

## REGULATORY MECHANISMS OF CELLULAR RESPIRATION

## II. THE RÔLE OF SOLUBLE SULFHYDRYL GROUPS AS SHOWN BY THE EFFECT OF SULFHYDRYL REAGENTS ON THE RESPIRATION OF SEA URCHIN SPERM\*

BY E. S. GUZMAN BARRON, LEONARD NELSON, AND MARIA ISABEL ARDAO

*(From the Chemical Division, Department of Medicine, The University of Chicago, Chicago, and The Marine Biological Laboratory, Woods Hole)*

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Since the discovery of glutathione by Hopkins (1) a large number of investigators have reported on the importance of sulfhydryl groups in cellular metabolism and in cell division and growth. Of these sulfhydryl groups, the rôle of the —SH groups in certain enzymes, demonstrated for the first time by Hellerman, Perkins, and Clark (2), was extensively investigated by Barron and Singer (3–5) who found that their presence was essential for the activity of a large number of enzymes concerned with the metabolism of carbohydrates, proteins, and fat. The rôle of sulfhydryl groups in cellular division and cell growth, postulated for the first time by Hammett (6), and confirmed by Voegtlin and Chalkley (7), and a number of other investigators (8–11), was demonstrated by quantitative measurements of the sulfhydryl groups by Rapkine (12) and by Chatton Lwoff, and Rapkine (13). More recently Bailey and Perry (14) have reported that the interaction of actin and myosin depends upon the presence of —SH groups in the myosin partner. Whether the —SH groups which are reported to be essential for cellular growth and division are of a protein nature or are peptides like glutathione or free amino acids like cysteine had not yet been determined, although Rapkine has suggested that cell division is preceded by “une denaturation des proteiques, celle-ci liberant des radicaux sulfhydryles qui en s’oxidant reduiront les groupements —S—S— en groupements —SH.” Oxidation of the —SH groups of enzymes by ionizing radiations (15), and mutations (similar to those found in irradiation) produced by —SH alkylating agents such as sulfur and nitrogen mustards (16, 17) has demonstrated the importance of sulfhydryl groups in biology. We present in this paper experiments on the effect of sulfhydryl reagents on the respiration of sea urchin sperm, which are, in our opinion, proof of the existence in these cells of soluble sulfhydryl groups acting as regulators of cellular metabolism.

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## EXPERIMENTAL

Shed sperm of *Arbacia punctulata* was centrifuged for 5 minutes, the supernatant coelomic fluid was pipetted off, and the sperm suspended in sea water in a ratio of one of sperm to 20 of filtered sea water. Of the sulfhydryl reagents used in these experiments, *p*-chloromercuribenzoic acid, 3 times crystallized, was prepared ac-

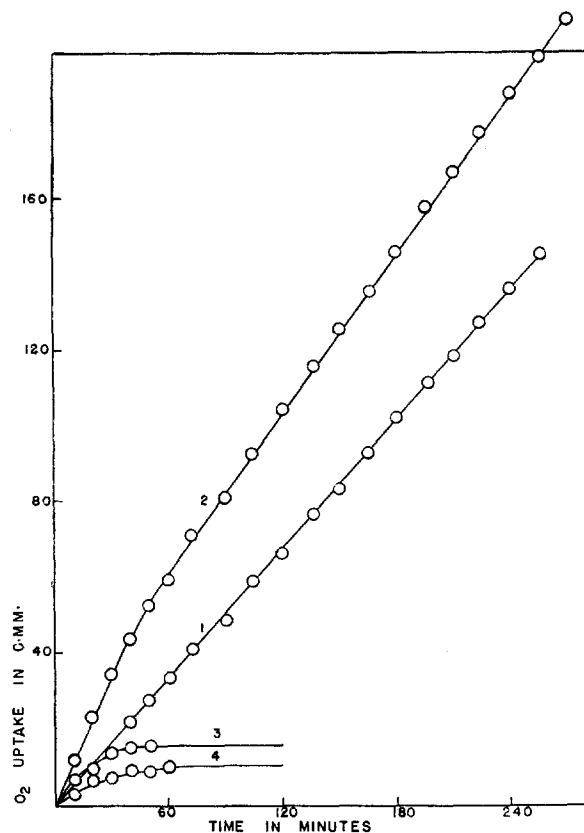


FIG. 1. Effect of iodosobenzoic acid on the respiration of sea urchin sperm. (1) Control; (2) iodosobenzoic acid,  $1 \times 10^{-4}$  M; (3)  $3 \times 10^{-4}$  M; (4)  $1 \times 10^{-3}$  M.

cording to Whitmore and Woodward (18); iodosobenzoic acid, according to Loevenhart and Grove (19); iodoacetamide, according to Anson (20). *p*-Carboxyphenylarsine oxide was kindly provided by Dr. Harry Eagle. The other substances used were good commercial products.

*Effect of Sulfhydryl Reagents on the Respiration of Sea Urchin Sperm.*—The presence of sulfhydryl groups in the cell can be recognized by the use of oxidizing agents, mercaptide-forming agents, and alkylating agents. None of these

reagents, except some mercaptide-forming agents, is specific for —SH groups. However, if the same results are obtained with the combined use of these three groups of reagents, there is reasonable certainty that the results obtained are due to action on the —SH groups. It is well known that the sperm cells are very rich in —SH groups (21). Furthermore, Barron and Goldinger (22) had shown that iodoacetate increased the respiration of sea urchin sperm. For these reasons, it was decided to use these cells to test the presence of soluble —SH groups acting as regulators of cellular respiration. If such groups did exist together with the fixed —SH groups of the protein moiety of enzymes, there

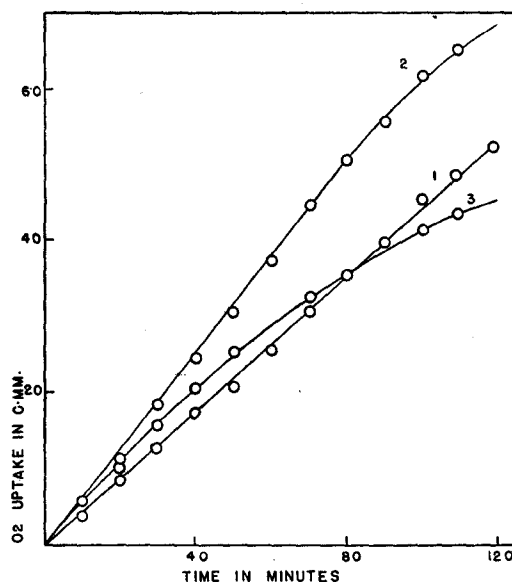


FIG. 2. Effect of iodoacetamide on the respiration of sea urchin sperm. (1) Control; (2) iodoacetamide,  $5 \times 10^{-4}$  M; (3)  $1 \times 10^{-3}$  M.

would occur, on addition of sulfhydryl reagents, at first an increase in the rate of O<sub>2</sub> uptake because of elimination of one of the regulators (the soluble —SH groups), to be followed by an inhibition because of combination with the fixed —SH groups of the sulfhydryl enzymes.

1. *Oxidizing Agents.*—Iodosobenzoic acid was chosen as the oxidizing agent for the —SH groups because Hellerman *et al.* (23) found that this mild oxidizing agent can be used for the titration of cysteine. Iodosobenzoic acid at a concentration of  $1 \times 10^{-4}$  M increased by 70 per cent the respiration of sea urchin sperm. When the concentration was increased to  $3 \times 10^{-4}$  M there was at first a slight rise in respiration, followed by inhibition. When it was increased to  $1 \times 10^{-3}$  M inhibition was the only effect observed (Fig. 1).

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2. *Alkylating Reagents*.—Barron and Goldinger (22) found that iodoacetic acid, an alkylating reagent of  $-SH$  groups, increased the respiration of sea urchin sperm; and Lardy and Phillips (24) confirmed these findings working with avian sperm. Iodoacetamide, another alkylating reagent, at a concentration of  $5 \times 10^{-4} M$  increased the respiration of sea urchin sperm. When the concentration was raised to  $1 \times 10^{-3} M$  there was at first some increase in the respiration, which at the end of 80 minutes was followed by inhibition (Fig. 2).

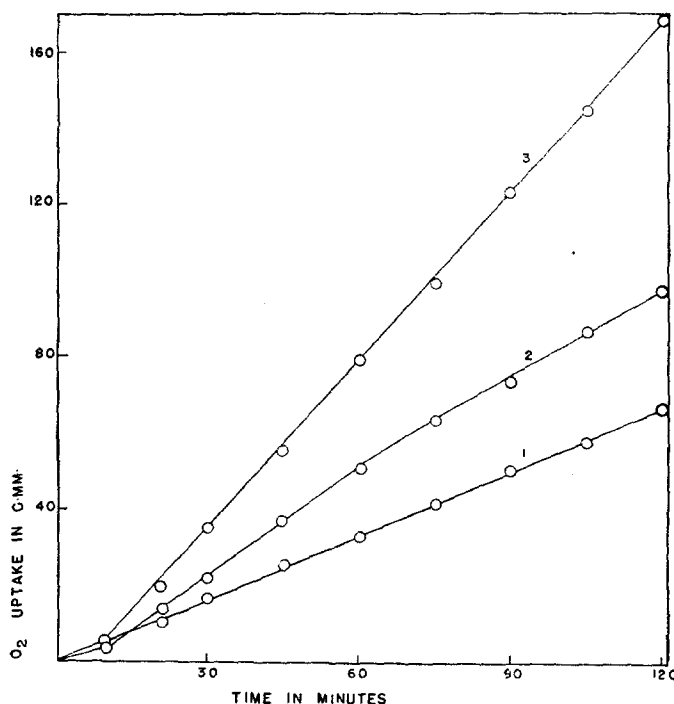
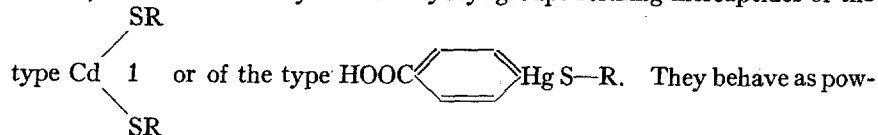


FIG. 3. The effect of  $CdCl_2$  on the respiration of sea urchin sperm. (1) Control, (2)  $CdCl_2$ ,  $1 \times 10^{-3} M$ ; (3)  $1 \times 10^{-4} M$ .

3. *Mercaptide-Forming Reagents*.—A large number of metals, and trivalent arsenic, combine reversibly with sulfhydryl groups forming mercaptides of the



erful inhibitors of  $-SH$  enzymes and may be considered as the most specific reagents for sulfhydryl groups.  $CdCl_2$  at a concentration of  $1 \times 10^{-4} M$  increased 140 per cent the respiration of sea urchin sperm. At the higher concentration of  $1 \times 10^{-3} M$ , (Fig. 3),  $CdCl_2$  increased the respiration only 51 per cent. *p*-Chloro-

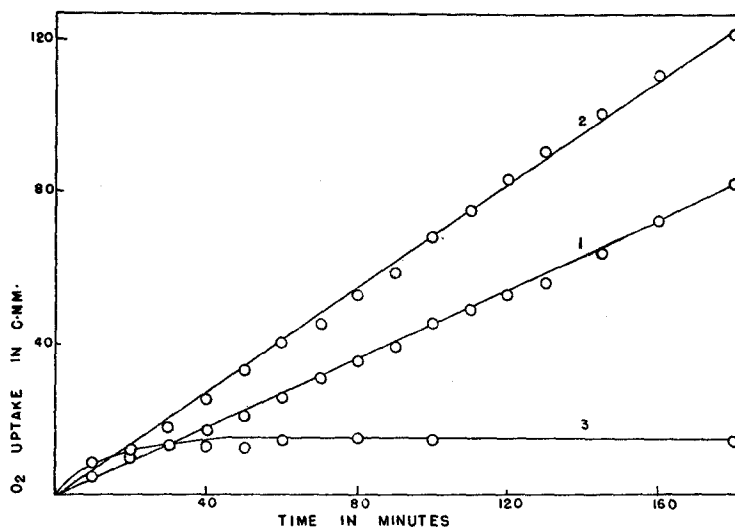


FIG. 4. Effect of *p*-chloromercuribenzoic acid on the respiration of sea urchin sperm. (1) Control; (2) *p*-Cl-Hg-benzoic acid,  $1 \times 10^{-4}$  M; (3)  $1 \times 10^{-3}$  M.

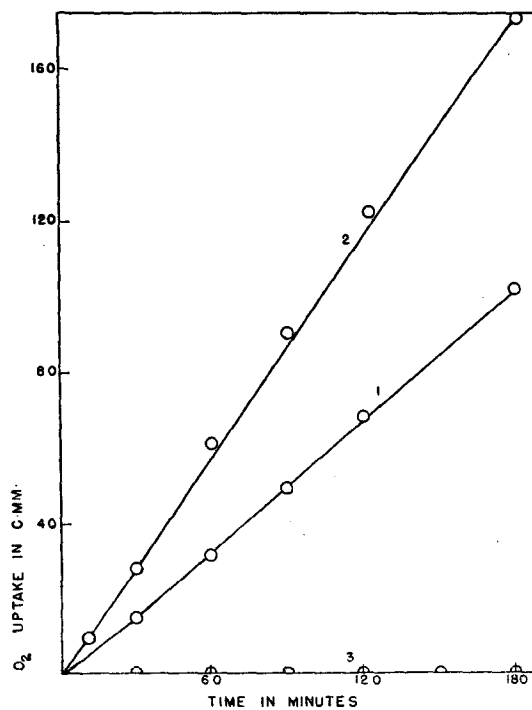


FIG. 5. The effect of  $\text{HgCl}_2$  on the respiration of sea urchin sperm. (1) Control; (2)  $\text{HgCl}_2$ ,  $5 \times 10^{-6}$  M; (3)  $1 \times 10^{-4}$  M.

mercuribenzoate at a concentration of  $1 \times 10^{-4}$  M increased it 57 per cent. When the concentration was increased to  $1 \times 10^{-3}$  M there was at first an increase in the respiration, to be followed by complete inhibition (Fig. 4).  $\text{HgCl}_2$  at a concentration of  $5 \times 10^{-6}$  M increased the respiration 88 per cent. When the concentration was raised to  $1 \times 10^{-4}$  M there was complete inhibition (Fig. 5). Sodium arsenite at a concentration of  $1 \times 10^{-5}$  M increased the res-

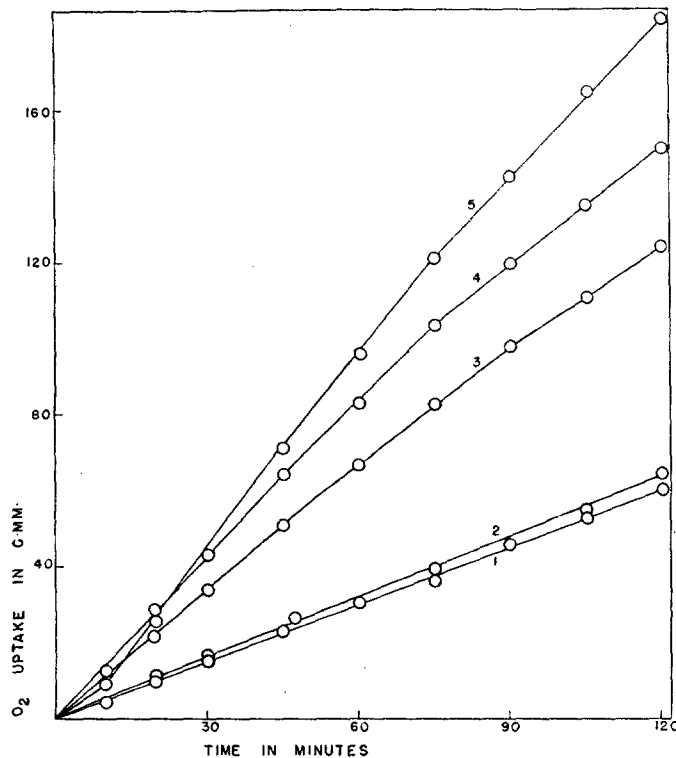


FIG. 6. The effect of Na arsenite on the respiration of sea urchin sperm. (1) Control; (2) Na arsenite,  $1 \times 10^{-3}$  M; (3)  $1 \times 10^{-4}$  M; (4)  $2 \times 10^{-5}$  M; (5)  $1 \times 10^{-5}$  M.

piration 263 per cent. This increase diminished as the concentration increased, so that when the concentration was raised to  $1 \times 10^{-3}$  M there was no effect at all (Fig. 6). The combined process of increase of respiration and inhibition could be studied in one single experiment with *p*-carboxyphenylarsine oxide, which seems to penetrate the cell membrane quite slowly. At a concentration of  $3 \times 10^{-3}$  M this arsenical produced a definite increase in respiration, which at the end of 30 minutes reached 100 per cent. From this time,  $\text{O}_2$  uptake decreased exponentially so that at the end of 120 minutes there was almost complete inhibition (Fig. 7).

A comparison of the effect of these three groups of sulfhydryl reagents was made with iodoacetamide, *p*-chloromercuribenzoate, and iodosobenzoate, all at a concentration of  $5 \times 10^{-4}$  M. Iodoacetamide maintained the increase in the respiration for the duration of the experiments (100 minutes); *p*-chloromercuribenzoate increased the respiration definitely for the first 30 minutes and produced complete inhibition 40 minutes later; iodosobenzoate produced only a slight increase in respiration, which at the end of 30 minutes was replaced by

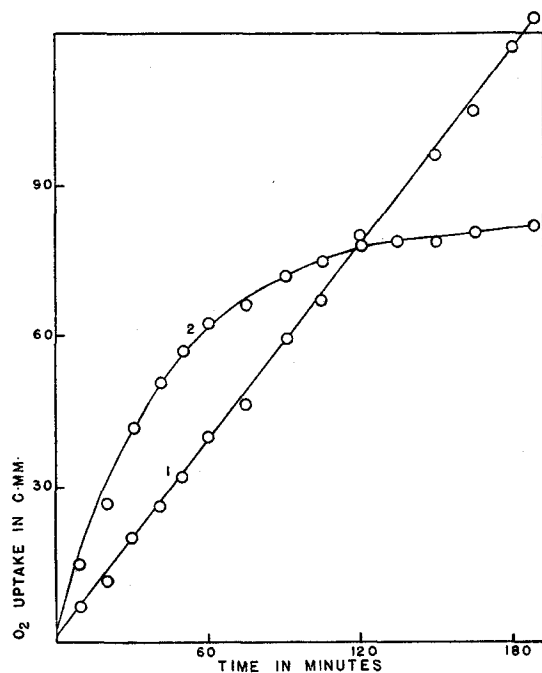


FIG. 7. Effect of *p*-carboxyphenarsine oxide ( $3 \times 10^{-3}$  M) on the respiration of sea urchin sperm. (1) Control; (2) *p*-carboxyphenarsine.

complete inhibition (Fig. 8). At equal concentrations the series of events (increase in respiration and inhibition), as well as the duration of the increased respiration, must be determined by the rate of penetration of the sulfhydryl reagents.

An attempt was made to reverse the increase in respiration produced by mercaptide-forming agents through the addition of sulfhydryl compounds which may combine with the reagents, thus liberating the cellular sulfhydryl groups. Since the inhibition of succinoxidase by  $\text{CdCl}_2$  is completely reversed on addition of 2,3-dimercaptopropanol (BAL) (25), the respiration of sea urchin sperm was measured in the presence of  $1 \times 10^{-4}$  M  $\text{CdCl}_2$ ,  $1 \times 10^{-3}$  M

BAL, and  $\text{CdCl}_2$  and BAL added 15 minutes later. The increase in respiration produced by  $\text{CdCl}_2$  (55 per cent) was reduced to 17 per cent on addition of

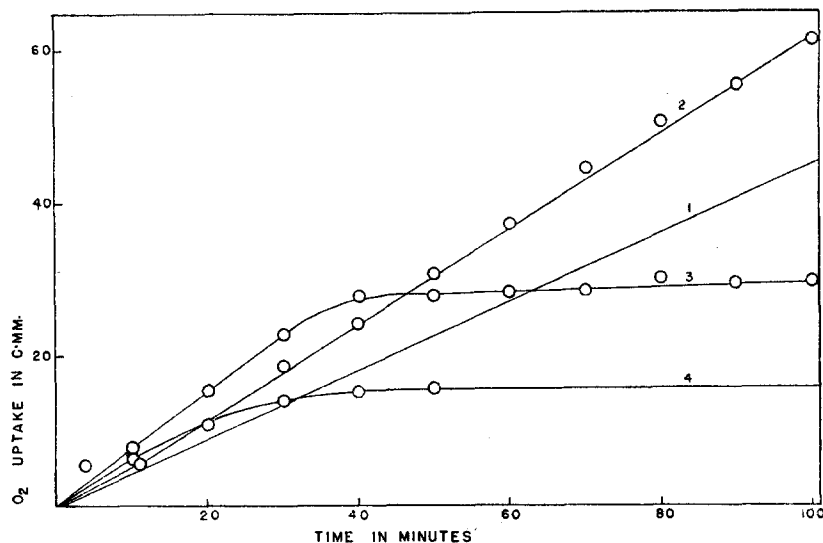


FIG. 8. Effect of sulfhydryl inhibitors on the respiration of sea urchin sperm. -SH inhibitor concentration,  $5 \times 10^{-4}$  M. (1) Control; (2) iodoacetamide; (3) *p*-chloromercuribenzoate; (4) iodosobenzoate.

TABLE I

*The Reversal of CdCl<sub>2</sub> Increase in Respiration by BAL*

Sea urchin sperm suspended in sea water.  $\text{CdCl}_2$ ,  $1 \times 10^{-4}$  M. 2,3-dimercaptopropanol (BAL),  $1 \times 10^{-3}$  M. BAL was added 15 minutes after the addition of  $\text{CdCl}_2$ . Temperature 25°. Duration of experiments, 90 minutes.

Additions	O <sub>2</sub> uptake	Increase
	<i>c.mm.</i>	<i>per cent</i>
None.....	45	—
$\text{CdCl}_2$ .....	70	55
BAL.....	60	33
$\text{CdCl}_2$ + BAL.....	54	17

BAL (Table I). If the O<sub>2</sub> uptake of BAL is subtracted, the reversal may be considered complete.

*Effect of Other Inhibitors of Cell Respiration.*—Other inhibitors of cellular respiration which do not combine with sulfhydryl groups were also tested. HCN was found to inhibit completely sea urchin sperm respiration at a concentration of  $1 \times 10^{-4}$  M. Even a concentration of  $1 \times 10^{-7}$  M inhibited the



TABLE II  
*Effect of HCN on the Respiration of Sea Urchin Sperm*  
 Sperm suspensions in sea water; temperature 25°. Duration of experiments, 60 minutes

HCN	O <sub>2</sub> uptake	Inhibition
<i>M</i>	<i>c.mm.</i>	<i>per cent</i>
None	35.3	—
$1 \times 10^{-4}$	1	97
$1 \times 10^{-5}$	4.8	86.4
$5 \times 10^{-6}$	13.5	61.7
$1 \times 10^{-7}$	20.4	42
$1 \times 10^{-8}$	35.0	None

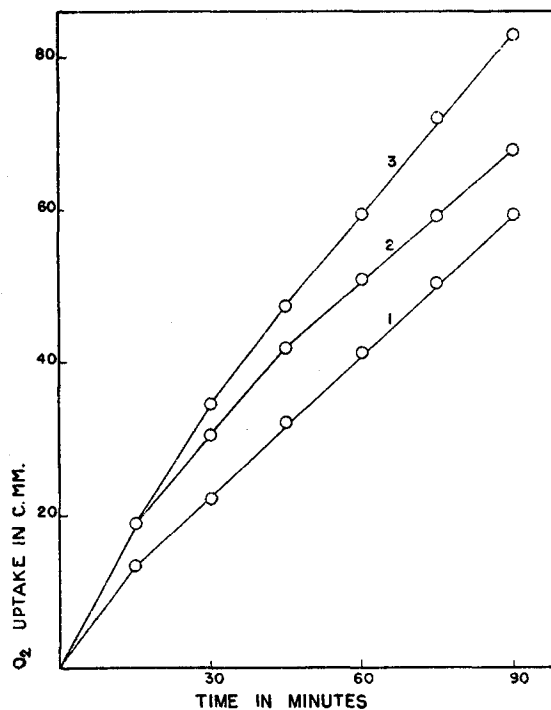


FIG. 9. The effect of Na azide on the respiration of sea urchin sperm. Sperm suspended in acetate buffer pH 6.8 (0.03 M). (1) Control; (2) Na azide,  $1 \times 10^{-3}$  M; (3) Na azide,  $5 \times 10^{-3}$  M.

respiration 42 per cent. When the concentration was diminished to  $1 \times 10^{-8}$  M HCN had no effect at all (Table II). In no case was there an increase in respiration. With sodium azide, under similar conditions (*i.e.* with the sperm cells suspended in sea water) no effect on the respiration of sea urchin sperm

was observed with concentrations varying from 0.01 M to 0.001 M. When the sperm cells were suspended in sea water containing acetate at a pH value of 6.8, sodium azide ( $5 \times 10^{-3}$  M) increased the respiration by 38 per cent. When the concentration was diminished to  $1 \times 10^{-3}$  M the increase diminished also (Fig. 9). Sperm cells utilize acetate, as shown by the increase in  $O_2$  uptake on addition of acetate (42 per cent). Perhaps sodium azide orients the acetate metabolism of sperm towards a more complete oxidation, as it does in yeast (26).

Urethanes were shown by Meyerhof (27) to inhibit the respiration of sea urchin eggs. A saturated solution of phenylurethane inhibited 60 per cent the respiration of sea urchin sperm; at half-saturation, the inhibition decreased to

TABLE III  
*Effect of Urethanes on the Respiration of Sea Urchin Sperm*  
Sperm suspended in sea water. Temperature 25°. Duration of experiments, 90 minutes.

Urethane	Concentration	$O_2$ uptake	Increase (+) or inhibition (-)
	M	c.mm.	per cent
None.....	—	43	—
Phenylurethane.....	Saturated solution	17	-60
Phenylurethane.....	$\frac{1}{2}$ saturation solution	26	-39
Phenylurethane.....	$\frac{1}{4}$ saturation solution	20.8	-12
Phenylurethane.....	$\frac{1}{10}$ saturation solution	43	None
None.....	—	62.4	—
Ethylurethane.....	0.1	76.8	+23
Ethylurethane.....	0.01	63	None
Ethylurethane.....	0.001	64	None

39 per cent; at one-fourth saturation the inhibition was only 12 per cent; at one-tenth saturation it had no effect at all. Ethylurethane at a concentration of 0.1 M increased by 23 per cent the respiration of sea urchin sperm; when the concentration was diminished to 0.01 M this small increase disappeared (Table III).

#### DISCUSSION

The experiments presented in this paper on the increase in the respiration of sea urchin sperm produced by small concentrations of sulfhydryl reagents, and inhibition of respiration when the concentrations are increased can be satisfactorily explained by assuming that there are in the cell two types of sulfhydryl groups: *soluble* sulfhydryl groups (glutathione and substances similar to it) distributed throughout the cell, and *fixed* sulfhydryl groups; *i.e.*, sulfhydryl groups in the side chains of the protein moiety of enzymes which are essential

for the activity of certain enzymes. The soluble sulfhydryl groups, being dissolved in the water phase of the cell, would contribute in a great measure to the maintenance of the oxidation-reduction equilibrium of the cell. By virtue of their very negative oxidation-reduction potential ( $E'_0$  of cysteine at pH 7 =  $-0.39$  v. according to Borsook *et al.* (28)) and their reversibility in the cell (29), the soluble sulfhydryl groups must regulate the rate of respiration by inhibiting the rate of reoxidation of the cytochrome system in the same manner as dimercaptopropanol (BAL) inhibits the rate of oxidation of reduced cytochrome C by cytochrome oxidase (30). Addition to the cells of sulfhydryl reagents in small concentrations will produce an increase in cellular respiration because of combination with these soluble sulfhydryl groups which readily react with them and thus produce an abolition of this regulating mechanism of cellular respiration. As the concentration of sulfhydryl reagents is increased they will combine with the fixed sulfhydryl groups belonging to the side chains of proteins and many of them essential for enzymatic activity. Inhibition of respiration will be the consequence. By this diminution in the rate of cellular oxidations, the soluble sulfhydryl groups contribute to the orientation of cellular metabolism towards synthesis towards anabolic processes. The reversible inhibition of cell division by  $\text{HgCl}_2$  (12) and by  $\text{Cu}^{++}$  (31) is evidence of the rôle of sulfhydryl groups in developmental growth. Further evidence for the influence of these compounds may be found in the increased concentration of sulfhydryl groups in fast growing cells and in cells during the process of division. We believe these sulfhydryl groups belong mainly to the type of *soluble* —SH groups and not to the type of *fixed* —SH groups of proteins, as was postulated by Rapkine (12). The rapid combination with the sulfhydryl reagents, as shown in these experiments long before there is combination with the —SH groups of enzymes, speaks in favor of this assumption.

#### SUMMARY

Oxidizing agents of sulfhydryl groups such as iodosobenzoate, alkylating agents such as iodoacetamide, and mercaptide-forming agents such as cadmium chloride, mercuric chloride, *p*-chloromercuribenzoate, sodium arsenite, and *p*-carboxyphenylarsine oxide, added in small concentrations to a suspension of sea urchin sperm produced an increase in respiration. When the concentration was increased there was an inhibition. These effects are explained by postulating the presence in the cells of two kinds of sulfhydryl groups: *soluble sulfhydryl groups*, which regulate cellular respiration, and *fixed sulfhydryl groups*, present in the protein moiety of enzymes. Small concentrations of sulfhydryl reagents combine only with the first, *thus* producing an increase in respiration; when the concentration is increased, the fixed sulfhydryl groups are also attacked and inhibition of respiration is the consequence.

Other inhibitors of cell respiration, such as cyanide and urethanes, which

do not combine with —SH groups, did not stimulate respiration in small concentration.

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