CORRELATION OF OXIDATION AND PHOSPHORYLATION IN HEMOLYZED BLOOD IN PRESENCE OF METHYLENE BLUE AND PYOCYANINE

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Mammalian erythrocytes though capable of glycolysis are incapable of oxidizing carbohydrates by molecular oxygen. They acquire this faculty on addition of methylene blue or other reversible dyestuffs of a similar range of oxidation-reduction potential (1, 2). On hemolysis both the faculty of producing lactic acid and, in presence of methylene blue, of oxidizing sugar is lost. When, however, hexosemonophosphate (Robison ester) is used as a substrate instead of glucose, even hemolyzed blood cells, in the presence of methylene blue, are capable of oxidizing the substrate, as Warburg, Kubowitz, and Christian (3) have shown.

Runnström, Lennerstrand, and Borei (4) found that in such a system consisting of hemolyzed blood, hexosephosphate ester (mono- or di-) and methylene blue, addition of cozymase from yeast cells has two effects: it increases oxidation and brings about a synthesis of inorganic phosphates to phosphate esters. These two effects go hand in hand; when respiration is suppressed by omitting methylene blue, phosphorylation does not occur either. On the contrary, the amount of inorganic phosphate increases in time. So, phosphate esters can in such a system either be synthesized or broken down according to whether or not respiration takes place. Obviously there is an energetic coupling of oxidation and synthesis quite analogous to what Warburg designated as Pasteur reaction, namely the coupling of oxidation and synthesis of carbohydrate from lactic acid as found by Meyerhof.

It has been shown already by Engelhardt (5) that a synthesis of pyrophosphate ester can take place in intact erythrocytes when their

faculty of respiration is artificially established by methylene blue. In muscle also, synthesis of a phosphate ester, namely creatin phosphate takes place under the influence of respiration. Because of the general importance of this coupling between respiration and synthesis of phosphate esters we have taken up this problem once more for the case of Warburg's system of hemolyzed blood as referred to above.

This system, as stated already is able to oxidize Robison ester but no synthesis of phosphate ester takes place unless cozymase is added. This holds when methylene blue is used as a catalyst. On several occasions it has been shown by Friedheim (6) that among the reversible dyestuffs of a potential range close to that of methylene blue, pyocyanine had not only a stronger but even a somewhat specific effect. For this reason Warburg's system of hemolyzed blood has been investigated replacing methylene blue by pyocyanine. It will be shown that in this case respiration is coupled with phosphate ester synthesis without addition of cozymase being necessary, though addition of cozymase increases the effect even to a higher extent.

The essential feature of this paper is to show that pyocyanine has an effect comparable to the one of a mixture of methylene blue and cozymase.

Methods

Horse blood corpuscles were used. The serum of defibrinated blood was removed by centrifuging. After two washings with 0.9 per cent NaCl the cells were hemolyzed by addition of 1½ volumes of distilled water and some drops of octyl alcohol were added to keep the fluid sterile. The hemolyzed cells were kept in the ice box for about 20 hours. Phosphate buffer, pH 7, was added to a final concentration M/20 to M/40 or in some experiments even lower. Under these conditions the stromata could not be removed by centrifuging (8000 R.P.M.). Oxygen consumption was measured in Warburg's apparatus at 37°C. Methylene blue or pyocyanine was used in a concentration of 0.008 to 0.01 per cent. The pyocyanine was a synthetic preparation. It was prepared as hydrochloride and the solution carefully neutralized before use. The dyestuffs were always added to the system of hemolyzed blood and buffer before it was introduced into the respiration chamber. So the formation of methemoglobin was brought to a standstill before the beginning of the measurements. The substrate, hexosemono- or diphosphate was introduced into the side arm of the vessel and after the first reading tipped into the main compartment. After a suitable time interval the reaction was interrupted by addition of a 20 per cent trichloracetic acid. The volume of each sample was diluted with water to 100 cc. The final concentration

of trichloracetic acid amounted to 1–1.2 per cent. Estimation of phosphorus was made according to Fiske and Subbarow (7) with the modification by Theorell (8) in which a colorimeter is replaced by a step photometer of Zeiss. The amount of P is expressed in milligrams per cubic centimeter of the mixture as used in the respiration vessel.

The organic acid-soluble phosphates were hydrolyzed according to the method of Lehnhartz in N-H_2SO_4 at 100°C. P estimations were made after boiling for 15, and for 120 minutes. Finally the total P was determined. So four different fractions of P are obtained which are designated below as I, II, III, IV. The amounts of the different fractions are recorded in per cent of the total P. The fractions are:

I. Inorganic P

II. Increase of inorganic P after 15 minutes hydrolysis (chiefly adenylpyrophosphate)

III. Further increase of inorganic P after 120 minutes hydrolysis (chiefly hexosediphosphate)

TABLE I

2 cc. hemolyzed cells with methylene blue or pyocyanine. At the time 0 0.2 cc. hexosemonophosphate added with or without cozymase (0.02 cc.).

	Methylene blue		Pyocyanine	
	——	Cozymase	——	Cozymase
Oxygen consumption 60 minutes, *c.mm.*.....	182	280	155	290
Increase through the addition of cozymase, *per cent*.....		55		87

IV. Further increase of inorganic P after complete combustion with concentrated H_2SO_4 (compounds highly resistant to hydrolysis: hexosemonophosphates, etc.)

The cozymase preparation used throughout the experiments was kindly forwarded to us by Professor K. Myrbäck. It was purified according to the method described by him (9). Its original strength was 330 Co units per cubic centimeter. It was kept in several sealed tubes. The activity may have somewhat decreased with aging but no current determinations of activity were made.

The Action of Methylene Blue and Pyocyanine

Pyocyanine and methylene blue do not differ essentially in their effect on oxygen consumption. On addition of cozymase there is an increase of oxygen consumption with either dye (Table I). There is, however, a distinct difference between methylene blue and pyocyanine as regards the distribution of the four P fractions. This is shown by

the record of a few experiments (Tables II and III) among the many carried out. The effect of pyocyanine alone is about the same or even somewhat stronger than that of methylene blue and cozymase, and a

TABLE II

2 cc. hemolyzed cells with methylene blue or pyocyanine. At the time 0 0.15 cc. hexosemonophosphate was added with or without cozymase (0.2 cc.).

		Methylene blue				Pyocyanine			
		——		Cozymase		——		Cozymase	
Duration of experiment, *min.*.....		0	180	0	180	0	180	0	180
P fractions in per cent of P	I	62	54.4	62.3	46.5	61.4	41.5	60.8	30.3
	II	2.6	3.9	0.54	6.3	0.7	6	2.3	6.75
	III	5.75	12.8	8.2	17.2	3.5	14	4	13.7
	IV	29.65	28.9	29	29	34.4	38.5	32.9	50.75

The total P was 1.1 mg. per 1 cc.

TABLE III

2 cc. hemolyzed cells with pyocyanine. At the time 0 0.2 hexosemonophosphate was added with or without cozymase (0.2 cc.).

		——			Cozymase		
Duration of experiment, *hrs.*.....		0	4	20	0	4	20
P fractions in per cent of total P	I	36.7	11.2	25	35.2	4.4	26
	II	3.7	21.6	10.9	3.1	20.2	11.3
	III	—	5.7	0.7	1.6	9.45	1.5
	IV	59.6	61.5	63.4	59.1	65.35	61.2

The total P was 0.945 mg. per 1 cc.

combination of pyocyanine and cozymase has a still higher effect. In the absence of a dyestuff no phosphate is bound,[1] on the contrary an increase of the inorganic phosphate may take place. It can also be

[1] The following experience may be worth communicating. As a rule, methylene blue without cozymase does not bring about any decrease of the inorganic phosphorus (Fraction I), sometimes even a small increase. In other cases there is a certain increase in Fraction I, though very small indeed. Table II represents such a case. This occurred particularly and regularly with the blood of the horse used for the experiment described in Table II. It is very likely that a substance analogous to cozymase is always present in the blood in a small concentration

seen from Table III that after a longer duration of the experiment (20 hours) a breakdown of P compounds takes place again. This is certainly due to the oxidation of the P compounds formed. However, even after 20 hours the inorganic P is far below its value at the time O.

In a series of experiments the influence of the concentration of the inorganic P was tested and it was found that the absolute amount of P combined is the same whether the initial concentration of the phosphate buffer was M/40 or M/20.

Table IV gives one of the experiments with hexosediphosphate as a substrate. There is a strong increase of Fraction IV during the experiment. The last three columns of the table show what happens

TABLE IV

2 cc. hemolyzed cells with pyocyanine or without pyocyanine (in this case corresponding amount of distilled water was added to the hemolyzed cells). At the time 0 0.2 cc. hexosediphosphate was added with or without cozymase (0.3 cc.).

		Pyocyanine						Without pyocyanine		
		—			Cozymase			—		
Duration of experiments, *hrs.*		0	3	20	0	3	20	0	3	20
P fractions in per cent of total P	I	38.8	28.5	30.2	38.5	24.4	24.6	38.2	44.4	62.8
	II	20.1	20.9	10.2	24.1	17.7	12.3	22.8	21.8	12.25
	III	22.9	16.9	7.7	18.8	17.7	7.7	23.2	20.7	10.7
	IV	18.2	33.7	48.1	18.6	40.2	55.4	15.8	13.1	14.25

The total P was 1.75 mg. per 1 cc.

when no dyestuff and no cozymase are present. In this case a strong increase of inorganic P (Fraction I) and no increase of Fraction IV takes place, and presence of cozymase without a dye does not change this result.

Glucose alone is not attacked in the system studied. To be sure, we found a slightly higher oxygen consumption on addition of glucose

varying with the individual horse. The actual result may be best stated as follows: in absence of methylene blue an increase in inorganic P with time always takes place; in presence of methylene blue the inorganic P is approximately constant with time; sometimes there is a very small increase and sometimes a very small decrease, as though the breakdown of esters to be expected were approximately compensated by a synthesis.

to the mixture of hemolyzed blood and pyocyanine and hexosemonophosphate; also a slight increase in formation of phosphate esters was observed. But these differences were very close to the experimental errors.

In some experiments it was revealed that both di- , and tetra-, methylparaphenylene diamine induce oxygen consumption in the same way as methylene blue. Phosphoglyceric acid and glycerophosphoric acid are not oxidized by hemolyzed cells in the presence of pyocyanine, a result which is in agreement with that obtained by Runnström, Lennerstrand, and Borei (4) with methylene blue as dye catalyst.

TABLE V

2 cc. hemolyzed cells with methylene blue + 0.3 cc. cozymase + 0.15 cc. hexosemonophosphate with or without urethane (0.2 cc.).

	——	1.5 per cent urethane	3 per cent urethane
Oxygen consumption, *c.mm. per 60 min.*.....	228	214	119
Decrease, *per cent*.....		6	42

Some Experiments with Inhibitory Substances

Table V shows an experiment on the influence of urethane on the oxygen consumption. Even in a concentration of 1.5 per cent urethane, there is no effect. At 3 per cent, however, there is a drop in oxygen consumption. But even at this concentration urethane did not interfere with the synthesis of P compounds. The decrease of inorganic P during 3 hours was the same as in the control, from 60 to 44 per cent of the total P which amounted to 1.1 mg. per cc.

Addition of neutralized KCN in concentrations that would strongly suppress respiration in normal cells did not affect the oxygen consumption in hemolyzed blood + methylene blue + cozymase + hexosemonophosphate. For instance in one experiment oxygen consumption was 234 c.mm. in 90 minutes in the control and 228 on addition of N/135 KCN. In either case the content of inorganic P was decreased after 3 hours from 64 to about 54 per cent (total P 1.1 mg. per cc.).

The effect of iodoacetic acid is quite different according to the par-

ticular nature of the system. In the system: hemolyzed blood + hexosemonophosphate + methylene blue, the oxygen consumption is scarcely decreased by iodoacetate. In a system containing cozymase in addition, the increase of oxidation which should have occurred due to the cozymase, does not take place in the presence of iodoacetate. The phosphate synthesis also, which should occur on addition of cozymase, is annihilated by iodoacetate. In the system: hemolyzed blood + hexosemonophosphate + pyocyanine, iodoacetate decreases respiration and entirely abolishes ester synthesis. The same holds true with hexosediphosphate as a substrate (Tables VII and VIII).

Sodium sulfite (neutralized) at concentrations of 0.15 to 0.3 M decreases, in the pyocyanine system, oxygen consumption by 15–20 per cent and also decreases the synthesis of esters. The effect is much less pronounced than with iodoacetate.

$CuSO_4$, at a concentration of 9×10^{-5} to 1.8×10^{-4} M, was tested in the methylene blue system. It does not decrease the oxygen uptake in absence of cozymase, but, in the presence of cozymase, decreases it to 25–30 per cent and prevents entirely the synthesis of phosphate esters.

DISCUSSION

(*A*) *Concerning the Effect of Pyocyanine.*—The main result of this investigation is the demonstration of the fact that pyocyanine not only acts as a catalyst for the oxygen uptake of the system: hemolyzed cells + hexosephosphate, but also brings about the synthesis of phosphate compounds in the system. This latter effect, to be sure, is distinctly increased by addition of cozymase, but is clearly noticeable without. No satisfactory explanation for the specific effect of pyocyanine can be offered as yet. It is, however, very suggestive to correlate it with its chemical behavior on reduction. Whereas methylene blue and similar dyes can be reduced only by a bivalent reduction, pyocyanine can be reduced by a monovalent or by a bivalent reduction. Though originally this two-step oxidation of pyocyanine had been demonstrated by Friedheim and Michaelis (10) only in strongly acid solution, its occurrence in physiological pH range could be later demonstrated by Michaelis, Hill, and Schubert (11). Here also, both steps of reduction are formed, but a considerable overlapping takes place.

Though no clear insight into the causal connection between the two-step reduction and the particular nature of the catalytic effect can be ascertained as yet, this property of pyocyanine can be no accidental

TABLE VI

2 cc. hemolyzed cells with methylene blue with or without iodoacetate. At the time 0 0.2 cc. hexosemonophosphate added with or without cozymase (0.3 cc.).

	—			Cozymase		
Iodoacetate	0	0.01 N	0.02 N	0	0.01 N	0.02 N
Oxygen consumption, *c.mm. per 60 min.*	154	140	146	228	151	146.5

TABLE VII

2 cc. hemolyzed cells with pyocyanine with or without iodoacetate (0.02 N). At the time 0 0.2 cc. hexosemonophosphate added with or without cozymase. (0.3 cc.).

	—	Cozymase	—	Cozymase
	—	—	Iodoacetate	
Oxygen consumption, *c.mm. per 60 min.*	135	193	65	65
Decrease, *per cent*			52	66.4

TABLE VIII

2 cc. hemolyzed cells with pyocyanine with or without iodoacetate. At the time 0 0.25 cc. hexosemonophosphate was added with or without cozymase (0.3 cc.).

		—		Iodoacetate	
		Cozymase	Cozymase	—	Cozymase
Duration of experiment, *min*		0	180	180	180
P fractions in per cent of total P	I	74.3	60.5	74.8	74.1
	II	0	6.7	0.5	2.5
	III	3.7	5.8	2.8	7.6
	IV	22	27	21.9	15.8

Total P was 1.77 mg. per 1 cc.

feature unrelated to its physiological effect. For not only certain dyestuffs closely related to pyocyanine, especially chlororaphine (12–13), and hallachrom (Friedheim (14)), but also the dyestuffs desig-

nated as "yellow respiration ferment," (O. Warburg) and flavines or lyochromes (Ellinger, and R. Kuhn) show the property of two-step reduction, as recently has been shown by R. Kuhn and Wagner-Jauregg (15), and by Barron and Hastings (16).

It is worth while emphasizing that KCN does not affect the influence of pyocyanine either on respiration or on phosphate synthesis.

(*B*) *Concerning the Effect of Iodoacetic Acid.*—The point of attack for iodoacetic acid can be safely regarded as primarily the sulfhydryl groups, as has been suggested by Quastel (17), and proven by Dickens (18), and Michaelis and Schubert (19). These latter authors, however, pointed out that according to concentration, pH, and other more chemical specificities, amino groups also are attacked by iodoacetic acid. With the possibility of these two modes of action, the effect of iodoacetic acid may be different according to circumstances, and such a consideration may account for discrepancies reported about the effect of the acid. Lundsgaard (20) claims that iodoacetic acid eliminates only the formation of lactic acid but has no influence on respiration. Nilsson, Zeile, and von Euler (21) claim that it decreases respiration also. Ehrenfest (22) assumed that respiration in the presence of iodoacetic acid takes place only at the expense of split products of carbohydrates already present. As the mode of action of iodoacetic acid may depend on concentration and other conditions, we wish to emphasize that the effect of the acid in our experiments should not be taken as the specific action of this acid in general, but holds for the concentration and other conditions prevailing in our experiment. The following effects were observed:

1. The respiration induced by methylene blue was not diminished by iodoacetate.

2. In a system containing methylene blue in addition to cozymase, iodoacetate had two effects: it cuts down just the increase of respiration otherwise brought about by cozymase; and it prevents the synthesis of phosphate esters otherwise brought about by cozymase.

The effect of iodoacetate in presence of pyocyanine was twofold also: (1) it decreases respiration; (2) it abolishes synthesis of phosphate esters. To appreciate the first of these two effects, it should be recalled that the respiration induced by pyocyanine (without cozymase) is not greater than the one induced by methylene blue (without

cozymase). Yet the first is decreased by iodoacetate, the latter is not. But, at any event, phosphate synthesis is always annihilated by iodoacetate.

(*C*) *Concerning the Phosphor-Ester Synthesis.*—The above data can only serve as a first rough orientation concerning the chemical changes involved in the processes leading to the increase of chemically bound phosphates. In the system hemolyzed cells + hexosemonophosphate + cozymase + Mb, or hemolyzed cells + buffer + hexosemonophosphate + pyocyanine, an increase of Fraction II is always found. From this it may be inferred that adenylpyrophosphate is built up. But also the Fraction III increases, indicating perhaps an increase of hexosediphosphates. In the system hemolyzed cells + hexosediphosphate + Mb or + pyocyanine (Table IV), there is a strong increase of Fraction IV, formed by compounds resistant to the hydrolysis in N sulfuric acid. It is difficult to understand this without assuming the breakdown of the hexosediphosphate to three- carbon compounds each carrying two phosphoric acid radicals (*cf.* glycerinediphosphoric acid found in blood by Greenwald (23) and by Jost (24)). In the system hemolyzed cells + hexosemonophosphate + cozymase + pyocyanine there is also an increase of Fraction IV. A more detailed discussion of the chemistry of the changes studied is, however, premature in view of the experimental material as yet available.

SUMMARY

1. The system: hemolyzed blood + glucose never exhibits glycolysis or, in the air, oxidation of glucose. When glucose is replaced by hexosephosphate ester, addition of methylene blue causes oxidation in air.

2. When cozymase is added also, the oxidation is increased, and a synthesis of hexosephosphate esters takes place.

3. When pyocyanine is used instead of methylene blue, the rate of oxidation is the same as with methylene blue, but a synthesis of phosphate esters takes place without addition of cozymase.

4. There is never a phosphate ester synthesis without oxidation going on, but oxidation does not necessarily go hand in hand with phosphate synthesis.

5. In order to couple the oxidation process with phosphate synthesis,

two methods are available: either to start oxidation by methylene blue and to add coenzyme from yeast cells; or to start oxidation by pyocyanine, in which case coenzyme is unnecessary, though it improves the effect.

6. Iodoacetate always suppresses synthesis, but only under certain conditions decreases oxidation. Cyanide has no effect upon either process.

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