINGANTI(1957) found that the respiration of the embryo in the early developmental stage and the unfertilized egg of an ascidian(Phallusia) was markedly stimulated by dithiocarbamate. He supposes that this agent may act as a chelating agent, so the effect is brought about by removing a block, which involves heavy metals such as copper, in the electron carriers between the substrates and the cytochromes.

On the other hand, many investigations on the relation of metal ions to the physiology of sea-urchin sperms have been performed hitherto. According to TYLER(1950, 1953), TYLER and ATKINSON(1950), the functional life-span of sperms is markedly prolonged and the fertilization reaction is improved when the sperms are suspended either in sea

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water with a metal chelating agent, such as some amino acids and peptides, ethylenediaminetetracetate, diethyldithiocarbamate etc., or in artificial sea water scacely containing hevy metal ions. There exist some evidences that the added amino acid is not utilized significantly as a nutrient by the sperms (TYLER and ATKINSON, 1950; TYLER and ROTHSCHILD, 1951). Furthermore, the latter authors found that the initial burst of oxygen uptake which usually occurs soon after dilution of semen with ordinary sea water was suppressed in the presence of amino acids. The total oxygen uptake of the amino acid-treated sperm during its prolonged life considerably exceeded that of the control. They concluded from these facts that the early death of the sperms in ordinary sea water should not be ascribed to the exhaustion of endogenous substrate for respiration. Thus, TYLER(1953) suggested that this early death may be caused by the toxic action of the metal ions in sea water. These toxic metals were also regarded as the important participants in the so-called respiratory dilution effect, and copper was supposed to be the most likely of them (ROTHSCHILD and TUFT, 1950; ROTHSCHILD and TYLER, 1954). In fact, an amount of copper was detected in semen, seminal plasm, coelomic fluid and sea water respectively (BARNES and ROTHSCHILD, 1950). In the stafish sperm, however, the situation seems somewhat different. It has been established that zinc concentrates in the middle piece and the acrosome and it more or less depresses the respiration and the movement of sperms. This depression is made to disappear by removing or masking the zinc with the addition of certain amino acids or some metal chelating agents (FUJII, 1954; FUJII et al., 1955 a,b; MIZUNO, 1956; KINOSHITA, 1956 a,b).

The present author has investigated the relation of metal ions to the respiration of sea-urchin sperms. In the present paper, it is reported that dithiocarbamate augments the sperm respiration but this effect depends both on the concentration of the agent and on the population density of sperms in the suspension. Analyses of the mode of the augmented resperation and the relation of this effect to some metal ions will be given in the succeeding papers.

MATERIALS AND METHODS

Sea-urchins, Hemicentrotus pulcherrimus and Pseudocentrotus depressus, were used as materials.

The testes were extirpated with a nonmetallic spoon and the semen was squeezed out through double silk cloth. The semen thus obtained was diluted about 15 times with either ordinary sea water or the artificial sea water, and centrifuged at 3,000 r.p.m. for 10 min.. The sediment was used as the standard dry sperm in the present experiments.

The filtered sea water or the artificial sea water was used to dilute the dry sperm. Both were buffered with M/50 H_3BO_3 and M/200 $Na_2B_4O_7 10H_2O$ at pH 8.1-8.2. The constituents of the artificial sea water were as follows; NaCl, 26.3g; KCl, 0.7g; $CaCl_2 2H_2O$, 1.5g; $MgSO_4 7H_2O$, 11.9g; $NaHCO_3$, 0.43g; deionized water, 1l.

The oxygen uptake was measured manometrically at 20°C with Warburg manometers run in duplicate or more. The vessel with side arm was conical and about 18 ml of capacity. The dry sperm was pipetted into the main compartment and diluted with the medium in the side arm at the initiation of measurement. The number of sperms was counted with

the hemacytometer of Bürker-Türk ruling.

Sodium-dimethyldithiocarbamate(DMDTC), sodium-diethyldithiocarbamate (DEDTC) and disodium-ethylenediaminetetracetate(EDTA) were used as metal chelating agents. The dithiocarbamate were washed several times with carbon tetrachloride in a few days before experiment and dried in air. All the agents were dissolved either in sea water or in artificial sea water to give a desired final concentration in sperm suspension.

RESULTS

The number of sperms in 1 ml of the standard dry sperm was always around 3×10^{10}. The dilution rate of the dry sperm was usually 1:6, 0.2 ml of the dry sperm being diluted with 1 ml of the suspending medium, so that the suspension contained 6×10^{9} sperms in total or 5×10^{9} sperms per ml. Only in the last experiment, dilution rates were 1:6, 1:24 and 1:48. To give the dilution at 1:24, 0.1 ml of the dry sperm in the main compartment was diluted with 2.3 ml of the medium in the side arm, and at 1:48, 0.05 ml of the dry sperm with 2.35 ml of the medium.

DMDTC in sea water. Figs. 1-a and 1-b show the effect of DMDTC in ordinary sea water on the respiration of the sperms of both species. In these diagrams, it is indicated that the effect of DMDTC on the respiration of sperms varies with the different concentrations of the agent. At a concentration of 10^{-2}M, the respiration was remarkably augmented and the total volume of oxygen uptake during two hours was nearly twice as much as that of the control. The respiration was more or less depressed at lower concentrations than 10^{-2}M, the rate

of depression being maximum at 10^{-3}M. The initial high rate of respiration was hardly affected at 10^{-2}M and the rate was maintained throughout the period under observation, although this initial high rate was evidently absent at any other concentrations of the agent.

DEDTC in sea water. The effect of DEDTC was found to be almost similar to that of DMDTC upon the sperm respiration (Fig. 2). Augmentation of respiration was gained at 10^{-2}M and inhibition occurred at lower concentrations but the maximum inhibition occurred at 10^{-4}M. Since the extent of augmentation in DEDTC seemed rather less than in DMDTC even at the concentration of 10^{-2}M, the latter was preferably used as the representative of dithiocarbamates in the following experiments to analyze the augmentative effect.

EDTA in sea water. Indeed the effect of DMDTC is remarkable, but it may be necessary to examine whether this effect commonly occurs also with other chelating agents or is characteristic of dithiocarbamate. The effect of EDTA, which easily combines with alkali earth ions, on the respiration of sperms was checked up for this reason (Fig.3). In any concentrations above 10^{-5}M, EDTA did not augment the respiration but inhibited it. This inhibition became larger with the increase of concentration of EDTA. This results agrees well with that of ROTHSCHILD and TYLER(1954). The effect of dithiocarbamate, therefore, should be regarded in connection with the metal which has higher affinity for dithiocarbamate or with the specific mode of action of this agent.

DMDTC in artificial sea water. If the effect of dithiocarbamate upon the sperm respiration can be assigned to the metal chelating action of this agent as surmised by MINGANTI(1957), the following question may naturally arise: which is the main participant, metals

in sea water or those contained in the sperm itself? To answer this question, the effect of DMDTC in the metal-free artificial sea water was examined. The semen was washed three times with the artificial sea water by means of centrifugation. As shown in Fig. 4, the respiration was augmented by 10^{-2}M DMDTC and depressed at lower concentrations. The fact that 10^{-2}M DMDTC similarly stimulates the respiration not only in sea water but also in the artificial sea water may suggest that the effect is principally participated in the metal involved not in sea water but in the sperm itself.

DMDTC in sea water under various densities of sperms. Finally, the effect of 10^{-2}M DMDTC in sea water on the respiration of sperms under various densities was examined (Figs. 5-a and 5-b). The dry sperm was diluted in three different rates, 1:6, 1:24 and 1:48, at the initiation of measurement. The amount of consumed oxygen in the normal respiration tended to increase as the density of sperms lowered. The rate of respiration was kept at high level, especially in dilute suspensions, only for a while after dilution and gradually became declined. By adding DMDTC at the final concentration of 10^{-2}M, the respiration was affected in different ways being dependent on the sperm densities of suspensions. In the sperm suspension of which the dilution rate was 1:6, the respiration was remarkably augmented. When the suspension was diluted to 1:24, augmentation still occurred but the rate of it was rather less than in the 1:6-suspension. On the other hand, the respiration in the suspension diluted to 1:48, was depressed by DMDTC. The rates of augmentation due to the addition of DMDTC were calculated from the differences of oxygen uptake during

two hours between DMDTC-respiration and controls at each density of sperms (see the note of Figs. 5-a and 5-b). The increase in respiration rate caused by diluting the sperm suspension and such a peculiar mode of DMDTC-effect may have some connection with the so-called respiratory dilution effect of sea-urchin sperms.

DISCUSSION

In the present experiments, the respiration of sea-urchin sperms is remarkably affected by the addition of dithiocarbamate. Changes in the respiration rate caused by the agent depend not only on the concentration of the agent but also on the density of sperms in the suspension. Augmentation of sperm respiration is obtained only when DMDTC is added at the concentration of 10^{-2}M to the sperm suspension, the density of which ranging from 1 to 5 x 10^{9} sperms per ml, but the respiration is depressed either when the agent is added at concentrations lower than 10^{-2}M or when the density of sperms is 6 x 10^{8} per ml even in the presence of the agent at 10^{-2}M. The effect of DEDTC is found to be almost similar to that of DMDTC, while EDTA, which is also one of the effective chelating agents, does not stimulate the respiration at any concentrations. Thus, the characteristic mode of action of dithiocarbamate should be taken into consideration when one discusses on the augmentative effect of this agent on the sperm respiration.

Concerning the action of dithiocarbamate in sperm suspension, two possible modes may be considered: the agent combines with certain metals to form complexes, or it is consumed as a respiratory substrate.

In this respect, the following facts should be noticed that by 10^{-2}M DMDTC the respiration of sperms is augmented only when the density of sperms is considerably high but it is inhibited in a dilute suspension, and that in the former cases the increment in oxygen uptake due to the addition of the agent sometimes exceeds the expectation from the complete oxidation of DMDTC into disulfides. If DMDTC is assumed to be utilized as a respiratory substrate, augmentation of respiration should occur independently on the density of sperms and increment in oxygen uptake should not exceed the amount of oxygen to be consumed by complete oxidation of the agent. There is also an additional evidence, which will be reported in the next paper, that the respiratory quotient in DMDTC-augmented respiration is found to differ insignificantly from that of the normal respiration. From these facts at hand, it would be difficult to assign the augmentation of the respiration to the oxidation of the agent added to the sperm suspension. Thus, the metal chelating action of dithiocarbamate should be taken into account.

Provided that the augmentative effect of dithiocarbamate on the sperm respiration is caused by the chelating action of the agent to some metals, such metals should mainly be included in the sperm body but not confined in sea water because DMDTC affects the respiration of sperms in the metal-free sea water as well as in ordinary sea water (cf. Figs. 1 and 3). Then, a question arises why such a high concentration as 10^{-2}M of the agent is needed to bring about the augmentation. This question may be answered on the basis of the following three possible suppositions: 1) since an amount of

alkali earth ions and the bivalent metal ions are contained in sea water, dithiocarbamate may also combine with them and the actual concentration may be lowered; 2) the rate of penetration of the agent into sperms may be restricted; 3) the agent may act as a narcotic at lower concentrations and the stimulating effect of the agent on the respiration is made to disappear even if the agent combines with some metals in the sperm body.

With regard to the last of these suppositions, it is necessary to refer here the experimental results of GOKSØYR(1955). He studied the effect of varying concentration of DMDTC on the acetate oxidation in yeast in the presence of low concentration of cupric- and zinc sulfate. The "inversion phenomenon" was found to occur, namely, a) at lower concentrations of DMDTC, inhibition due to the formation of Cu-DMDTC(1:1) complex, b) reversal of the inhibition by transforming the complex to DMDTC-Cu-DMDTC(1:2), c) at further addition of DMDTC, new inhibition due to the formation of Zn-DMDTC(1:1). He also described that the 1:1 complexes have high fungicidal activity but the 1:2 complexes are inactive. On referring to his data, it may be supposed in the present work that dithiocarbamate at lower concentrations acts as a narcotic forming 1:1 complexes with some kinds of metals so that the inhibitory effect surpasses the augmentative effect attributed to the chelating action of the agent. If the concentration of the agent is enough high to form 1:2 complexes, the narcotic action can be reversed and only the augmentative effect is made its appearance.

There still remains another problem to be discussed why the augmentative effect of 10^{-2}M DMDTC on the respiration decreases as the sperm density becomes lower. It might be correlated with the "dilution effect",

of which mechanism has long been discussed by many workers but not yet been settled clearly. If sperms undergo some physiological changes by dilution, these changes may have some connection with certain metals. As to this problem, the explanation will be given in following papers.

In conclusion, dithiocarbamate are able to augment the respiration of sea-urchin sperms in considerably dense suspension probably by chelating to some metals which may be involved in the sperm body. Since this effect can not be brought about by EDTA, it may be rather characteristic of dithiocarbamate, although it has not yet been known whether this characteristic is due to the species of metal combined with this agent or to some other mode of action, if any, ascribed to the chemical configuration, sulfhydryl groups for instance, of dithiocarbamate.

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SUMMARY

1. DMDTC and DEDTC at 10^{-2}M remarkably augmented the respiration of sea-urchin sperms in dense suspension, 5×10^9 sperms per ml, while lower concentrations of the agent depressed it even at the same sperm density.

2. These effects occurred not only in sea water but also in

metal-free artificial sea water.

3. EDTA did not augment the respiration but inhibited it as the concentration increased.

4. The augmentative effect of 10^{-2}M DMDTC was not so large in the sperm suspension of 10^{9} sperms per ml as in more dense suspension. The inhibition was observed in the dilute suspension of 6×10^{8} per ml.

5. The reason why such a high concentration of the agent was needed to augment the respiration and that why this effect decreased in sperm suspension of lower density were discussed

Fig. 1-a. Effect of DMDTC on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control, ○ : DMDTC 10^{-2}M, ◑ : 5×10^{-3}M, △ : 10^{-3}M, + : 5×10^{-4}M.

Fig. 1-b. Effect of DMDTC on the respiration of sperms of Pseudocentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control, ○ : DMDTC 10^{-2}M, △ : 10^{-3}M, □ : 10^{-4}M.

Fig. 2. Effect of DEDTC on the respiration of sperms of Pseudocentrotus. Sperm density: 5×10^9 sperms per ml. ● : Control, ○ : DEDTC 10^{-2}M, △ : 10^{-3}M, □ : 5×10^{-4}M, + : 10^{-4}M.

Fig. 3. Effect of EDTA on the respiration of sperms of Hemicentrotus. Sperm density: 5×10^9 sperms per ml. ● : Control, ○ : EDTA 10^{-2}M, △ : 10^{-3}M, □ : 10^{-4}M, + : 10^{-5}M.

Fig. 4. Effect of DMDTC on the respiration of sperms of Hemicentrotus in artificial sea water. Sperm density : 5×10^9 sperms per ml. ● : Control in artificial sea water, ○ : DMDTC 10^{-2}M, △ : 10^{-3}M, □ : 10^{-4}M; ◒ : Control in sea water.

Fig. 5-a. Effect of 10^{-2}M DMDTC on the respiration of sperms of Hemicentrotus under different rates of dilution. Sperm densities : I, 5×10^9 sperms per ml; II, 1.25×10^9 per ml; III, 6.25×10^8 per ml. D : With 10^{-2}M DMDTC. Augmentation rate = $\{(D\text{-Control}) / \text{Control}\} \times 100(\%)$: I-D, +167 %; II-D + 106 %; III-D, -7%.

Fig. 5-b. Effect of 10^{-2}M DMDTC on the respiration of sperms of Pseudocentrotus under different rates of dilution. Sperm densities : I, 5×10^9 sperms per ml; II, 1.25×10^9 per ml; III, 6.25×10^8 per ml. D : With 10^{-2}M DMDTC. Augmentation rate = $\{(D\text{-Control}) / \text{Control}\} \times 100(\%)$: I-D, +89%; II-D, +34%; III-D, -13%.

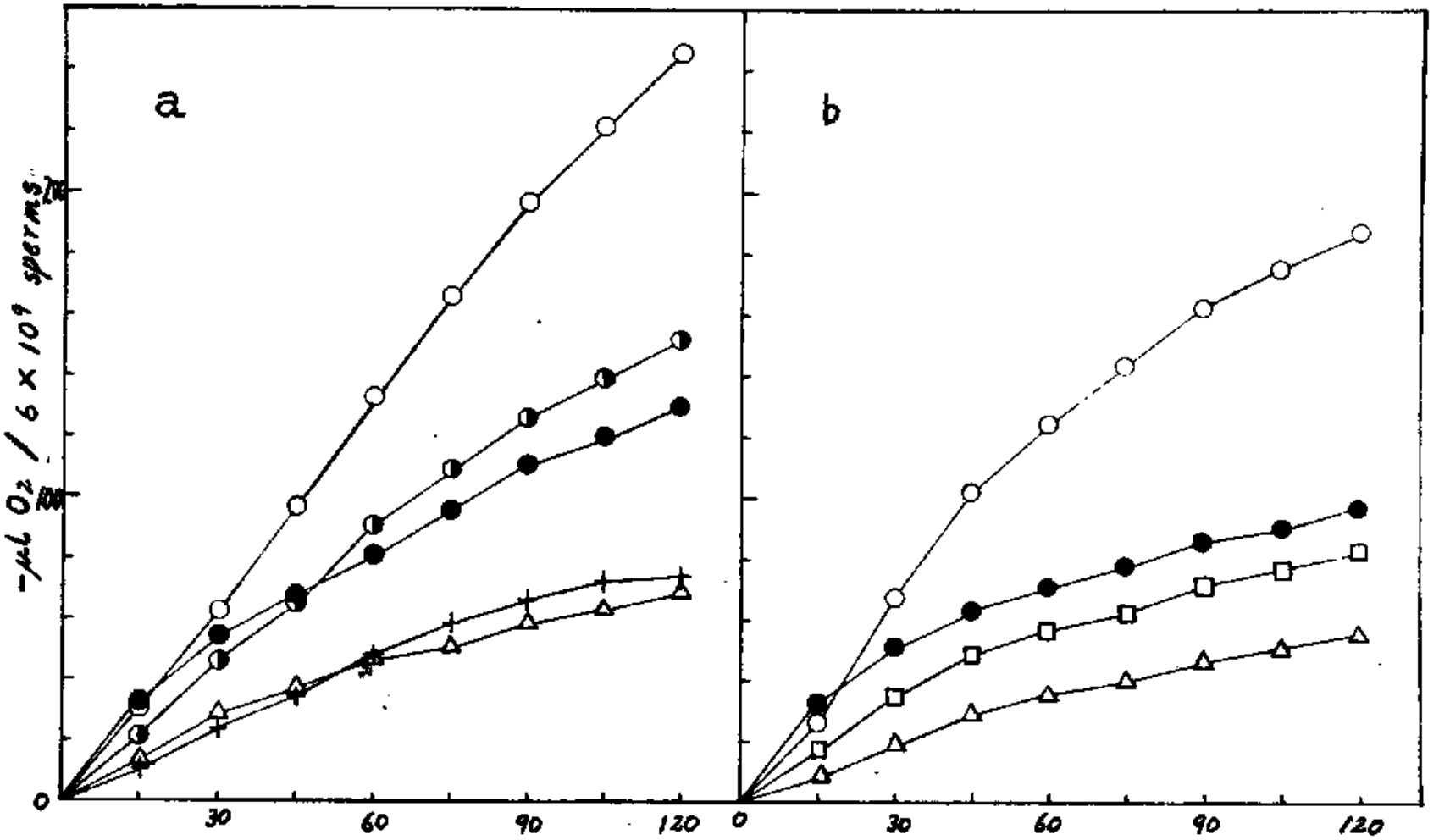

Fig. -1

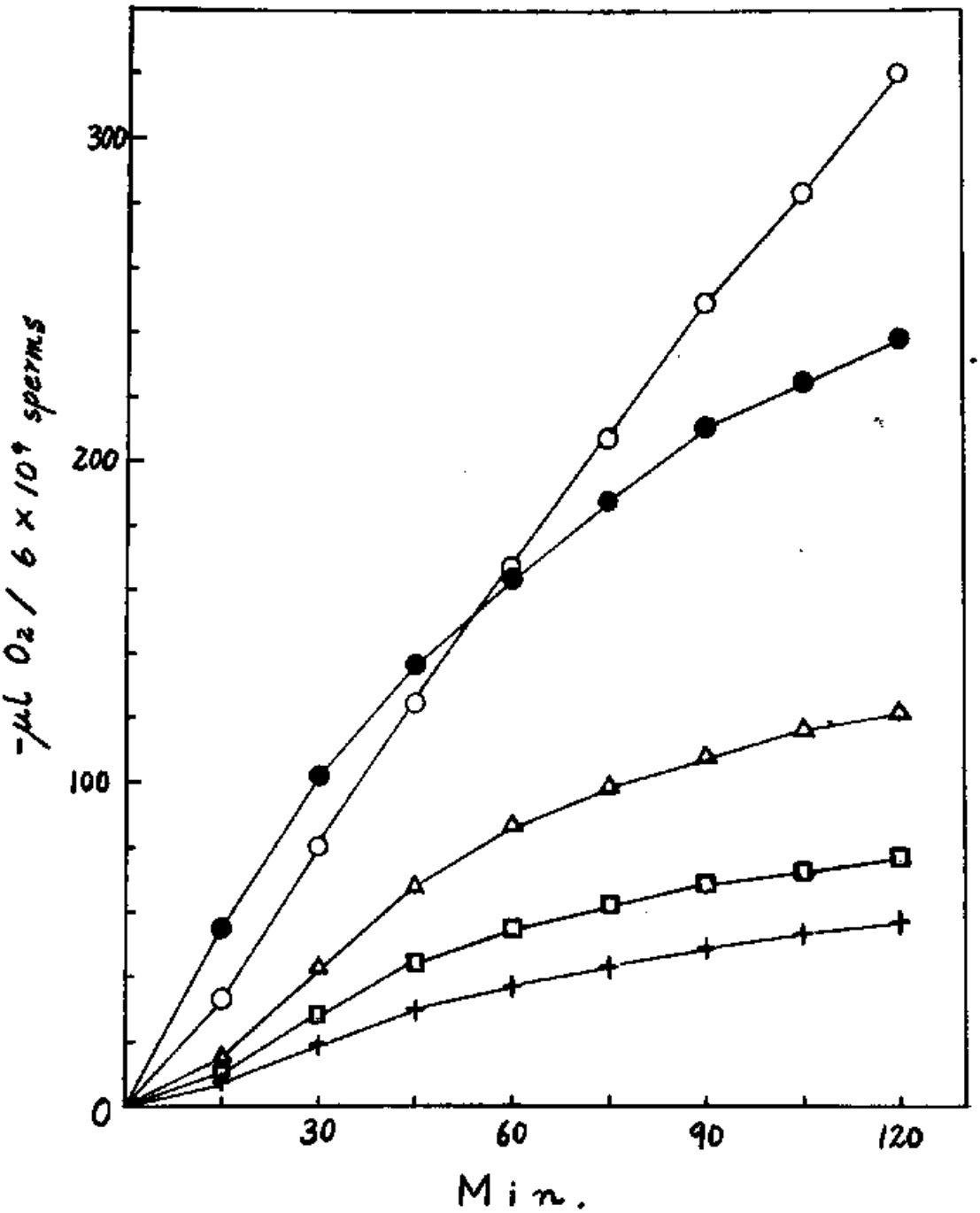

Fig-2

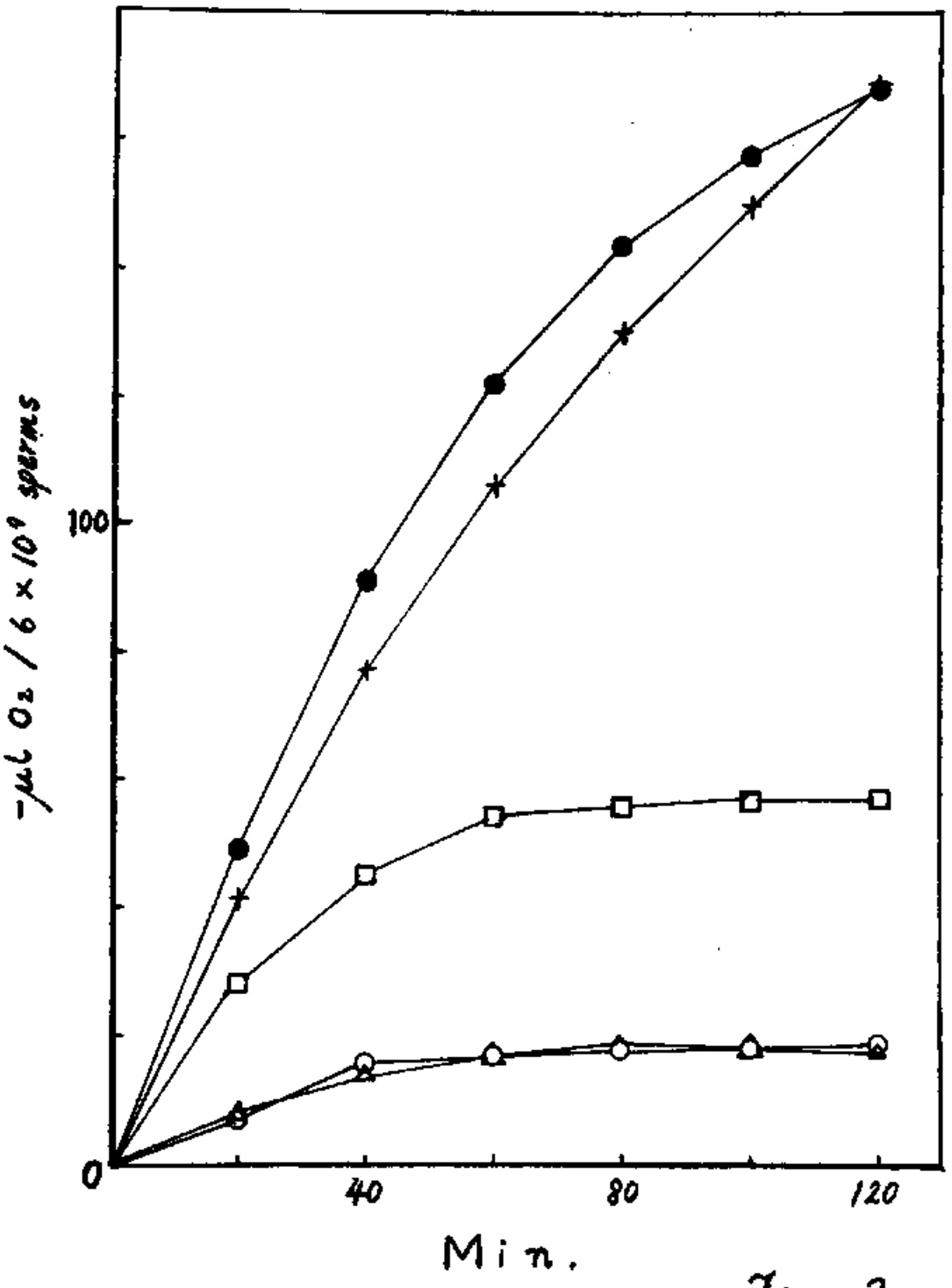

Fig. - 3

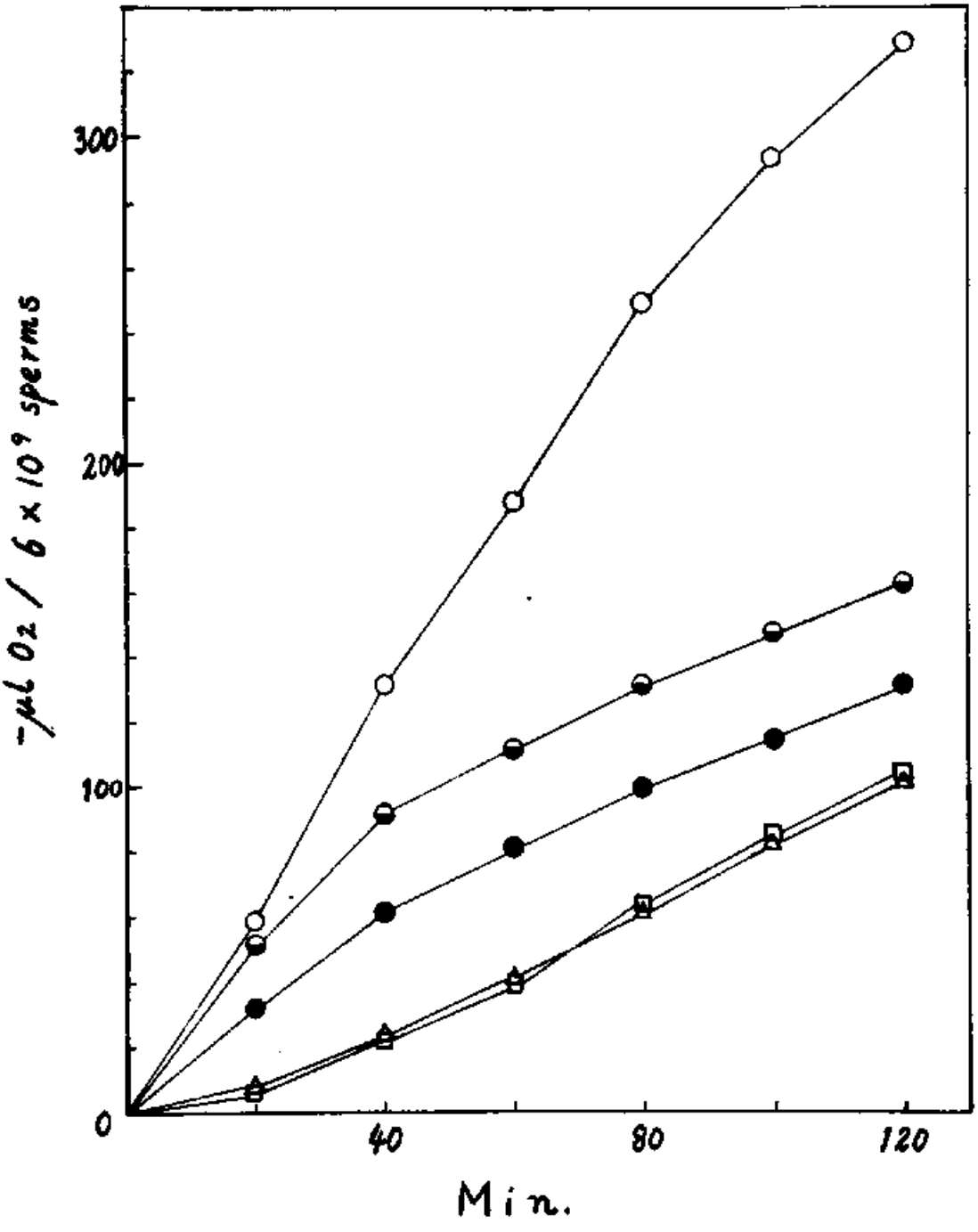

Fig-4

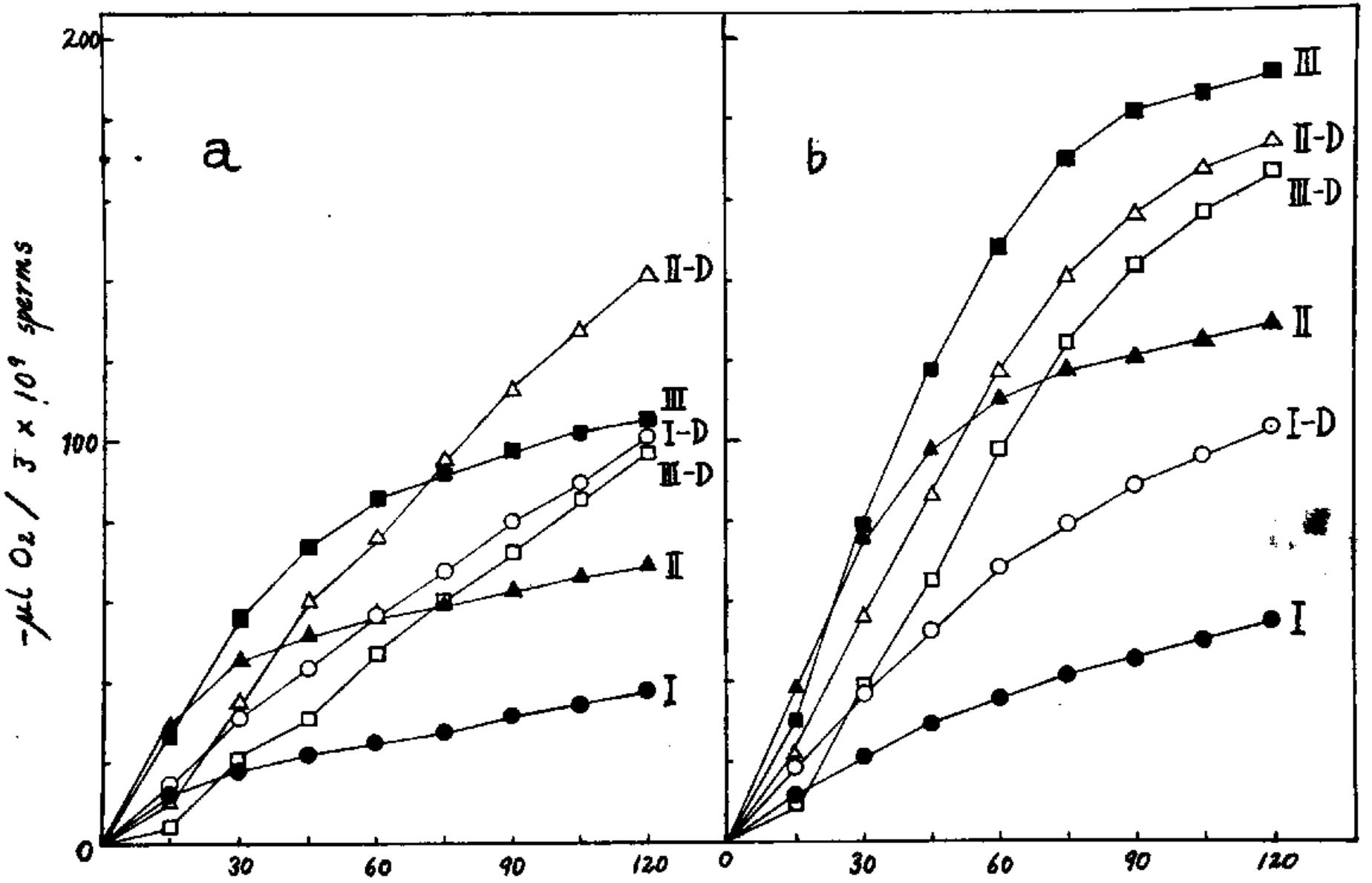
a
b
200
100
0
30
60
90
120
Min.
-μl O2 / 3 x 10⁹ sperms
I
II
III
I-D
II-D
III-D

Fig-5

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主論文 第二部

STUDIES ON THE EFFECT OF DITHIOCARBAMATE ON THE RESPIRATION OF SEA-URCHIN SPERMS. II. THE MODE OF THE AUGMENTED RESPIRATION.

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INTRODUCTION

In the preceding paper (MURAMATSU, 1962), the author reported that dithiocarbamate augmented the respiration of sea-urchin sperms suspended in sea water at high densities. He ascribed this phenomenon to the chelating action of the agent to some kind of metals probably included in the sperm. Since rather high concentration of the agent, 10^{-2}M, was needed to augment the respiration efficiently, another possibility that the agent might be oxidized by the respiratory machinery in sperm could not be excluded. However, the author pointed out in the paper that this seemed unlikely, because the rate of augmentation at a given concentration of the agent was dependent upon the density of sperms in the suspension, and moreover, the increment in the oxygen uptake sometimes exceeded the amount expectable from the complete oxidation of the agent into disulfide form.

The present experiments have been performed to elucidate further the mechanism involved in the phenomenon and to verify the hypothesis presented in the preceding paper. Thus, the respiratory quotient(R. Q.)

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in the augmented respiration was estimated and the effects of some respiratory inhibitors, such as carbon monoxide, cyanide and azide, and 2,4-dinitrophenol, as an uncoupler for oxidative phosphorylation, upon the augmentative action of dithiocarbamate were examined respectively.

MATERIALS AND METHODS

Sea-urchins, Hemicentrotus pulcherrimus and Pseudocentrotus depressus were used as material. The standard dry sperm containing 3×10^{10} sperms per ml was prepared following the procedure mentioned in the previous paper (MURAMATSU, 1962). O_2 uptake and CO_2 output were measured at 20°C using Warburg manometers run in duplicate or more. The dry sperm was diluted at the initiation of measurement with filtered sea water buffered at pH 8.1-8.2 with M/50 H_3BO_3 and M/200 $Na_2B_4O_7 10H_2O$. In most of the experiments, 0.2 ml of dry sperm in the main compartment was diluted six times with 1 ml of diluent in the side arm.

Respiratory quotients (R. Q.) were estimated by the direct method. In the CO_2 output estimation, CO_2 retained in the suspension was liberated to the gas phase by adding 3N H_2SO_4.

To test the inhibitory effect of CO, the vessels were kept in darkness being packed with aluminium foils. The photo-reversibility of the effect was examined by illumination through a mirror under the vessels with a 500 watt projector lamp set at some distance away from the water bath. Gas exchange by evacuation was repeated four times per one vessel. CO was prepared by decomposing sodium formate with warm concentrated H_2SO_4.

In the cyanide experiment, 0.2 ml of KCN-KOH mixture at a suitable concentration (ROBBIE, 1946) was put in the center well to give a desired concentration of cyanide in the suspension instead of adding cyanide directly to the suspension.

Sodium dimethyldithiocarbamate(DMDTC) was washed with carbon tetrachloride and dried in air a few days before use.

RESULTS AND DISCUSSIONS

Respiratory quotient (R. Q.) As mentioned above, the augmentative effect of DMDTC on the sperm respiration has been assumed not to be caused by the oxidation of the agent as a respiratory substrate. To give an evidence in support of this presumption, the R. Q. values in the DMDTC-augmented respiration were estimated to compare with those in the normal respiration. ROTHSCHILD(1948 a) pointed out that the direct method is not adequate to estimate the R.Q. of sea-urchin sperms very accurately, because the respiration is so sensitive to the increase of CO_2 tension in the suspension that there is a tendency to decrease in the oxygen uptake in the vessel which does not contain KOH in the center well. However, the R.Q. values obtained in the present experiment, although they might not indicate what kinds of substrate are utilized, should make it possible to answer the question whether there are any differences between the substrates in the DMDTC-respiration and in the normal one, or at least whether DMDTC is utilized as a substrate. If DMDTC added to the suspension was oxidized to disulfide form to augment the respiration, the R.Q. values in the DMDTC-respiration should be lower

than in the normal one. The results obtained are shown in Fig. 1 and Table 1.

By 10^{-2}M DMDTC the respiration was markedly stimulated; not only O_2 uptake but also CO_2 output were twice as much as those in the normal respiration. Concerning the respiratory substrate of sea-urchin sperms, phospholipids have been established to be the main substance ~~of them~~ (ROTHSCHILD and CLELAND, 1952; MOHRI, 1957 a,b, 1959, 1961 a,b), so that around 0.7 of R.Q. value should be expected. Disagreeding with this expectation, the values in Table 1 ranges from 0.78 to 0.99, so they seems not to be adoptable to indicate the kind of substrate used.

From a survey of the values obtained, it may be concluded that there are hardly any differences between the substrate for the DMDTC -respiration and for the normal one, or at least that DMDTC cannot be a substrate, because the R.Q. values in the DMDTC respiration do not differ significantly from those of the normal respiration at any time after dilution. Here, a possibility that DMDTC is oxidized by sperms to cause the augmentation of respiration may be excluded. The results in this experiment seems to give one of the evidences for the alternative possibility that the augmentation of respiration by DMDTC is caused by the chelating action of the agent to metals in sperms.

Carbon monoxide The respiration of sea-urchin sperms has been established to be inhibited by CO in darkness photo-reversibly (ROTHSCHILD, 1948 a,b), and the cytochrome-cytochrome oxidase system is considered to be involved in the process of respiration. Such

an effect of CO was confirmed to occur in the present experiment on the respiration in the suspension containing 1.25 x 10^9 sperms per ml (1:24 dilution).(Fig. 2). In a preliminary test, the comparison was made between the respiration rate in 5% O_2-95% N_2 mixture and that in air. The result indicated that the presence of 5% O_2 in the gas phase was sufficient for sperms to perform the normal respiration. So, O_2 was mixed at 5% with CO, and air was used for controls .

Fig. 3 shows the effect of CO on the DMDTC-respiration and the normal one in the suspension containing 5 x 10^9 sperms per ml (1:6 dilution) under alternation of dark and light periods. The rate of DMDTC-respiration was remarkably reduced by CO in darkness, down to as much as the normal rate in air, and it was completely restored by the illumination. Without DMDTC, the respiration rate was not reduced so conspicuously by CO even in darkness but it was rather stimulated in light. The latter result essentially agrees with that of ROTHSCHILD(1948 a,b) that the sperm respiration is sharply inhibited by CO in dilute suspension but this inhibition is not so large in dense suspension. He supposed that the reduction of the inhibition in dense suspension may be ascribed to some differences in the cytochrome-cytochrome oxidase system between dense and dilute suspension. It was reported previously (MURAMATSU, 1962) that the rate of normal respiration increased as the density of sperms decreased and that DMDTC augmented the respiration remarkably in dense suspension but less in dilute suspension. From these facts, it can be said that the respiration in high rate, such as the normal respiration in dilute suspension and the DMDTC-augmented respiration in dense

suspension, is inhibited by CO in darkness, while the respiration in low rate like the normal respiration in dense suspension is hardly affected by CO. Standing only on the basis of the sensitivity to CO, it may be considered that DMDTC seems to shift the metabolic patterns of sperms in dense suspension to the similar condition of them in dilute suspension.

The CO-sensitivety of the normal respiration in dilute suspension and that of the DMDTC-respiration in dense suspension indicate that the respiration in such cases is mediated through the cytochrome-cytochrome oxidase system. In dense suspension, the normal sperms may respire in another way, since the respiration is not inhibited by CO even in darkness. The data of MINGANTI(1957) that the respiration of the ascidian egg is sometimes stimulated by CO may ~~have~~ Have some connection with this insensitivity of the sperms.

Cyanide The effect of HCN at ~~the~~ concentrations of 10^{-3}-10^{-5}M on the DMDTC-respiration was examined comparing with that on the normal respiration (Fig. 4). The normal respiration was reduced by HCN at any concentrations applied here and the inhibition rate increased as the concentration of HCN increased. These facts agree with those of ROBBIE(1948) and BARRON et al.(1949). The augmentative effect of DMDTC was also suppressed by HCN in the same manner as in the normal respiration, and DMDTC hardly influenced on the inhibitory effect of HCN. Though it may be inclined to suppose that the respiration is mediated by the cytochrome-cytochrome oxidase system if the respiration is sharply inhibited by HCN, this supposition should never be so simply applied to the normal respiration in the present experiment

because of the insensitivity to CO even in darkness.

Sodium azide In the experiment of BARRON et al.(1949) and UTIDA and NANAO(1954), the respiration of sea-urchin sperms was not affected by NaN_3. MOHRI(1956 a,b) found on the other hand that the respiration rate was reduced by NaN_3 only while it proceeded in a high level during a few hours after dilution of semen, down to as low as the rate of steady respiration which was preceded by the high rate respiration and hardly affected by NaN_3. Thus, he assumed that the sperm involved two separable components in its respiratory machinery, one sensitive and the other insensitive to NaN_3, and the former might be an important participant in the high rate respiration. Such a hypothesis may be considered in analogy with the result of UTIDA and NANAO(1954) that the highly activated respiration by DNP was depressed by NaN_3 down to the level of normal respiration even when the latter was not affected by the agent.

As shown in Fig. 5, NaN_3 at 10^{-2} and 10^{-3}M reduced the initial high rate in the normal respiration for about one hour after dilution, while at 10^{-4} and 10^{-5}M it rather stimulated the respiration. When NaN_3 at 10^{-2}M was added with DMDTC, the augmentation of respiration was completely made to disappear and the rate of respiration became as low as at the single administration of NaN_3. NaN_3 at 10^{-3}M was less inhibitory to the normal respiration than at 10^{-2}M, so that the augmentative effect of DMDTC was partially reduced. By the lower concentrations, the augmentative effect of DMDTC was hardly influenced.

The reduction of the DMDTC-effect caused by the addition of NaN_3 suggests that the DMDTC-augmented respiration may be analogous

to the activated respiration, or NaN_3-sensitive respiration mentioned above, which is seen either in the initial period after dilution of semen or by the addition of DNP. The normal respiration, in the present experiment, though the sperm density in the suspension was considerably high, was also reduced by NaN_3, but the rate of reduction was not so large as in the DMDTC-respiration. If the steady rate respiration is completely insensitive to NaN_3, the normal respiration may undergo a slight activation for a while after dilution even at such a high density of sperms. In the DMDTC-respiration, the activation was maintained for a long time so that the inhibitory effect of NaN_3 could be made its appearance during the cource of measurement. At any rate, since the DMDTC-respiration is sensitive to the inhibitors for aerobic respiration, such as CO in darkness, HCN and NaN_3, it may be mediated by the cytochrome-cytochrome oxidase system as well as the high rate respiration in the normal condition. This system is not evident in the low rate respiration, such as the normal respiration in dense suspension, because of the very low sensitivity to CO and to NaN_3.

2,4-Dinitrophenol(DNP) It is well known that DNP, one of the uncoupling agents for oxidative phosphorylation, stimulates the sperm respiration (UTIDA and NANAO, 1954, 1956; MOHRI, 1956 a; ROTHSCHILD, 1956; etc.). According to ROTHSCHILD, DNP was more effective in dense suspension of sperms than in dilute one, and ethylenediaminetetracetate(EDTA) acted antagonistically against DNP. He assumed that both DNP and EDTA acted on the same enzyme system and the existence of the trace metal is necessary to uncouple the oxidative phosphorylation in sea-urchin sperms. In the same time, the rate

of normal respiration was increased as the density of sperms decreased. Based on the analogy between the effect of DNP and the dilution effect on sperm respiration, he also supposed that the dilution effect would be resulted from the uncoupling of oxidative phosphorylation. To examine whether the augmentative effect of DMDTC on the sperm respiration has the common basis with that of DNP, DNP was added to the sperm suspension with 10^{-2}M DMDTC. As shown in Figs. 6-a and 6-b, DNP at 10^{-3}-10^{-5}M stimulated the respiration. The rate of normal respiration at 10^{-4}M was highest exceeding the rate augmented by 10^{-2}M DMDTC. When DNP at 10^{-4}-10^{-6}M was added with DMDTC, the respiration was always augmented beyond the level of that obtained by either the single addition of DNP or of DMDTC. Thogh DNP at 10^{-6}M did not manifest any stimulating effect on the normal respiration, it elevated the DMDTC-respiration as well as at 10^{-5}M. By 10^{-3}M DNP, the normal respiration was also slightly augmented, but the DMDTC-respiration was somewhat depressed. The concentration of 10^{-3}M was so high that it might act rather toxically on the high rate respiration such as the DMDTC-respiration but not on the low rate respiration such as the normal one in dense suspension.

Generally surveying the present results, it seems difficult to assume that DNP and DMDTC act together through the identical way. If these two agents act on the same enzyme system relating to the oxidative phosphorylation, and if the existence of the metal which is to be chelated by DMDTC is essentially concerned with the action of DNP, the DNP effect should be influenced by DMDTC. The results do not seem to meet this expectation.

The stimulating effect of DNP may be caused by the uncoupling of the oxidative phosphorylation, while the effect of DMDTC may be caused by the matal chelating action. These two effect resemble only in appearance and the agents probably behave in different ways in the respiratory process.

CONCLUSION

As a consequence of present experiment, the follbwing points may be presented. 1) The augmentation of respiration by DMDTC is never attributable to the utilization of the agent as a respiratory substrate. 2) The augmented respiration may be mediated through the cytochrome-cytochrome oxidase system, since it is sensitive to CO in darkness, to HCN and also to NaN_3. 3) The dense suspension is hardly sensitive to CO, but more dilute one is sensitive, so the analogous feature of metabolism may exist between in the DMDTC-treated sperm in dense suspension and in the normal sperm in dilute one. 4) DNP may act through a different way from DMDTC, so the uncoupling of oxidative phosphorylation cannot be so easily connected with the heavy metal ion. 5) Finally, the metal which is chelated by DMDTC cannot be iron, since if it be so, the augmentation should occur by CO in darkness.

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SUMMARY

1. The mode of the augmented respiration, caused by 10^{-2}M DMDTC, of sea-urchin sperms was investigated in comparison with that of the normal respiration at a dilution 1:6, or at a density of 5 x 10^9 sperms per ml of the suspension.

2. There were no significant differences in the R.Q. values between the DMDTC-respiration and the normal one.

3. The normal respiration at a dilution 1:6 was hardly sensitive to CO even in darkaness but it was slightly stimulated in light. The DMDTC-respiration at that density and the normal one at a dilution 1:24 were similarly inhibited by CO in darkness and they were restored by illumination.

4. HCN reduced both the DMDTC-respiration and the normal one. The inhibitory effect of HCN was not influenced by DMDTC.

5. NaN_3 at 10^{-2}M acted in a similar manner as HCN. At 10^{-3}M, it hardly affected the normal respiration but somewhat reduced the DMDTC-respiration.

6. DMDTC seemed not to interfere with the action of DNP.

7. From these results, the DMDTC-respiration in dense suspension of sperms was assumed to be mediated through the cytochrome-cytochrome oxidase system as well as the normal respiration in dilute suspension. A working hypothesis as to the analogy between the effect of DMDTC and the dilution effect on the sperm respiration was presented.

Table 1. R. Q. of sperms of Hemicentrotus.

Sperm density: 5 x 10^9 sperms per ml. (See Fig. 1).

Time in minutes after dilution of semen	30	60	120
Control	0.78	0.95	0.93
10^{-2}M DMDTC	0.80	0.94	0.99

Fig. 1. Effect of 10^{-2}M DMDTC on O_2 uptake and CO_2 output of sperms of Hemicentrotus. Sperm density: 5×10^9 sperms per ml. ● : Control, ○ : 10^{-2}M DMDTC, ——— : O_2 uptake, ----- : CO_2 output.

Fig. 2. Effect of CO on the respiration of sperms of Hemicentrotus. Sperm density: 1.25×10^9 sperms per ml. ——— : in air, ----- : in 95% CO-5% O_2. Black and white blocks indicate dark and light periods respectively.

Fig. 3. Effect of CO on the DMDTC-augmented respiration of sperms of Hemicentrotus. Sperm density: 5×10^9 sperms per ml. ● : Control, ○ : 10^{-2}M DMDTC, ——— : in air, ----- : in 95% CO-5% O_2. Black and white blocks indicate dark and light periods respectively.

Fig. 4. Effect of HCN on the DMDTC-augmented respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control, ○ : 10^{-2}M DMDTC, △ : 10^{-3}M HCN, □ : 10^{-4}M HCN, + : 10^{-5}M HCN, ⊘ : 10^{-2}M DMDTC + 10^{-3}M HCN, ◎ : 10^{-2}M DMDTC + 10^{-4}M HCN, ⊕ : 10^{-2}M DMDTC + 10^{-5}M HCN.

Fig. 5. Effect of NaN_3 on the DMDTC-augmented respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control, ○ : 10^{-2}M DMDTC, △ : 10^{-2}M NaN_3, □ : 10^{-3}M NaN_3, + : 10^{-4}M NaN_3, ▽ : 10^{-5}M NaN_3, ⊛ : 10^{-2}M DMDTC + 10^{-2}M NaN_3, ◎ : 10^{-2}M DMDTC + 10^{-3}M NaN_3, ⊕ : 10^{-2}M DMDTC + 10^{-4}M NaN_3, ⊜ : 10^{-2}M DMDTC + 10^{-5}M NaN_3.

Fig. 6-a. Effect of DNP on the DMDTC-augmented respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control, ○ : 10^{-2}M DMDTC, △ : 10^{-3}M DNP, □ : 10^{-4}M DNP, + : 10^{-5}M DNP, ▽ : 10^{-6}M DNP, ⊛ : 10^{-2}M DMDTC + 10^{-3}M DNP, ◎ : 10^{-2}M DMDTC + 10^{-4}M DNP, ⊕ : 10^{-2}M DMDTC + 10^{-5}M DNP, ⊜ : 10^{-2}M DMDTC + 10^{-6}M DNP.

Fig. 6-b. Effect of DNP on the DMDTC-augmented respiration of sperms of Pseudocentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control, ○ : 10^{-2}M DMDTC, △ : 10^{-3}M DNP, □ : 10^{-4}M DNP, + : 10^{-5}M DNP, ⊛ : 10^{-2}M DMDTC + 10^{-3}M DNP, ◎ : 10^{-2}M DMDTC + 10^{-4}M DNP, ⊕ : 10^{-2}M DMDTC + 10^{-5}M DNP.

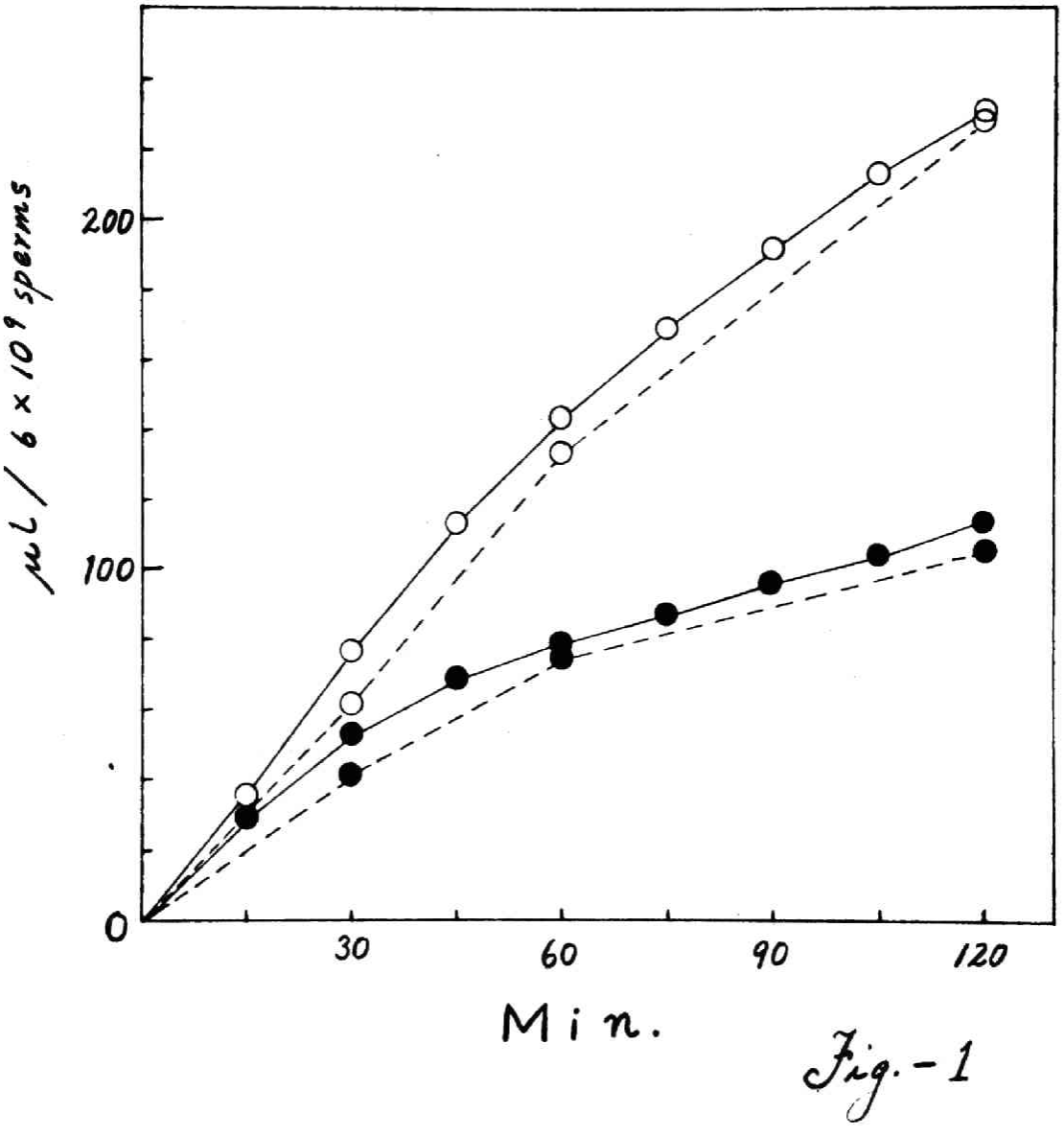


Fig. - 1

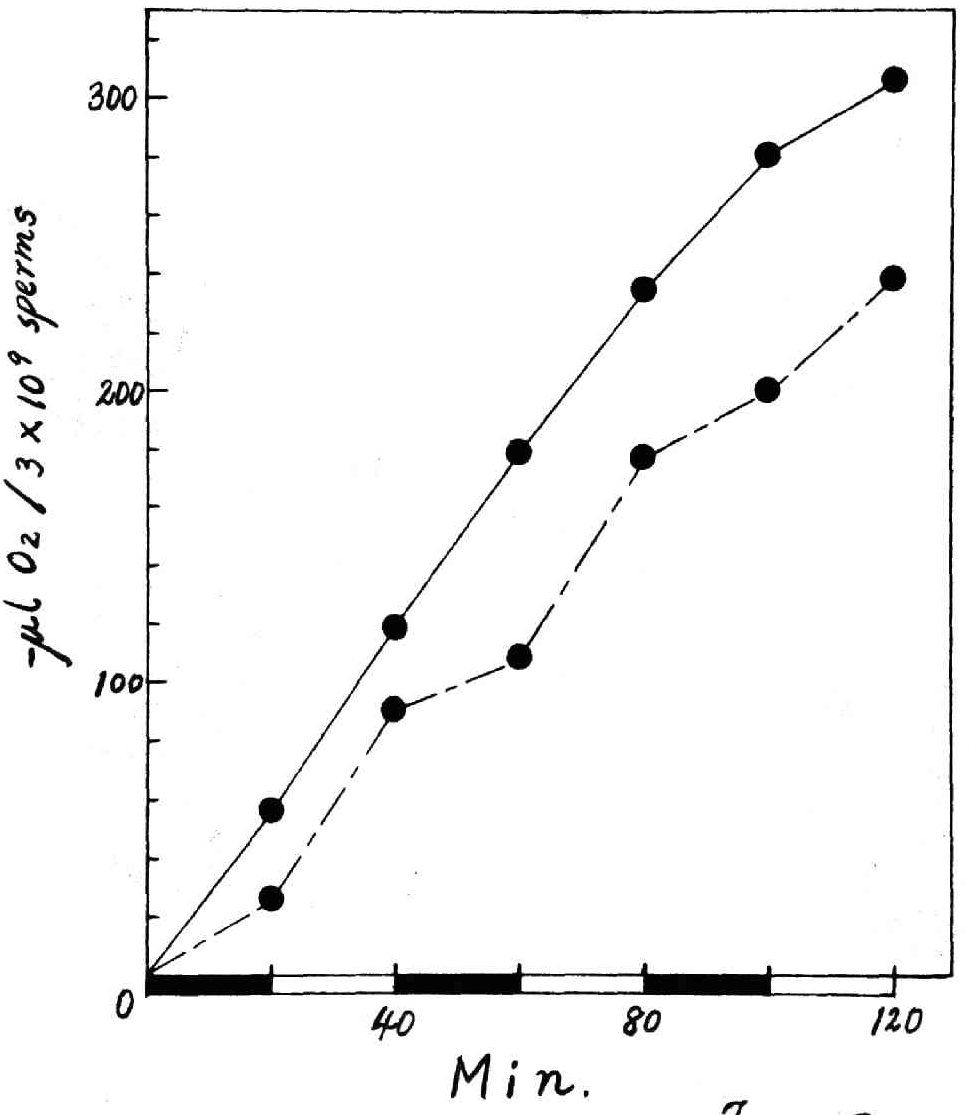


Fig. -2

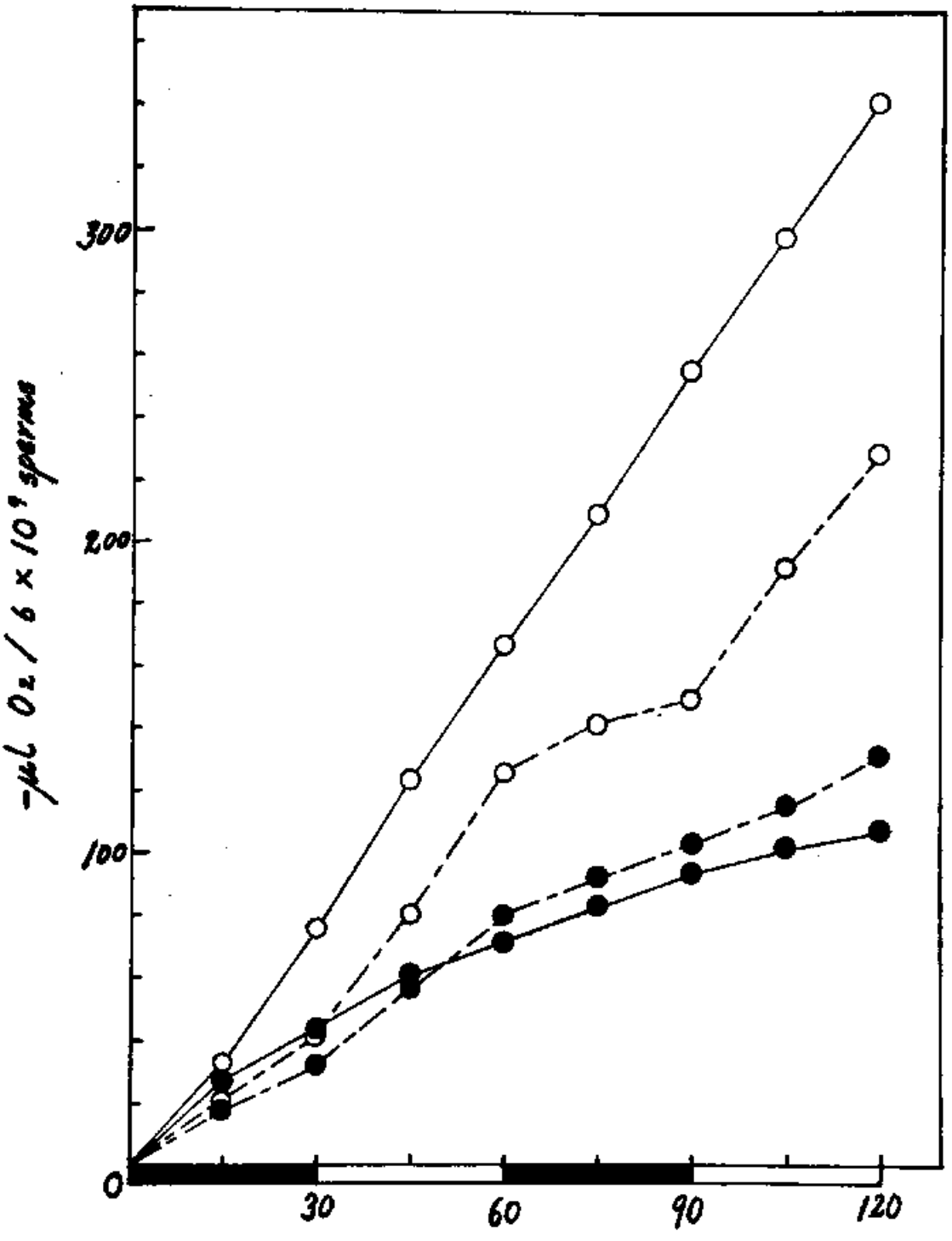


Fig. -3

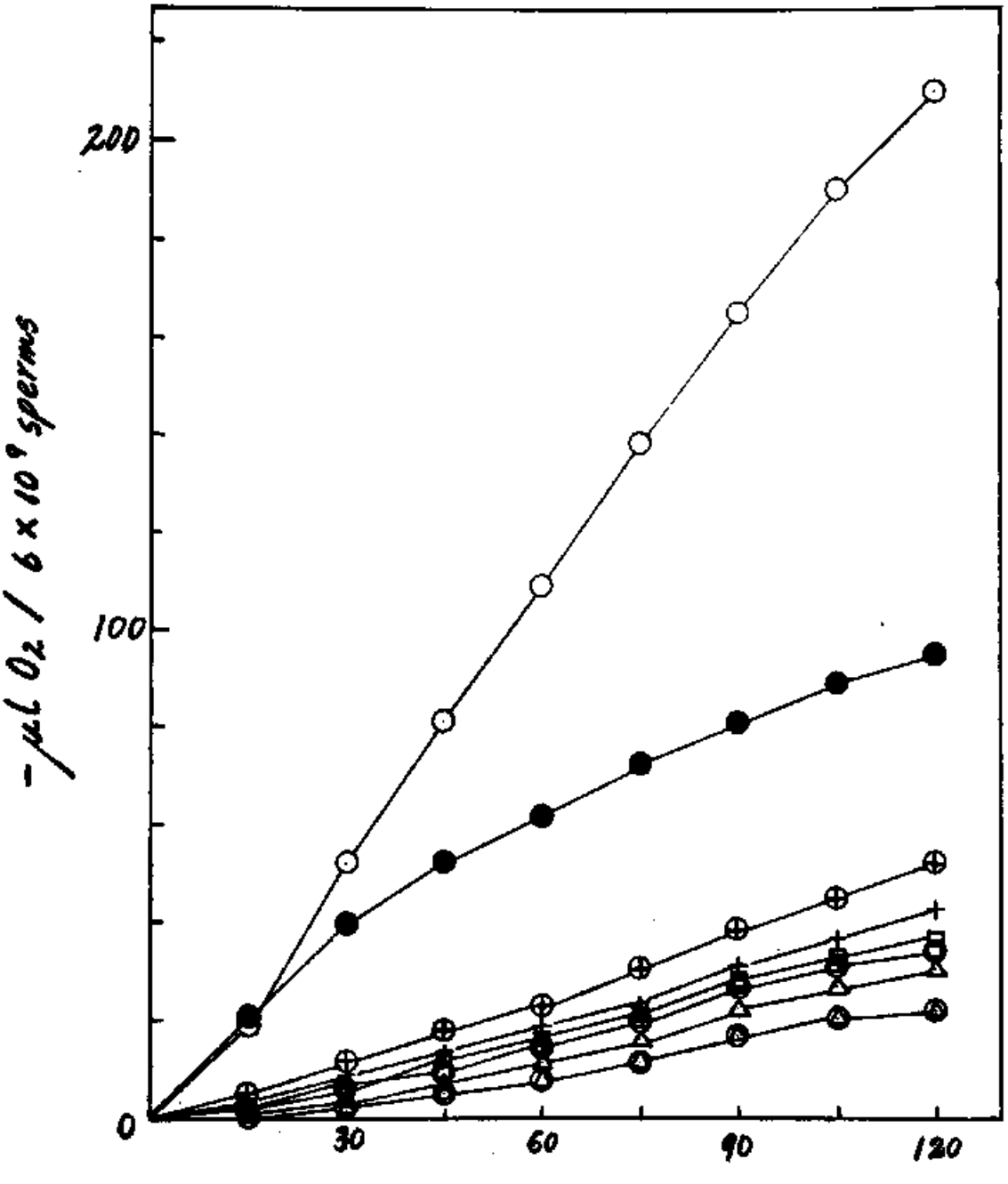


Fig. - 4

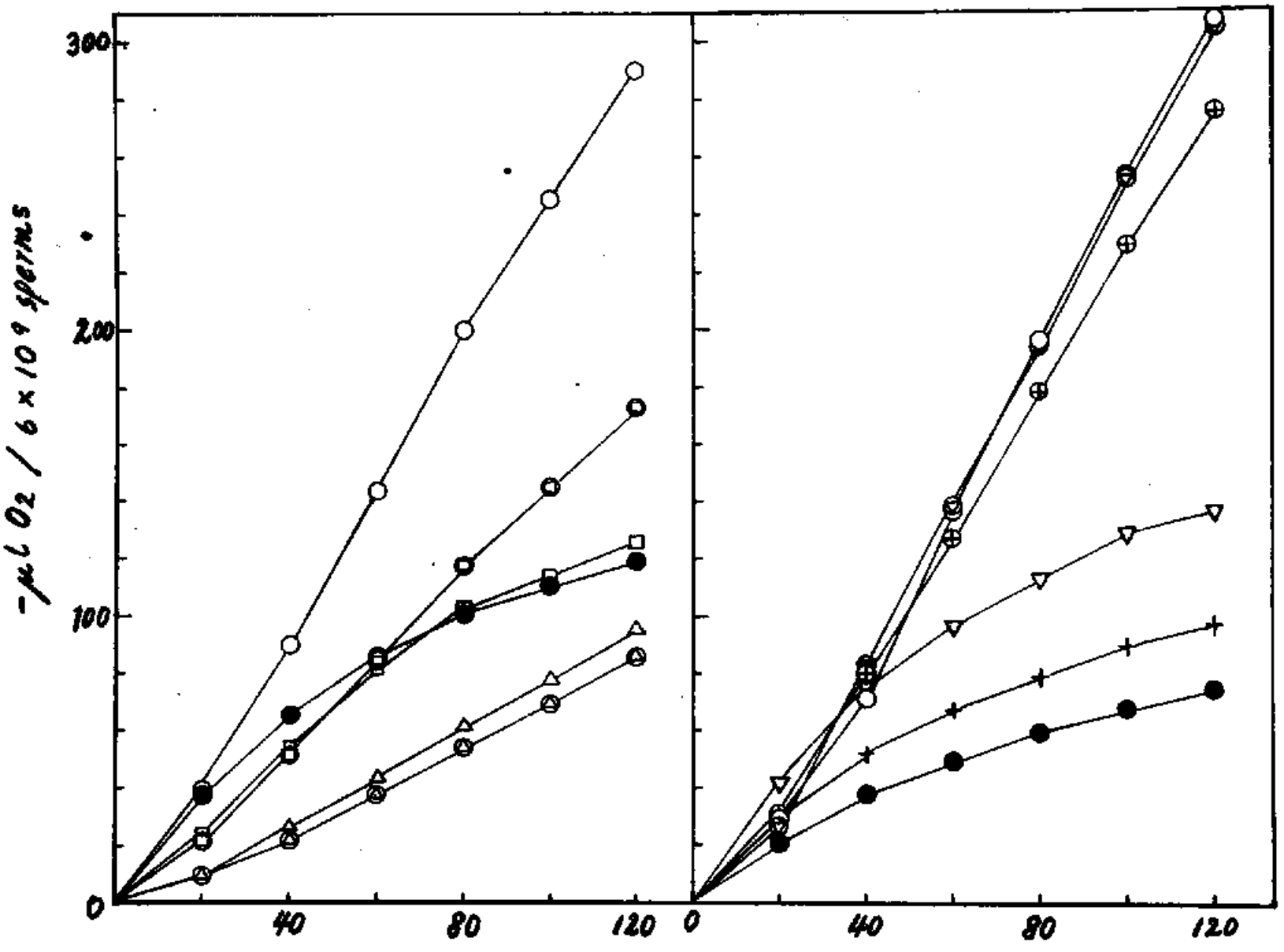
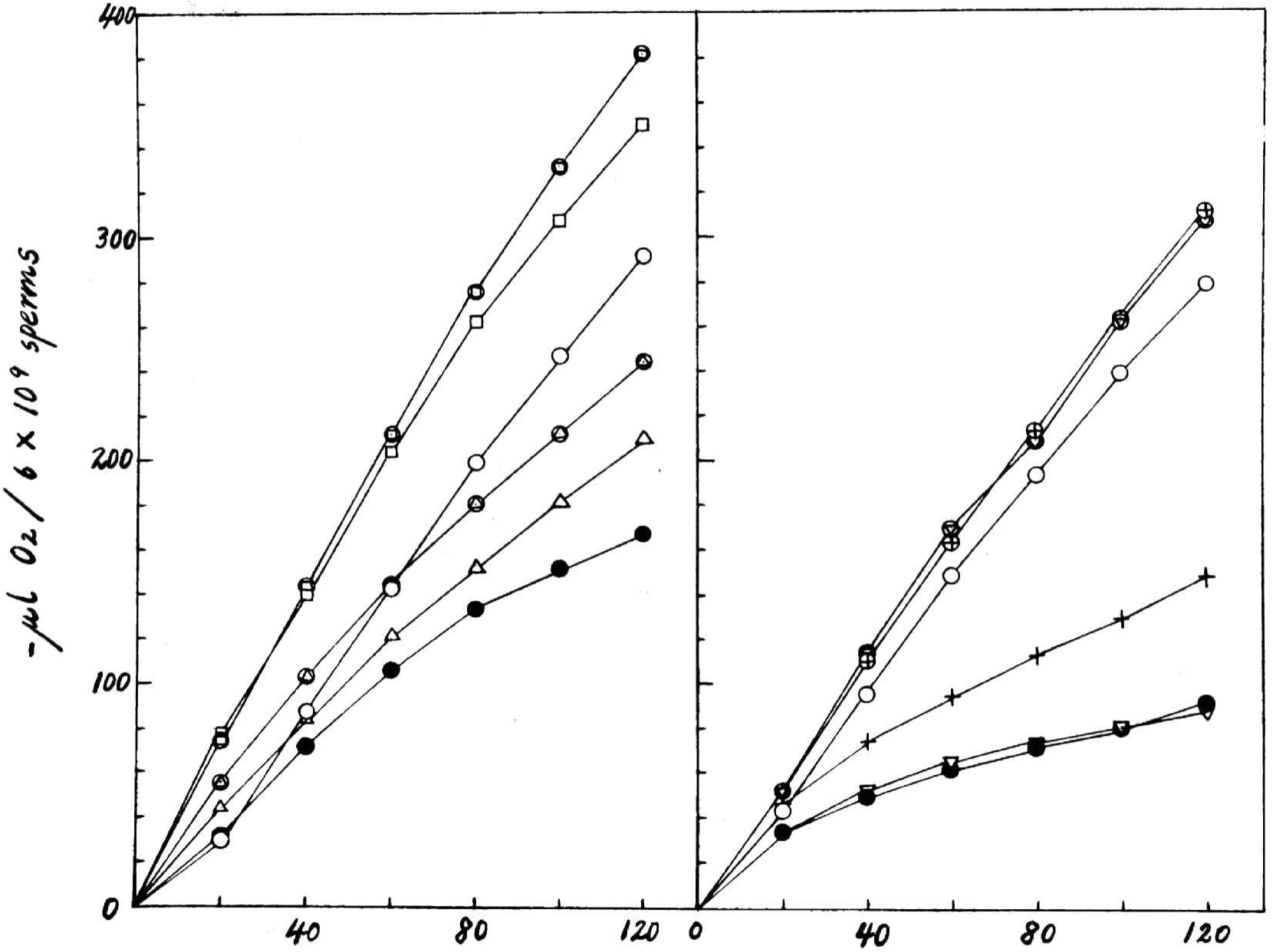


Fig. - 5


-μl O2 / 6 × 10⁹ sperms
400
300
200
100
0
40
80
120
0
40
80
120

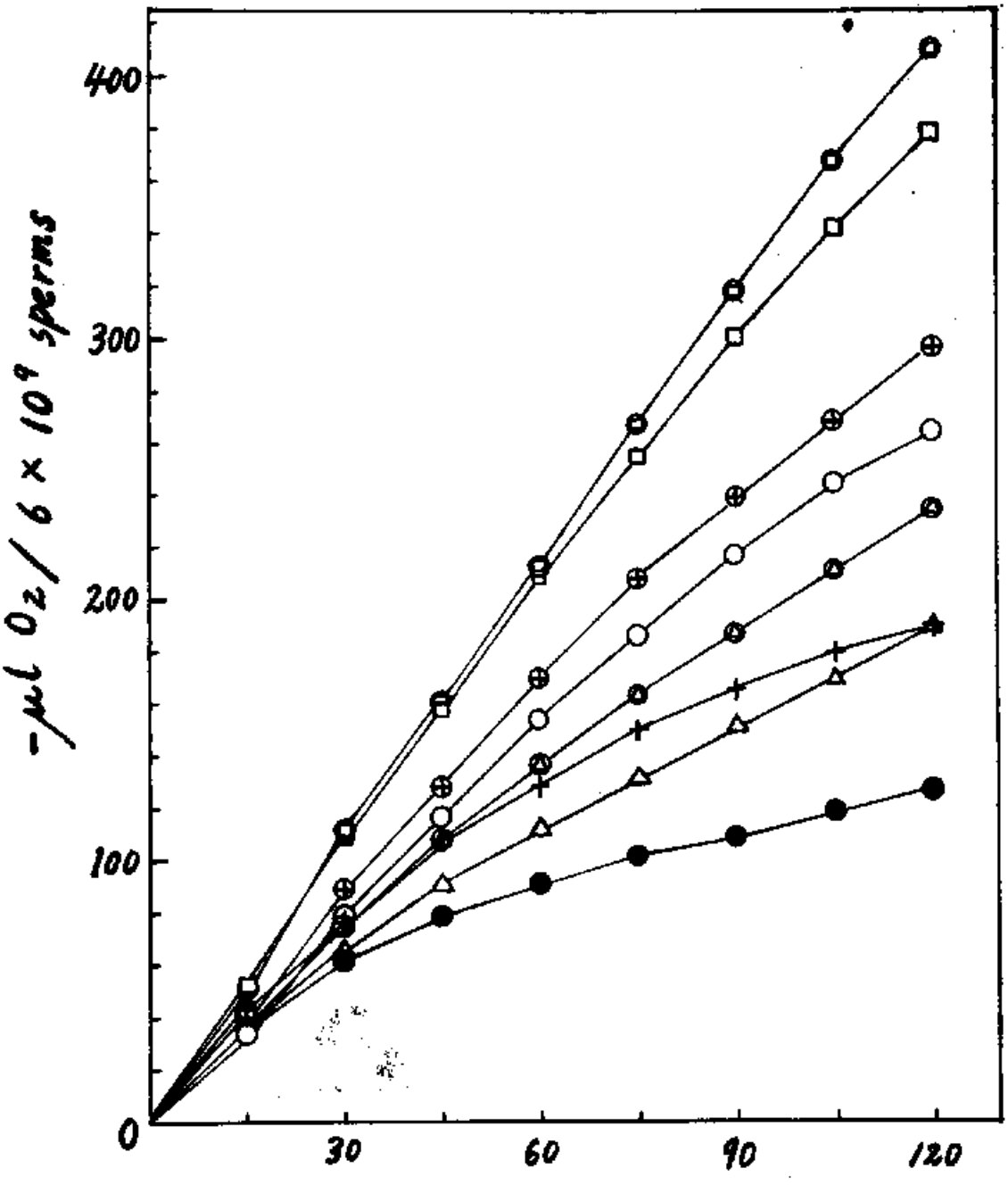


Fig.-6-b

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主論文第三部

STUDIES ON THE EFFECT OF DITHIOCARBAMATE ON THE RESPIRATION OF SEA-URCHIN SPERMS. III. THE RELATION OF SOME METAL IONS TO THE ACTION OF DITHIOCARBAMATE.

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INTRODUCTION

In this series of experiments, it has already been reported (MURAMATSU, 1962 a,b) that dithiocarbamate at 10^{-2}M remarkably augments the respiration of sea-urchin sperms being suspended at a rather high density. This phenomenon has been considered to be caused by the chelating action of the agent to some metal ions probably involved in the sperm itself and never to be due to the oxidation of the agent like as a respiratory substrate. The respiration augmented by dithiocabamate seems to be mediated through the cytochrome-cytochrome oxidase system, since it was evidently inhibited by CO, HCN and NaN_3. This may not be applicable to the normal respiration in such a dense suspension because of the very low sensitivity to CO even in darkness. When the suspension is more diluted, however, the normal respiration also becomes sensitive to CO. Thus, a working hypothesis as to the mode of action of dithiocarbamate has suggested that the agent may shift the metabolic pattern of sperms from the state natural in dense suspension to the more activated one which is analogous to the metabolic state in dilute suspension. This appears to conflict with the hypothesis for the respiratory dilution effect provided by ROTHSCHILD

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et al. (ROTHSCHILD and TUFT, 1950; ROTHSCHILD, 1950; ROTHSCHILD and TYLER, 1954; ROTHSCHILD, 1956). They insisted that the augmentation of the sperm respiration by diluting the sperm suspension may be due to the increase in the ratio of the amount of metal ions in sea water to the number of sperms. These metal ions seemed to act catalytically on the sperm respiration.

At any rate, since the activation of the sperm respiration seems to have some connection with metal ions, it should be fruitful to investigate the relations of some ions to the augmentative effect of dithiocarbamate on the sperm respiration. Moreover, as mentioned above, since the sensitivity to CO seems an index of the characteristics of the sperm metabolism in dilute suspension, the effects of CO on the respiration stimulated by the dilution or by adding either Cu or Zn ions were examined. Some possible explanations as a consequence of this series of experiments will be offered concerning the mode of action of dithiocarbamate and also the respiratory dilution effect, that is, the activation of the sperm respiration by the dilution of suspension.

MATERIALS AND METHODS

Sea-urchins, Hemicentrotus pulcherrimus and Pseudocentrotus depressus were used as materials. The procedure for preparing the standard dry sperm containing 3 x 10^{10} sperms per ml and the constituents of the artificial sea water as the suspending medium were the same as described in the previous paper(MURAMATSU, 1962 a). The medium was buffered with M/50 H_3BO_3 and M/200 $Na_2B_4O_7 10H_2O$ at pH 8.2. When Ca or Mg ion was freed from the artificial sea water, the reduction of

the original tonicity was compensated with NaCl. The ordinary sea water was never used as a suspending medium. The dry sperm was always washed three times with the artificial sea water by means of centrifugation before use.

Oxygen uptake was measured manometrically at 20°C using Warburg manometers run in duplicate or more. In the experiment with CO, all the procedures were the same as in the previous work (MUKAMATSU, 1962 b). The dilution rate of the dry sperm was usually 1:6, i.e. 0.2 ml of the dry sperm in the main compartment of the vessel was diluted with 1 ml of the suspending medium in the side arm at the initiation of measurement. When the rate 1:24 was required, 0.1 ml of the dry sperm was diluted with 2.3 ml of the medium.

All the chemicals were dissolved in the artificial sea water to give the desired final concentrations in the sperm suspension. Sodium dimethyldithiocarbamate(DMDTC) was washed with carbon tetrachloride several times in a few days before use and dried in air.

RESULTS AND DISCUSSIONS

Ca^{++}, Mg^{++} and DMDTC Salts of Ca and those of Mg are contained in sea water approximately at the concentrations of 10^{-2}M and 5×10^{-2}M respectively, and they may play some role in the sperm respiration. Before investigating the relation of the action of DMDTC to alkali earth ions, the effects of these ions under various concentrations on the sperm respiration were examined. $CaCl_2$ was added to Ca^{++}-free sea water and $MgSO_4$ was added to Mg^{++}-free sea water. The results

are shown in Fig. 1 and Fig. 2. The rate of respiration seems dependent on the concentration of Ca^{++} and scarcely on that of Mg^{++}. When either Ca^{++} or Mg^{++} was freed from the medium, the respiration was somewhat reduced; the rate of reduction was larger in the Ca^{++}-free sea water than in the Mg^{++}-free sea water. The excess of Ca^{++}-concentration over the standard artificial sea water, i.e. at 2×10^{-2}M, resulted in the stimulation of respiration and the lack of it, i.e. at 5×10^{-3}M, rather reduced the respiration, while the excess of Mg^{++} (see Fig. 5, at 10^{-2}M added to the artificial sea water) or the lack of it, i.e. at 6×10^{-3}M, hardly influenced on the respiration rate.

These preliminary tests were followed by the experiments for estimating the relation of such ions to the action of DMDTC. If the changes in the concentrations of either Ca^{++} or Mg^{++} could influence on the DMDTC-respiration in a different way comparing with on the normal respiration, such an ion would be assumed to be concerned with the action of DMDTC. From this point of view, DMDTC was added to the sperm suspensions which either contained an excess of the ions over the artificial sea water or did not contain these ions. When the ions were contained excessively, either $CaCl_2$ or $MgSO_4$ was added at 10^{-2}M respectively to the standard artificial sea water. The excess or the absence of Ca^{++} influenced on the DMDTC-respiration, increasingly or decreasingly, in a similar manner as on the normal respiration (Fig. 3 and Fig. 4); the excess of Mg^{++} also could not modify the DMDTC-respiration (Fig. 5). The absence of Mg^{++} scarcely affected the normal respiration in Fig. 6, but the DMDTC-respiration

was somewhat reduced at that time. This might indicate that the DMDTC-respiration required the aid of Mg^{++} to keep its high rate of respiration. It seems difficult to hastily conclude so, however, since the reduction of the normal respiration by the absence of Mg^{++} was seen in Fig. 2. From the above facts, at any rate, it may be reasonable to assume that alkali earth ions such as Ca^{++} and Mg^{++} do not very closely related to the action of DMDTC. An additional evidence for this assumption is presented in Fig. 7 in which both Ca^{++} and Mg^{++} were freed from the medium so that the DMDTC-respiration was reduced as well as the normal respiration.

The experiments in Fig. 4 and Fig. 6 were carried out also with another purpose to analyze the mode of action of DMDTC. The augmentative effect of DMDTC on the sperm respiration has been found to occur at rather high concentration such as 10^{-2}M, and the agents acted inhibitory at a lower concentration such as 10^{-3}M (MURAMATSU, 1962 a). If the necessity of such a high concentration of DMDTC to augment the sperm respiration is attributed to the reduction of the actual concentration of DMDTC in sea water caused by the combining of the agent with alkali earth ions, the lower concentrations of the agent than 10^{-2}M should be satisfactory to reveal the similar augmentative effect when either Ca^{++} or Mg^{++} was freed from the medium. This presumption seemed to be somewhat proved by the rather augmentative effect of 10^{-3}M DMDTC, so that the necessity of high concentration of DMDTC to augment the respiration may be ascribed, at least in part, to the reduction of the actual concentration of the agent in sea water, though the inhibitory effect at lower concentrations has not been

settled yet. Another interpretation to answer the problem, perhaps it may be more important, has been presented in the previous paper in relation to the differences in the effects of the agent due to the ratio of the amount of the agent to that of metal elements in the complex salts.

Cu^{++}, Zn^{++} and DMDTC The stimulating effect of Cu^{++}(ROTHSCHILD and TUFT, 1950) and that of Zn^{++}(UTIDA and NANAO, 1956) on the respiration of sea-urchin sperms have been established. In the experiments shown in Fig.8 and Fig. 10, the similar results as that were obtained. Both $CuSO_4$ and $ZnSO_4$ were most efficient at 10^{-4}M. The rather less stimulation at 10^{-3}M might be due to the toxic action on the sperm metabolism, an excess of the metal ions being present. A peculiar feature in the time course of the respiration was seen when 10^{-5}M $ZnSO_4$ was added; the initial rate of respiration was as high as at 10^{-4}M but it tended to decline thereafter. The concentration of Cu salts in sea water is around 10^{-7}M (BARNES and ROTHSCHILD, 1950) and that of Zn salts is around 10^{-6}M (KINOSHITA, 1956 a) so that the most effectively stimulative concentrations of Cu^{++} and Zn^{++} were many times higher than in the natural condition. At 10^{-6}M, both the stimulating effect of Cu^{++} and that of Zn^{++} were very slight, so perhaps 10^{-7}M Cu^{++} may be expected to scarcely stimulate the respiration. If the respiratory dilution effect could be just ascribed to the increase in the ratio of the number of sperms to that of metal ions, the sperm suspension should be very much diluted, approximately more than 100 times more diluted than the sperm density in the present

experiment, to bring about the conspicuous augmentation in the respiration rate. When the dilution rate is lower than such a value, the dilution effect will be difficult to made its appearance. In practice, the rather remarkable increase usually occurs in the respiration rate even when the dilution rate is only four times higher, i.e. 1:24 (see Fig. 13), than the usual rate in the present work, i.e. 1:6; this facts seems not to meet such an expectation mentioned above.

Though both Cu^{++} and Zn^{++} can stimulate the sperm respiration in dense suspension, the following question should arise which ion is more closely related with the action of DMDTC and with the process of normal respiration. Here let us compare Figs. 9, 11 and 12 with each other. Either $CuSO_4$ at 10^{-3} and 10^{-4}M (Fig. 9) or $ZnSO_4$ at 10^{-4} and 10^{-5}M (Fig. 11) was added with 10^{-2}M DMDTC to the sperm suspension. The augmentative effect of DMDTC exceeded that of Cu^{++}, and DMDTC could elevate the respiration rate revealed at the single addition of Cu^{++}. The reduction of the rate of DMDTC-respiration by 10^{-3}M $CuSO_4$ may be caused by the toxic action of Cu^{++}. $ZnSO_4$ at 10^{-4}M was more efficient to augment the respiration than DMDTC, and even at 10^{-5}M, the initial rate exceeded that of DMDTC-respiration. Such modes of action of Zn^{++} were made to disappear completely by the addition of DMDTC, the rate of respiration revealed at the co-addition of Zn^{++} and DMDTC being almost equal to that at the single addition of DMDTC. From these facts, it may be assumed that Cu^{++} and DMDTC act rather independently and that Zn^{++} has some close connection with the action of DMDTC. Both DMDTC and Zn^{++} may act on the same engyme system and

the catalytic action of added Zn^{++} on the respiration may be completely suppressed by the chelating action of DMDTC. This consideration was also confirmed by the experiment of Fig. 12 in which both $CuSO_4$ and $ZnSO_4$ at 10^{-4}M, which was the most efficient concentration to stimulate the respiration, were added simultaneously to the suspension and the effect of DMDTC under this condition was examined. When only one of these agents was added to the suspension, the order concerning the effectiveness for augmentation was shown as follows; 10^{-4}M $ZnSO_4$, 10^{-2}M DMDTC, 10^{-4}M $CuSO_4$. When Cu^{++} and Zn^{++} were added together, the single effect of Cu^{++} was made to disappear and replaced by the single effect of Zn^{++}. When DMDTC was given in addition to them, the respiration rate revealed by Cu^{++} and Zn^{++} was considerably reduced to that slightly less than the DMDTC-respiration. These results may indicate that Zn^{++} is more closely concerned than Cu^{++} not only with the DMDTC -respiration but also with the normal respiration. The consideration that Zn^{++} may be very closely related with the sperm respiration does not agree with that of ROTHSCHILD et al., for they insisted on Cu^{++} to be the main participant. However, since their conclusion has been drawn only from the experiment with Cu^{++} and they did not compare the effect of Cu^{++} with that of Zn^{++}, the present author's conclusion seems rather reasonable.

Remarks on the respiratory dilution effect Throughout the series of experiments (MURAMATSU, 1962 a, b), the author has paid his attention to the augmentative effect of DMDTC on the respiration of sea-urchin sperms and has ascribed the respiratory dilution effect, that is, the increase in respiration rate caused by the dilution of

sperm suspension, to the removal or masking of some metal included in the sperm body. This presumption seems to very much conflict with another hypothesis presented so far by several workers. They insisted on the catalytic action of Cu ions in sea water to be a main cause to increase the respiration rate. According to ROTHSCHILD and TUFT (1950), the dilution effect did not occur when sperms were suspended in NaCl solution isotonic to sea water, but it was made its appearance by the addition of 1 p.p.m. $CuCl_2$. The characteristic decline in O_2 uptake in the dilute suspension was somewhat arrested by adding Cu salts. By diethyldithiocarbamate at 10^{-3}M or below, the O_2 uptake in sea water was reduced but it could be restored again by the addition of Cu salts. ROTHSCHILD(1950) could elevate the respiration rate in dense suspension by the addition of Cu salts to sea water to the equal level seen at the dilute suspension so that the dilutionneffect seemed not to occur. On the contrary, diethyldithiocarbamate at 10^{-3}M or below acted antagonistically against Cu^{++} and reduced the respiration rate in dense suspension again. ROTHSCHILD and TYLER (1954) found that 10^{-3}-10^{-6}M ethylenediaminetetracetate reduced or abolished the dilution effect in dilute suspension. As a consequence of these experiments, they have assumed that the dilution effect should be caused by the catalytic action of Cu^{++} in sea water on the respiratory system resulting from the increase in the ratio of the number of sperms to that of thesmetal ions in sea water.

Though Cu ions in sea water were assumed to cause the respiratory dilution effect, such ions have well been established to influence toxically on the life-span of sperms for maintaining their fertilizing

capacity. TYLER(1950, 1953), TYLER and ATKINSON(1950) found the prolongation of the life-span by the addition of some metal chelating agents to the sperm suspension. The short life-span in natural condition could not be associated to the exhaustion of the endogenous substrate but to the toxic action of the heavy metals (TYLER and ROTHSCHILD, 1951). That Ca ions were not responsible for such a toxic action was affirmed by TYLER(1950), ROTHSCHILD and TYLER(1954), the life-span being longest at the Ca-concentration of 10^{-2}M which is aboutathe value in sea water.

As mentioned above, the addition of metal ions stimulate the respiration of sea-urchin sperms, while in the starfish sperms,it seems not to be the case. The existence of an amount of Zn in the sperms of this animal was reported by FUJII(1954). Starfish sperms are usually motionless being suspended in sea water, but they are made to move by the addition of some metal chelating agents resulting in the increase of the O_2 uptake (FUJII et al., 1955). Zn was found to be contained in lipoprotein in the sperm (KINOSHITA, 1956 b), and it was released out of the sperm to the surrounding medium when the sperm motion was induced by histidine (MIZUNO,1956; KINOSHITA, 1956 a). This was not be able to observed in the case of sea-urchin sperms in which the content of Zn was lower than starfish sperms. UTIDA and NANAO(1956) found that the rapid fall of initial high rate of respiration in sea-urchin sperms was restored by adding Zn^{++} and this high rate might be comparable to the steady low rate of respiration of starfish sperms. By the addition of Zn^{++}, the apyrase activity in the sperm tails was inhibited in the starfish but it was rather

activated in sea-urchin (UTIDA et al., 1956 a,b), and such a difference between two animals was considered to correspond to the difference in the effect of Zn^{++} on the respiration.

Gathering from the facts described above, the respiration of sperms both of sea-urchin and of starfish seems to be closely concerned with certain metal ions. According to the hypotheses presented so far, the activation of the respiration should be attributed to the addition of metals to sperms in the case of sea-urchin, while in the case of starfish it should be to the removal of metals from the sperms, though these two animals belong to the same phylum, echinoderm. If the hypothesis of the present author, on the other hand, can be accepted to be reasonable, the mechanism in the activation of sperm respiration of both animals will be explained in a similar way.

From the present knowledge, it may be reasonable to assume that to become sensitive to CO is one of the characteristic changes in the respiration associated with diluting the suspension of sea-urchin sperms (MURAMATSU, 1962 b). Since both the DMDTC-augmented respiration in dense suspension and the normal respiration in dilute suspension were sensitive to CO, while the normal one in dense suspension was hardly reduced by CO, an analogy in the metabolism was expected between the DMDTC-treated sperms and the normal ones. When the sperm respiration in dense suspension is augmented by certain treatments, and if this augmentation is caused by the metabolic changes analogous to the natural ones associated with the dilution of dense suspension with sea water, the respiration should be sensitive to CO in darkness.

In other words, at least the CO-insensitive respiration should not have the same respiratory process as in the normal respiration in dilute suspension. From this point of view, the effects of CO on the sperm respiration augmented by either the dilution with artificial sea water or by the addition of Cu^{++} or Zn^{++} were investigated. The gas mixture used for the tests was composed of 95% CO and 5% O_2. Air was employed for control, since in the preliminary test the difference in the O_2 uptake was not seen between in air and in the mixture of 95% N_2 and 5% O_2.

In the experiment of Fig. 13, the dry sperm was diluted either 1:6 or 1:24. Comparing the normal respiration between at these different densities, the rate of it far exceeded in the dilute suspension over that in the dense suspension. This indicated the occurrence of the dilution effect even without the addition of heavy metal ions. The result appears to disagree with that of ROTHSCHILD and TUFT(1950): the dilution effect did not occur if the sperms were suspended in the NaCl solution isotonic to sea water at a certain dilution rate at which the dilution effect occurred if sea water was employed. It seems , however, doubtful to convince only from their result that heavy metal ions are essential to increase the respiration rate by means of dilution, because their result should be attributed not only to the absence of heavy metal ions but also to that of the other important ions such as Ca^{++} and Mg^{++}. As already shown in Figs. 1 and 2, the lack of alkali earth ions reduces the sperm respiration. If the stimulating trigger in the respiratory system was pulled by the dilution even with NaCl solution, the high rate respira-

tion should not be able to take place because of the lack of Ca^{++} and Mg^{++}. When only the heavy metal ions are subjected for investigating the sperm respiration, only such ions should be excluded from the medium but other ions should be included. Hence, the present result can be available as a reliable evidence to put the work forward. The respiration of sperms diluted 1:6 was hardly reduced by CO, except at the initial high rate, even in darkness but slightly stimulated. In more dilute suspension (1:24), the sperms were very sensitive to CO in darkness, especially at the initial high rate, and restored completely by illumination. A somewhat over compensation due to illumination was seen about one hour or more after dilution of semen, at which the normal respiration became declined. From these results, a similar feature of respiratory process may be expected between in the sperms diluted with sea water and with artificial sea water.

To add the evidences for the author's working hypothesis, the experiments in Figs. 14 and 15 were carried out in which the sensitivity to CO of the high respiration caused by the addition of either Cu^{++} or Zn^{++} was examined. If such a high rate respiration is hardly affected by CO, it should proceed in a different way from the natural high respiration. The results did not meet the expectation; the high rate respiration in both cases also sensitive to CO photo-reversibly. The CO-sensitivity of Cu^{++}- and Zn^{++}-respiration might appear favorable for the hypothesis that the dilution effect may be caused by the addition of metal ions to sperms, but this is unreasonable because of the occurrence of the dilution effect

without the addition of metal ions as shown in Fig. 13. At any rate, the respiration in high rate, whatever the cause may be, is sensitive to CO and the cytochrome-cytochrome oxidase system may be a rate limiting factor in it.

Now the present author wishes to approach the task for explaining the respiratory dilution effect as a consequence of the series of experiments. Since the dilution effect also can take place even without the aid of heavy metal ions, the hypothesis presented by ROTHSCHILD et al. should be excluded. If the theories of FUJII et al. that the activation of the respiration of starfish sperm may be caused by the release of Zn out of the sperm body can be admitted to be reasonable, a similar mechanisms are expected also in sea-urchin sperms. Although MIZUNO(1956) has reported that starfish sperms contained a considerable amount of Zn and it released out when they were made to be active in the respiration and that Zn was very small in sea-urchin sperms and the release of it could not be detected at the dilution of sperm suspension with sea water, the possibility that Zn might release from the sea-urchin sperms would not be completely excluded. The present author does not intend to ignore the catalytic action of the exogenous metal ions on the sperm respiration , which has gave the favorable basis for the hypothesis presented so far. However, it seems unnatural to suppose that the metal ions which play an important role in the activation of sperm respiration acts on the other hand toxically on the physiology of the sperm as considered by TYLER et al.. Whereas, if the activation is really due to the release of metal from the blocking system in respiratory

process, the catalytic but toxic action of the exogenous metal ions cannot be taken part in the activation in natural condition. This presumption may be partly proved from the fact that the catalytic action of Zn^{++} is completely masked by adding DMDTC (Fig. 11); when the metal in blocking system is removed by DMDTC or also perhaps by dilution so that the respiration becomes to be stimulated, the catalytic action of Zn^{++} on the respiratory system cannot make its appearance at the dilution.

A hypothesis of the present author for the mechanism of the dilution effect on the respiration of sea-urchin sperms is as follows: the blocking system or compound involving the heavy metal probably Zn may intervene in the electron carriers between the substrate and cytochromes, and it is removed or masked, though this mechanism has not yet been known, by the dilution with sea water resulting in the activation of the respiration. This can be artificially realized when the dense suspension, in which the respiration rate is rather low, is treated with dithiocarbamate which is one of the metal chelating agents having a high affinity to some transition metals such as Cu or Zn. The activation of sperm respiration by the addition of metal ions should be only an artificial phenomenon so that they do not seem to be concerned with the dilution effect in practice. Heavy metal ions in sea water act negatively and injuriously to the sperm function for acheiving the fertilization.

To make sure this working hypothesis, it may be necessary to investigate the effect of dithiocarbamate on the motility and the fertilizing capacity of the sperms and further to obtain the entity of such

a presumptive blocking system or compound probably involving Zn from the respiratory system in sea-urchin sperms.

CONCLUSION

The augmentative effect of DMDTC on the respiration of sea-urchin sperms in dense suspension is closely related with Zn ions. The respiratory dilution effect also takes place without the addition of heavy metal ions from the surrounding medium, so that the hypothesis presented so far that the catalytic action of the exogenous metal ions would be a main cause to activate the sperm respiration should be excluded. The removal or masking of a blocking system or compound involving some metal in the electron carriers at the dilution of semen may be more preferable theory to interpret the dilution effect.

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SUMMARY

1. The relations of some bivalent metal ions, such as Ca^{++}, Mg^{++}, Cu^{++} and Zn^{++}, to the action of DMDTC, and the effects of CO on the sperm respiration activated either by dilution or by the addition of Cu^{++} or Zn^{++} were examined. The artificial sea water containing no heavy metal ions was always used as the suspending

medium. The dry sperms were diluted usually 1:6 unless otherwise indicated.

2. The excess amount of Ca^{++} over natural sea water slightly stimulated and the absence of it rather reduced both the DMDTC-respiration and the nomal one. The influences of the changes in the concentration of Mg^{++} on the respiration was far less than those of Ca^{++}.

3. Both Cu^{++} and Zn^{++} were able to augment the sperm respiration; Zn^{++} was more effective than Cu^{++}. When 10^{-2}M DMDTC was added either with Cu^{++} or with Zn^{++}, the stimulated respiration by Cu^{++} was further augmented, while the single effect of Zn^{++} was made to disappear and replaced by that of DMDTC. When Cu^{++} and Zn^{++} were added simultaneously, the effect of Zn^{++} was dominant; and when DMDTC was added besides, the result was almost similar to the one in the case when Zn^{++} and DMDTC were added together.

4. The dilution effect occurred even when the dry sperms was diluted 1:24 with the heavy metal-free sea water, the respiration rate far exceeding that of the sperms diluted 1:6. The respiration was sensitive to CO at 1:24 dilution, while it was hardly affected at 1:6 dilution. The augmented respiration caused by either Cu^{++} or Zn^{++} was also sensitive to CO photo-reversibly.

5. From the results obtained in this and the preceding papers (MURAMATSU, 1962 a, b), the author discussed as to the relation of metal ions to the action of DMDTC and a new reasonable hypothesis for the mechanism involved in the respiratory dilution effect was presented.

Fig. 1. Effect of various concentrations of $CaCl_2$ on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water) ; $CaCl_2$-concentration, ◐ : 0(Ca^{++}-free), Δ : 2×10^{-2}M, □ : 10^{-2}M, + : 5×10^{-3}M.

Fig. 2. Effect of various concentrations of $MgSO_4$ on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water) ; $MgSO_4$-concentration, ◐ : 0(Mg^{++}-free), □ : 6×10^{-2}M, + : 6×10^{-3}M.

Fig. 3. Effect of DMDTC in the presence of excess of Ca^{++} on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-2}M DMDTC in artificial sea water, ▲ : 10^{-2}M $CaCl_2$ added to artificial sea water, Δ : 10^{-2}M DMDTC added with 10^{-2}M $CaCl_2$ to artificial sea water.

Fig. 4. Effect of DMDTC in Ca^{++}-free sea water on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-2}M DMDTC in artificial sea water, ◐ : Ca^{++}-free sea water ; DMDTC-concentration in Ca^{++}-free sea water, ◑ : 10^{-2}M, ◒ : 5×10^{-3}M, ◒ : 10^{-3}M.

Fig. 5. Effect of DMDTC in the presence of excess of Mg^{++} on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-2}M DMDTC in artificial sea water, ▲ : 10^{-2}M $MgSO_4$ added to artificial sea water, △ : 10^{-2}M DMDTC added with 10^{-2}M $MgSO_4$ to artificial sea water.

Fig. 6. Effect of DMDTC in Mg^{++}-free sea water on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-2}M DMDTC in artificial sea water, ◐ : Mg^{++}-free sea water; DMDTC-concentration in Mg^{++}-free sea water, ◑ : 10^{-2}M, ◓ : 5×10^{-3}M, ◒ : 10^{-3}M.

Fig. 7. Effect of DMDTC in Ca^{++}, Mg^{++}-free sea water on the respiration of sperms of Hemicentrotus. Sperm density: 5×10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-2}M DMDTC in artificial sea water, ◉ : Ca^{++}, Mg^{++}-free sea water, ◎ : 10^{-2}M DMDTC in Ca^{++}, Mg^{++}-free sea water.

Fig. 8. Effect of $CuSO_4$ on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water); $CuSO_4$-concentration, △ : 10^{-3}M, □ : 10^{-4}M, + : 10^{-5}M, ▽ : 10^{-6}M.

Fig. 9. Effect of $CuSO_4$ on the DMDTC-augmented respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-2}M DMDTC, △ : 10^{-3}M $CuSO_4$, □ : 10^{-4}M $CuSO_4$, ⓐ : 10^{-3}M $CuSO_4$ + 10^{-2}M DMDTC, ⊕ : 10^{-4}M $CuSO_4$ + 10^{-2}M DMDTC.

Fig. 10. Effect of $ZnSO_4$ on the respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water); $ZnSO_4$-concentration, △ : 10^{-3}M, □ : 10^{-4}M, + : 10^{-5}M, ▽ : 10^{-6}M.

Fig. 11. Effect of $ZnSO_4$ on the DMDTC-augmented respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-2}M DMDTC, □ : 10^{-4}M $ZnSO_4$, + : 10^{-5}M $ZnSO_4$, ⓟ : 10^{-4}M $ZnSO_4$ + 10^{-2}M DMDTC, ⊕ : 10^{-5}M $ZnSO_4$ + 10^{-2}M DMDTC.

Fig. 12. Effect of $CuSO_4$ and $ZnSO_4$ on the DMDTC-augmented respiration of sperms of Hemicentrotus. Sperm density : 5×10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-2}M DMDTC, ▲ : 10^{-4}M $CuSO_4$, △ : 10^{-4}M $ZnSO_4$, ◭ : 10^{-4}M $CuSO_4$ + 10^{-4}M $ZnSO_4$, ⓐ : 10^{-4}M $CuSO_4$ + 10^{-4}M $ZnSO_4$ + 10^{-2}M DMDTC.

Fig. 13. Effect of CO on the respiration of sperms of Pseudocentrotus under different densities. Sperm densities : 5 x 10^9 sperms per ml at the dilution 1:6, 1.25 x 10^9 sperms per ml at the dilution 1 : 24. ● : Dilution 1:6, ○ : Dilution 1:24, ——— : in air, ----- : in 95% CO-5% O_2. Black and white blocks indicate dark and light periods respectively.

Fig. 14. Effect of CO on the respiration stimulated by 10^{-4}M $CuSO_4$ of sperms of Pseudocentrotus. Sperm density : 5 x 10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-4}M $CuSO_4$, ——— : in air, ----- : in 95% CO- 5% O_2. Black and white blocks indicate dark and light periods respectively.

Fig. 15. Effect of CO on the respiration stimulated by 10^{-4}M $ZnSO_4$ of sperms of Pseudocentrotus. Sperm density : 5 x 10^9 sperms per ml. ● : Control (Artificial sea water), ○ : 10^{-4}M $ZnSO_4$, ——— : in air, ----- : in 95% CO-5% O_2. Black and white blocks indicate dark and light periods respectively.

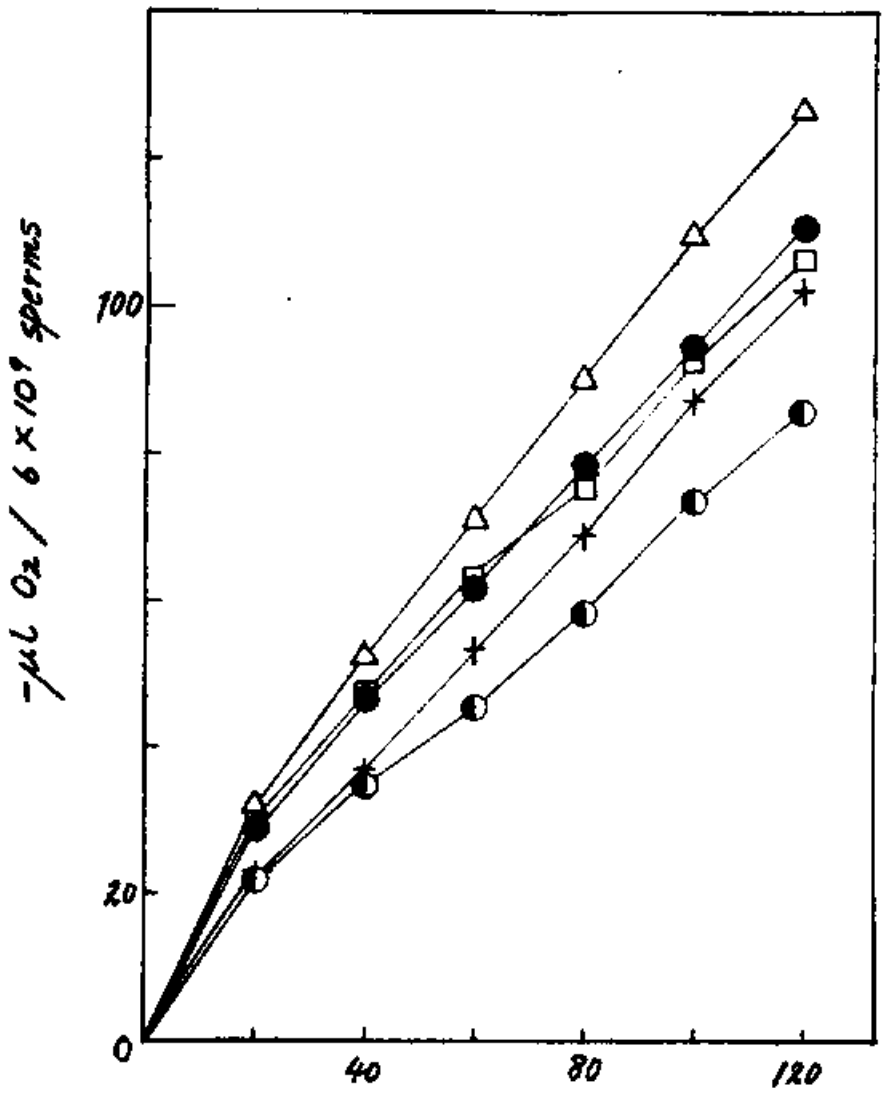
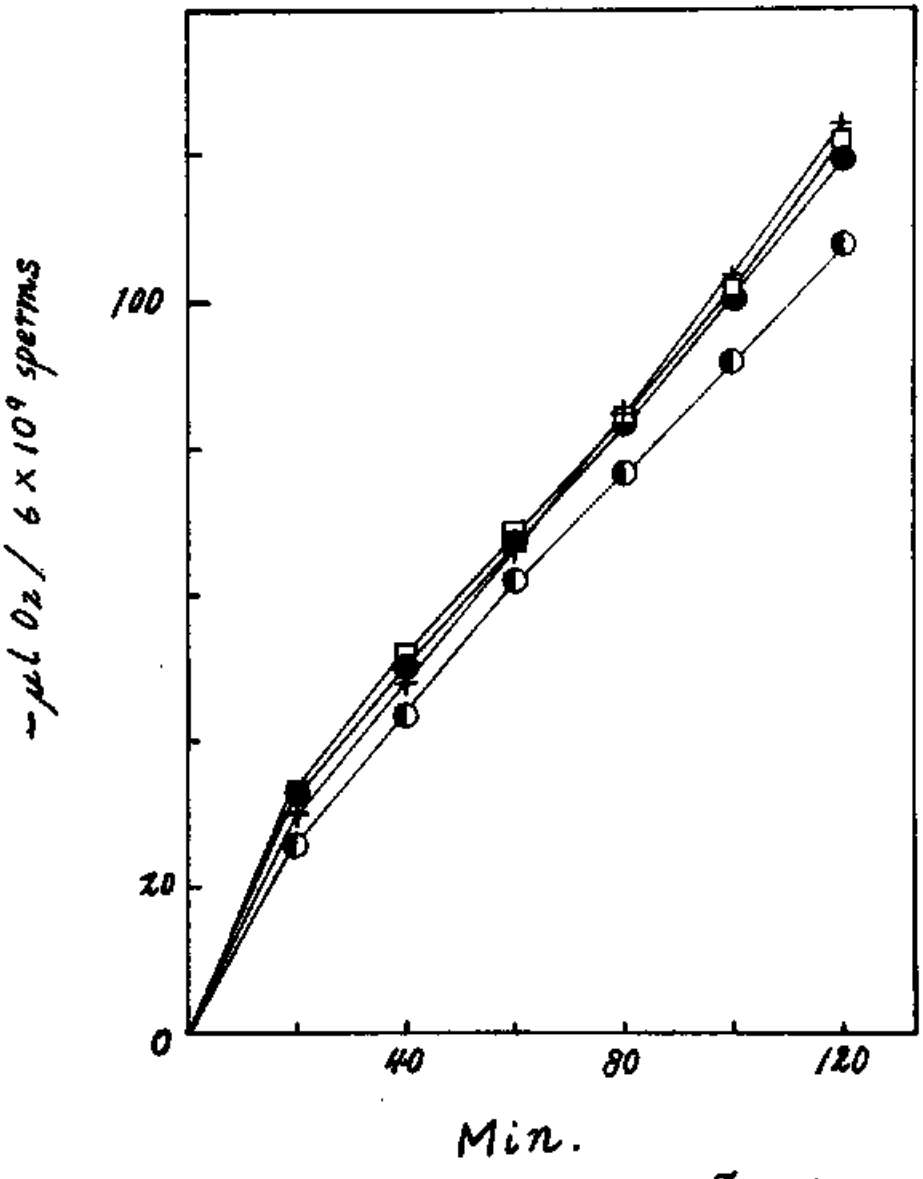


Fig. 1



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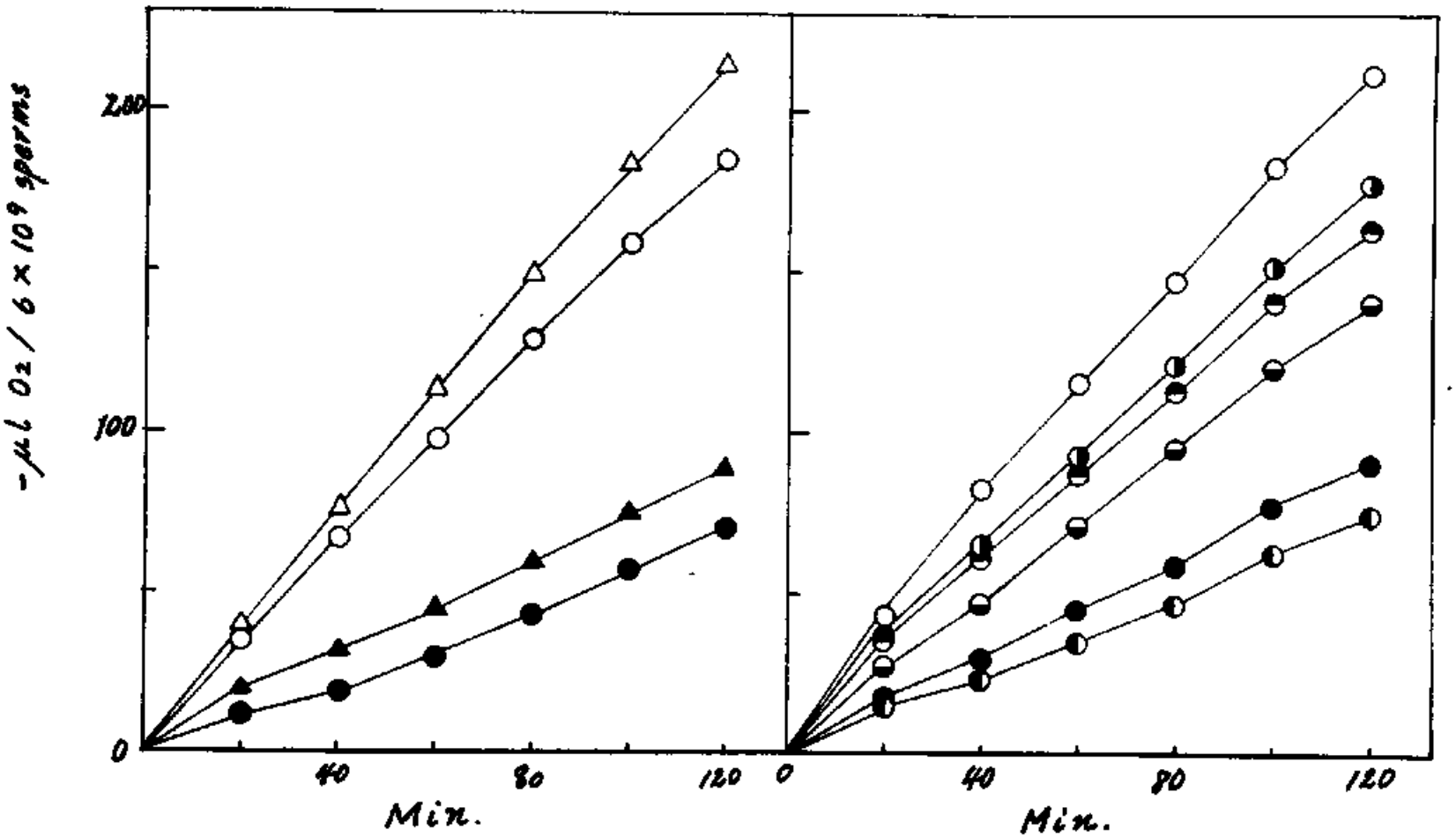

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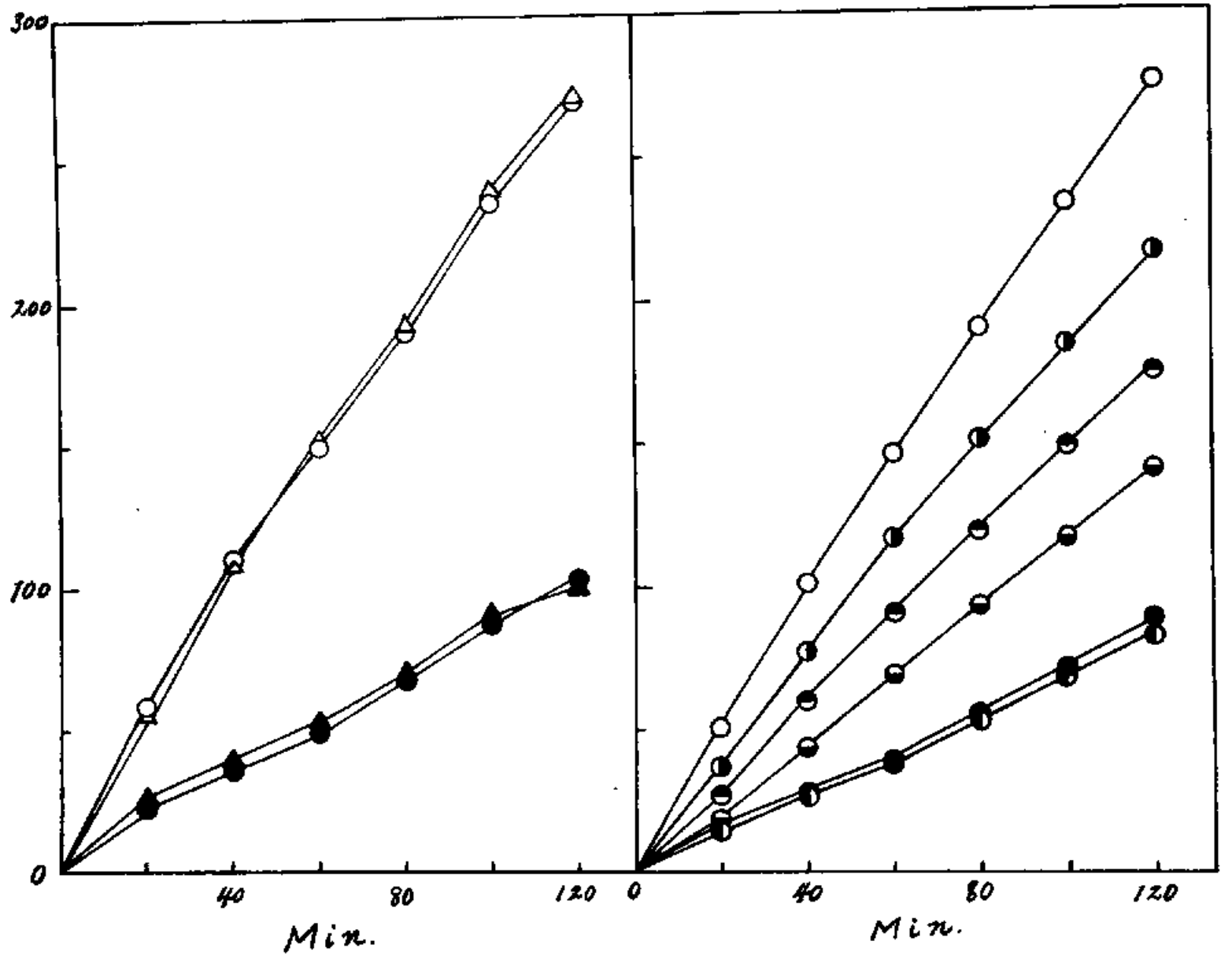
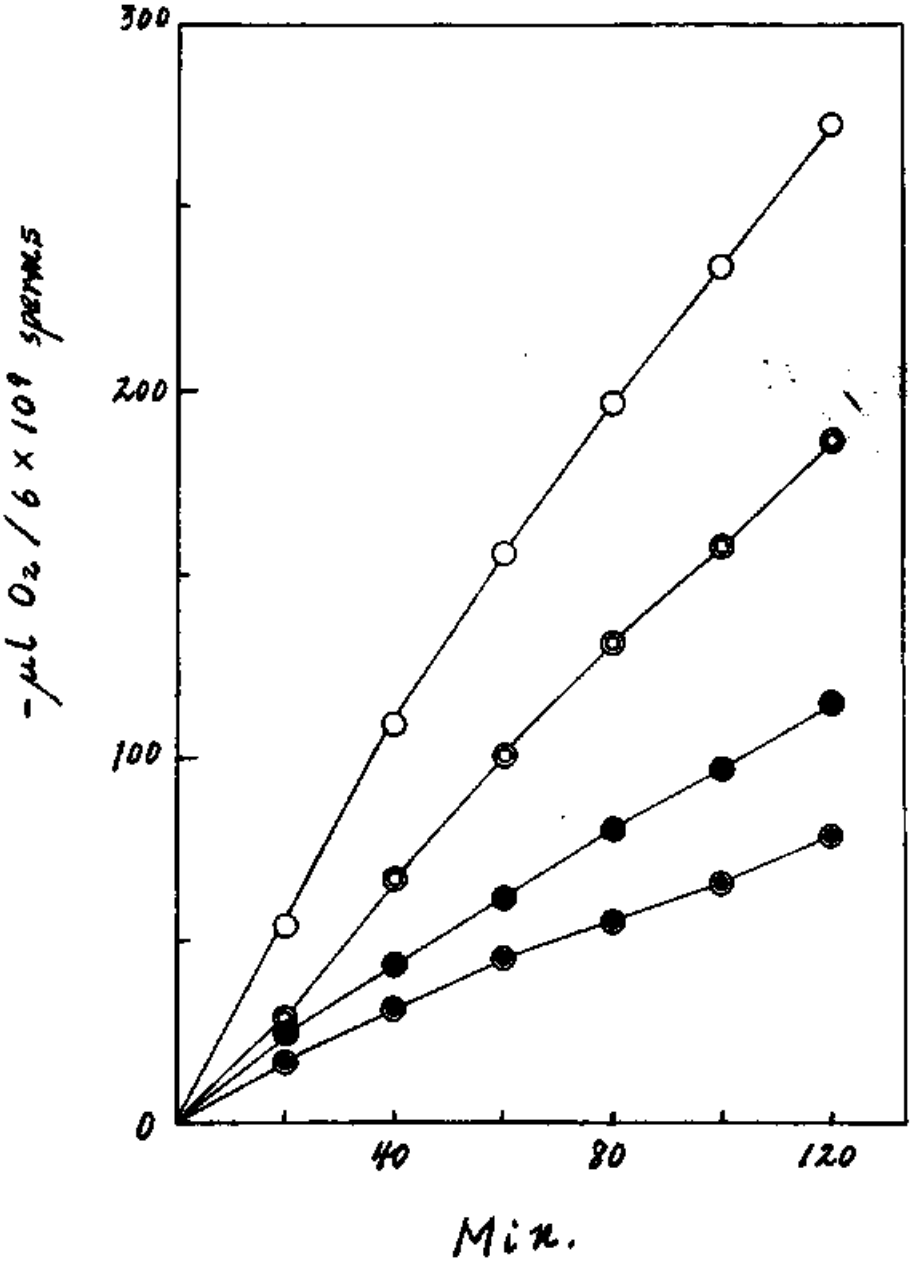


Fig. 5

Fig. 6



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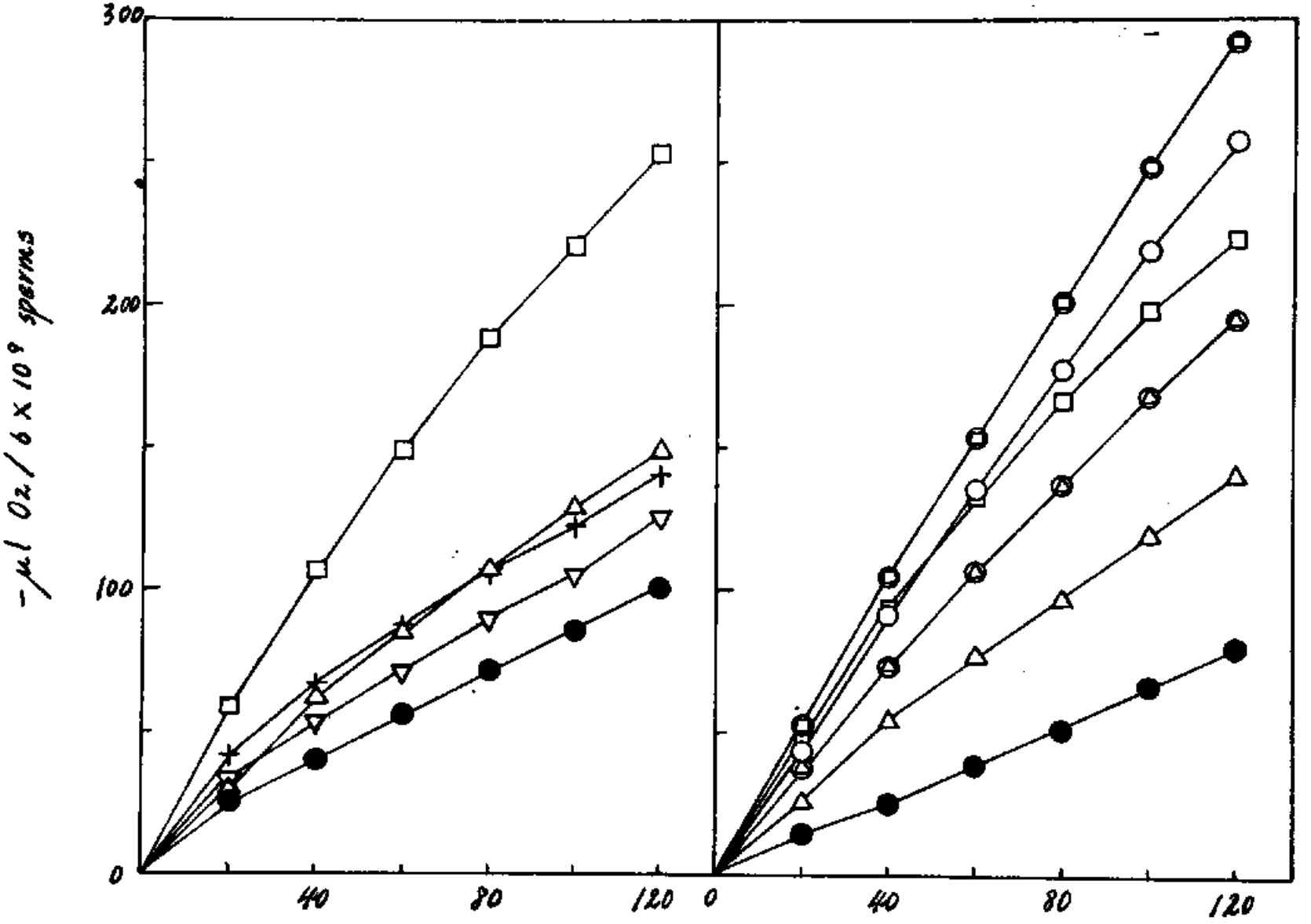


Fig. 8

Fig. 9

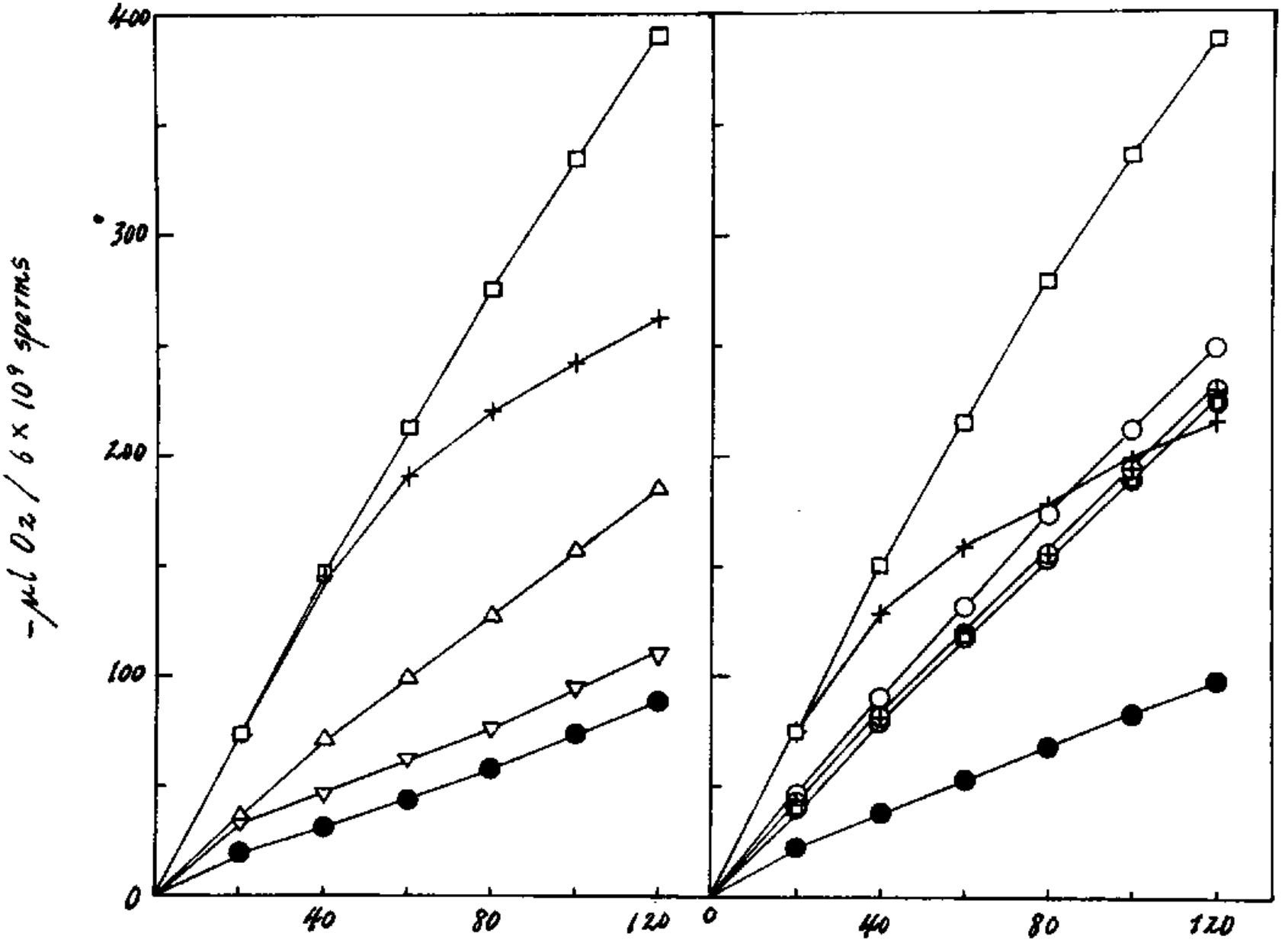
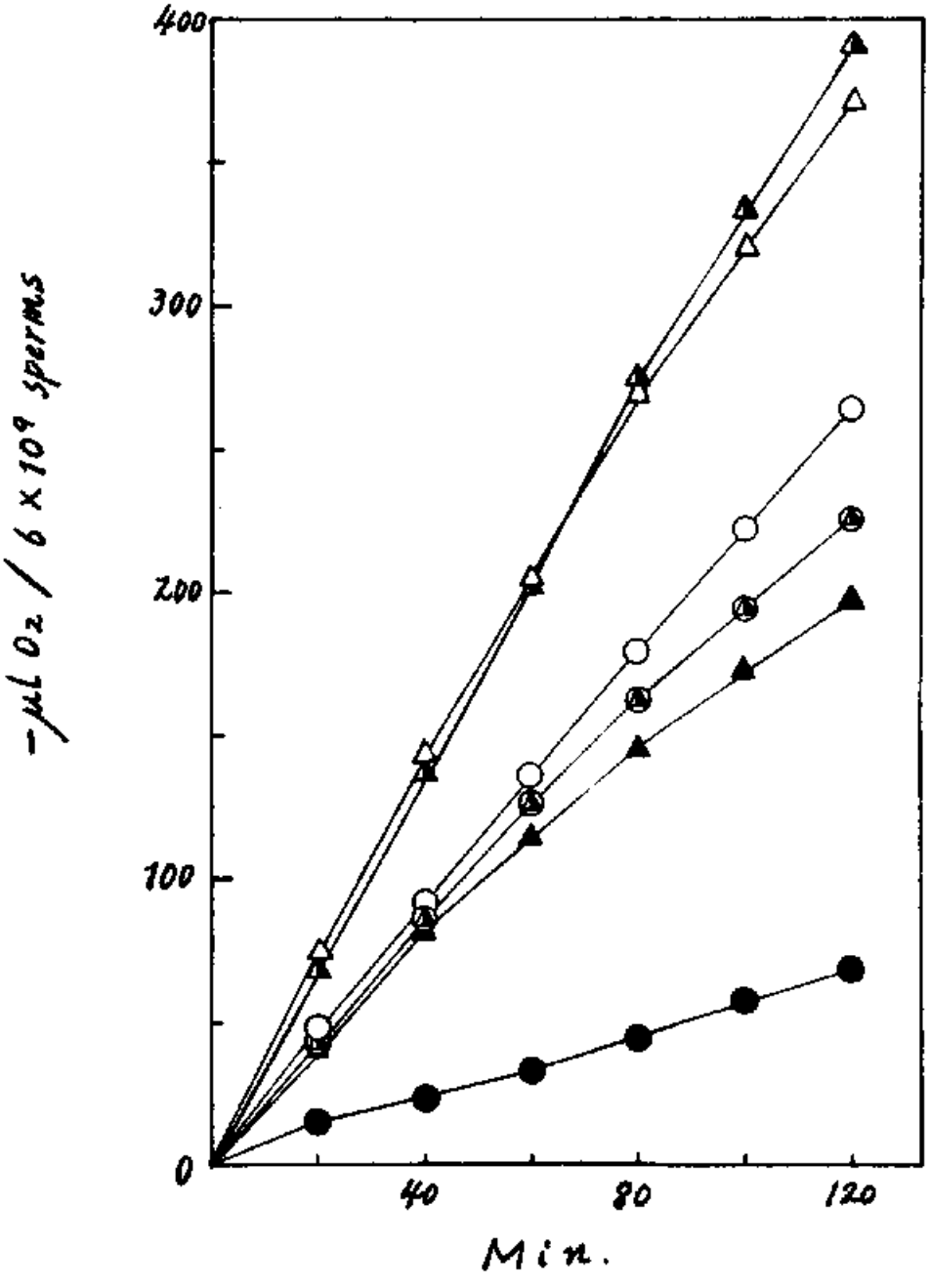


Fig. 10

Fig. 11



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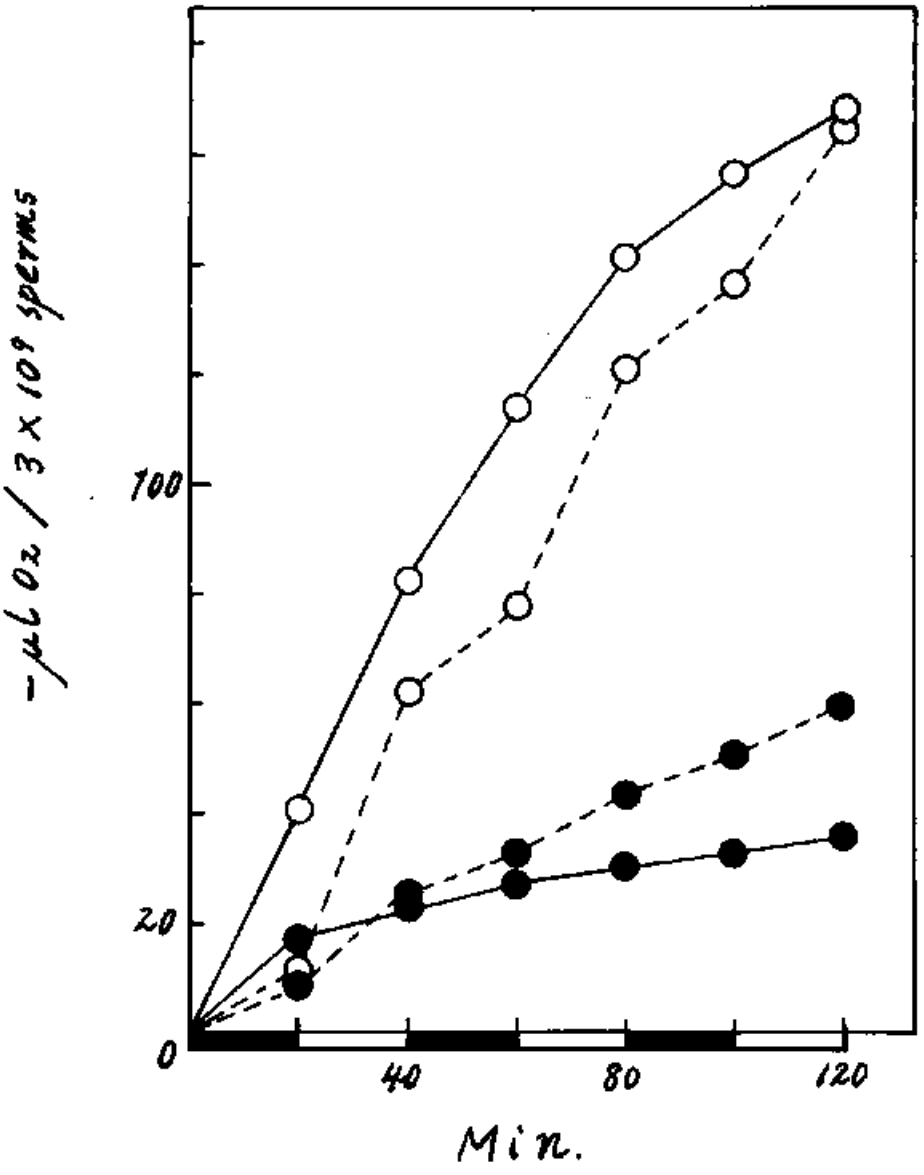

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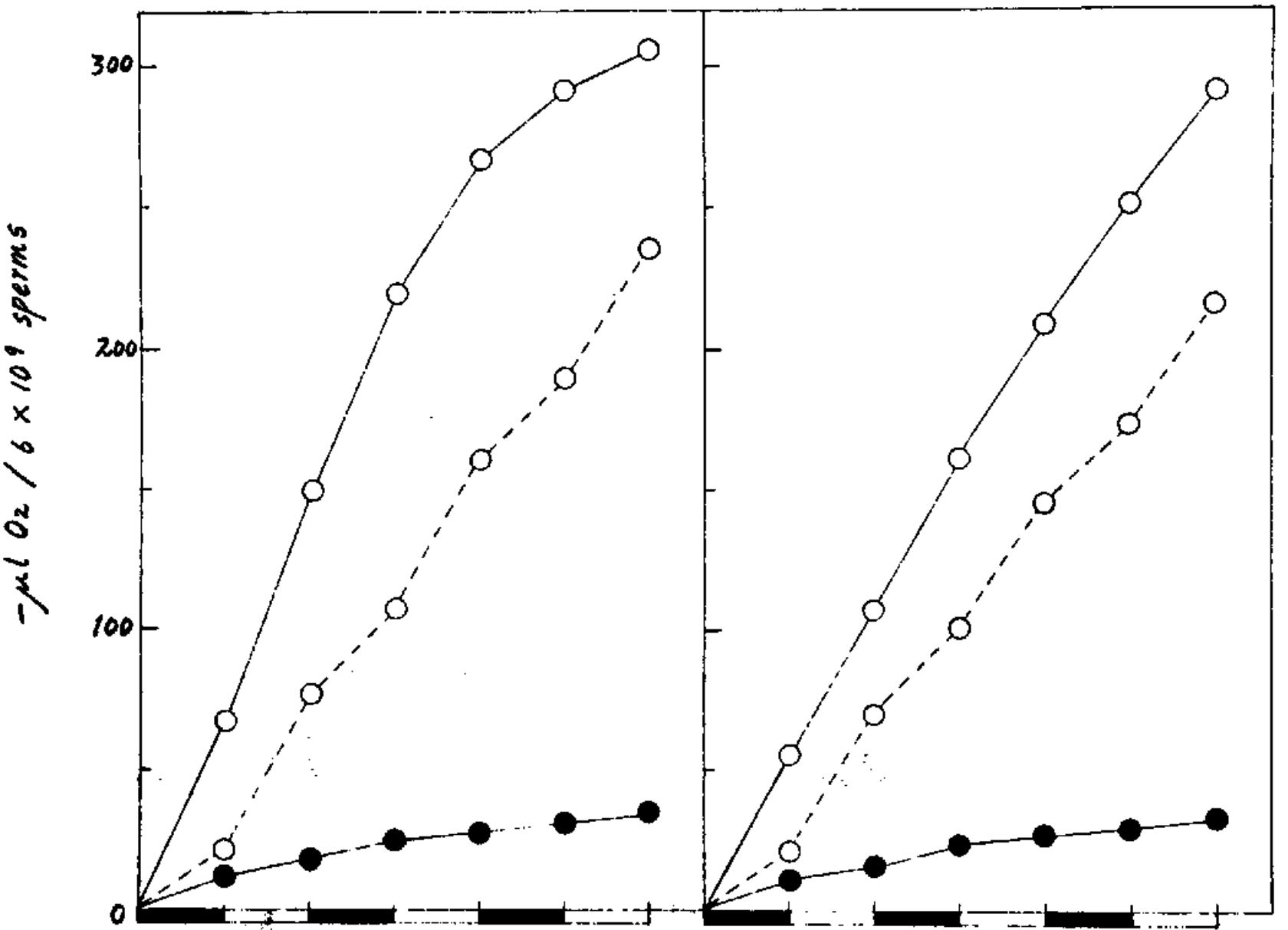

Fig. 14

Fig. 15

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