XXV. THE EFFECT OF PYOCYANINE ON THE RESPIRATION OF SOME NORMAL TISSUES AND TUMOURS¹.

By ERNST A. H. FRIEDHEIM.

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WARBURG's theory states that the tumour cell suffers from a relative insufficiency of respiratory enzyme, so that even under aerobic conditions the balance of the Pasteur-Meyerhof reaction shows an excess of lactic acid. This suggests that the lack of enzyme might be compensated by the addition of an accessory catalyst. This working hypothesis gains support from the evidence that a similar mechanism actually occurs in nature [Friedheim, 1931]. The *Bacillus pyocyaneus* contains, besides cytochrome, a respiratory ferment similar to that found in higher animals in that it is sensitive to CO and KCN. However, even with free access of molecular oxygen this respiratory enzyme proves insufficient in the absence of assimilable extraneous substances; the oxygen consumption is minimum, the respiratory quotient is almost zero and the oxidation incomplete. Corresponding to those conditions described by Warburg as being characteristic of the tumour cell, *B. pyocyaneus* lives almost as an anaerobic organism even in the presence of air. Under certain conditions it produces the blue pigment, pyocyanine, which, owing to the reversibility of its oxidation and reduction, acts as an accessory oxidation catalyst. In its presence oxygen consumption is increased as much as twenty-four fold and the R.Q. approximates to unity.

It has previously been shown [Friedheim, 1931] that the stimulating effect of pyocyanine on respiration is in no way species-specific but is also to be observed in the red blood corpuscles of rabbits. In the following the effect of pyocyanine on the respiration of some normal and tumour tissues will be described.

EXPERIMENTAL.

Procedure. The Barcroft-Warburg manometric method was employed. Tissue sections 0.2 mm. thick were put with 2 ml. of Ringer's solution into the main chamber of a 10 ml. respiration vessel. For the absorption of CO₂ the inner vessel contained 0.2 ml. of 6 % NaOH, the accessory bulb 0.2 ml. M/50 solution of recrystallised pyocyanine. The thermostat was kept at 37.5°. The manometer was shaken at the rate of fifty complete oscillations per minute. The oxygen consumption in Ringer's solution was measured for a first period varying from 30 to 60 minutes. The pyocyanine was then tipped from the accessory vessel into the main chambers, and the observations were continued during a second equal period. This method suffers from the unphysiological condition that the partial pressure of CO₂ is held at approximately zero; but against this drawback stands the great advantage of extraordinary simplicity and the identity of the tissue fragment in the experiment and the controls.

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Tissue sections which were introduced into the previously warmed respiration vessels within a period of less than four minutes after the death of the animal showed the oxygen consumption to be strictly proportional to the time for a period of at least 100 minutes. The deviations to be expected with exclusion of bacterial growth are in the direction of a decrease in respiration and cannot exaggerate any observed increase in respiration. The Ringer's solution contained 0.0025 *M* bicarbonate. In the experiments where sugar was added, a concentration of 0.2 % glucose was used. In those in which phosphate-Ringer was employed, the phosphate concentration was 0.01 *M* and the p_H 7.4.

Results. As can be seen from Tables I–III, the respiration of rat kidney in carbonate-Ringer is either uninfluenced or slightly impeded by pyocyanine; whereas the respiration of testicular tissue, and more so that of tumours, is very

Table I. *Respiration of rat kidney.*

Corrected manometric values for mm.³ O₂ absorbed obtained in two periods of equal duration, the first without, and the second with, pyocyanine.

Duration of exp.				30–60 mins.		
Thickness of section				0.2 mm.		
Glucose concentration				0.2 %		
Atmosphere				O ₂		
Temperature				37.5°		
Final pyocyanine concentration				<i>M</i> /500		
Absorption of CO ₂ by 6 % NaOH						
In carbonate-Ringer 2.5/1000 <i>M</i>				In phosphate-Ringer (1/100 <i>M</i>) p_H 7.4		
Exp. No.	Without* pyocyanine	With pyocyanine	Difference %	Without pyocyanine	With pyocyanine	Difference %
1	26.0	25.6	– 1.5	18.0	22.1	+ 22.7
2	19	16.5	– 13.15	22	29	+ 31.8
3	32	26	– 12.5	31	31.8	+ 2.5
4	18.5	18.0	– 2.7	25	36	+ 3.6
5	35	31	– 11.4	35	37.5	+ 7.1
6	35	37	+ 5.7	50	53.0	+ 6.0
7	30.4	30.7	+ 1.9	54.0	60.2	+ 11.5
Mean difference			– 0.5 %	Mean difference		+ 16.8 %

* Mean value of Q_{O_2} , 14.2.

Table II. *Respiration of testicular tissue (rat).*

Corrected manometric values for mm.³ O₂ absorbed obtained in two periods of equal duration, the first without, the second with, pyocyanine.

Testicular tubuli carefully dissociated to form only one layer.

Ringer solution		NaHCO ₃ 2.5/1000 <i>M</i>	
Duration of exp.		30–60 mins.	
Glucose		0.2 %	
Final pyocyanine concentration		<i>M</i> /500	
Atmosphere		O ₂	
Temperature		37.6°	
Absorption of CO ₂ by 6 % NaOH			
Experiment No.	Without* pyocyanine	With pyocyanine	Difference %
1	59	67	+ 30.5
2	43	49	+ 13.9
3	53	58	+ 9.4
4	59	68	+ 15.2
5	22	30	+ 30
Mean difference			+ 19.8 %

* Mean value of Q_{O_2} , 9.4.

Table III. *Respiration of tumour tissue and rat testicle in Ringer solution 2.5/1000 M NaHCO₃.*

(Conditions the same as in Table I).

Tissue	Experi- ment No.	With 0.2 % glucose			Without glucose		
		Without pyo- cyanine	With pyo- cyanine	Differ- ence %	Without pyo- cyanine	With pyo- cyanine	Differ- ence %
Mouse sarcoma S 37	1	23	32.7	+42	35	23.5	-25.7
	2	20	32	+60	39	33	-15.3
	3	39	54	+38.5	25	28	+12
	4	23	62	+170	20	20	0.0
	5	23	31.3	+36	27	24.1	-11
	6	57	78	+36.9			
	7	28	34	+23.5			
	8	29	61.6	+105	26	30.3	+16.5
Mean values				+63.8			-4 %
Mouse sarcoma S 159	1	16.4	24	+46.3	25	27	+8
	2	19	33	+73.7	23	27	+17.4
	3	18	24	+33.7	18	16	-11
	4	29	39	+37.9			
	5	18	22	+22.2	23	23	0.0
	6	22	32	+45.4	26	28	+7.7
Mean values				+43.2			+2.4
Human breast carcinoma	1	9	23	+155	7	8	+12.5
	2	22.5	29.6	+31.8	18	18	0.0
	3	10	24	+140			
Spindle-cell sar- coma of humerus	1	23.5	42	+78.8	14	14	0.0
Neuro-blastoma of retina	1	12	27	+125	20	37	+75
Rat testicle (mm. ³ O ₂ /hr./mg.)	1	9.02	11.65	+29.7	7.4	7.4	0.0
	2	9.72	12.31	+26.6	7.2	7.2	0.0

considerably enhanced. It is worthy of note that the presence of glucose is essential for the increase of respiration observed with pyocyanine. This is contrary to the findings in the case of bacteria, where the catalytic effect of pyocyanine is exercised on the substance of the cell itself only in the absence of extraneous assimilable substances.

The relative toxicity of pyocyanine and methylene blue.

Harrop and Barron [1928] were the first to show that the respiration of living cells can be increased by the catalytic action of reversible dyestuffs such as methylene blue. These authors found, in accordance with the theory, that the accessory catalyst could only enhance the respiration in cases where there is aerobic glycolysis, *i.e.* where the respiration is insufficient to bring about the disappearance of all lactic acid in the Pasteur-Meyerhof reaction. Our experiments confirm in principle those of Barron [1930], as they show that kidney, a tissue without aerobic glycolysis, is uninfluenced by pyocyanine in its respiration, or if anything, slightly hindered; whereas in tumours with positive aerobic glycolysis the respiration is considerably increased. In accordance with their theoretical considerations the above-mentioned authors expected to find that in the case of testicular tissue, which exhibits aerobic glycolysis, the respiration would be increased on the addition of methylene blue. However, methylene blue

failed to bring about this effect, which, as can be seen from Table II, was brought about by pyocyanine. As the potential range of the two dyestuffs is of the same order of magnitude (E_0 pyocyanine = -34 mv. E_0 methylene blue = $+11$ mv. at p_H 7.0), the cause of the dissimilarity in their actions is to be sought in a difference in toxicity. In the case of testicular tissue, the catalytic action seems to be almost exactly compensated by the toxic action. Pyocyanine, probably owing to its small affinity for proteins and high affinity for lipoids, is much less toxic than methylene blue. In the previous experiment with rabbit erythrocytes, pyocyanine caused an increase in respiration of 1250 % (duration of experiment, one hour), a figure which is just above the best values reported by Harrop and Barron [1928] and Michaelis and Salomon [1930] with methylene blue.

In normal rat kidney, which shows practically no glycolysis, the toxicity cannot be masked by any catalytic action. In spite of this fact the average inhibition of respiration brought about by pyocyanine is only 5.8 %, while with methylene blue, according to Harrop and Barron, this amounts to 13.8 %. It is to be emphasised that our experiments were carried out with carbonate-Ringer, while those of Harrop and Barron were done with phosphate-Ringer.

Influence of phosphate.

In opposition to Dixon and Elliott [1929], Warburg [1931] has strongly emphasised the unphysiological action of phosphates. Using phosphate solution, Dixon found that the inhibition of tissue respiration by cyanide was incomplete; in other words, the phosphate solution caused a relative increase in the respiration. An effect of phosphate in the same sense was apparently observed by Barron, since he reports that in phosphate solution the catalytic action of methylene blue on the respiration of rabbit erythrocytes is increased. Table I shows that in normal rabbit kidney in the presence of phosphates pyocyanine causes a considerable increase in respiration which does not occur in carbonate-Ringer.

In Barron's [1930] experiments methylene blue inhibited the respiration of rat kidney even in a phosphate-Ringer solution, which in itself tends to increase the catalytic action. On the other hand, under the same conditions pyocyanine induces an increase of 16.8 %. Hence the difference in toxicity of the two dyestuffs is readily demonstrated.

Recently Dickens and Šimer [1930] have used phosphate-Ringer for determining the R.Q. in tissue sections. In control experiments these authors have compared the standard values of the respiration (Q_{O_2}) in carbonate- and in phosphate-Ringer. These authors, having obtained results which coincide within the limits of experimental error, feel justified in assuming the conditions in phosphate-Ringer to be physiological and in according an important rôle to the values of the R.Q. determined in such solutions. In view of the above experiments it seems to us that the comparability of the standard values of respiration in carbonate- and phosphate-Ringer offers no guarantee that the tissues in phosphate-Ringer are under physiological conditions. The great increase in respiration caused by pyocyanine in phosphate- but not in carbonate-Ringer in normal rat kidney seems strongly to indicate that in phosphate solution the oxidation mechanism of the cell is fundamentally different.

The mode of action of pyocyanine.

Increase in depth of respiring layer of tissue sections. Warburg [1923] has drawn attention to the fact that the metabolic exchange is restricted to a more or less thick cortical layer, while the central portion remains inactive. The thickness of

the layer where molecular oxygen is available can be calculated according to Warburg as

$$d = \sqrt{8c_0 \frac{D}{A}}$$

where d = thickness of layer where oxygen is available,

c_0 = oxygen concentration at the limits between section and solution,

D = diffusion constant of oxygen for tissue substance,

A = the oxygen consumption of the tissue per unit volume and time.

If we regard D as the sum of the diffusion constants of all substances which can act with respect to the tissue as hydrogen acceptors, then D will be increased on addition of pyocyanine by a value equivalent to the diffusion constant of the dye solution in the tissue. As pyocyanine, and to a lesser extent methylene blue, are extremely lipid-soluble, they have a tendency to accumulate in the tissues to a concentration exceeding the value which would be reached by simple diffusion. Thus there results a considerable increase in D with a corresponding increase in d according to the formula. So far this would only signify an increase in the depth of the tissue layer taking an active part in oxidation metabolism but have no bearing on the measurable oxygen consumption. But as this process goes on, the reduced form of the dye tends to accumulate in the depth of the tissue and a diffusion gradient of the leuco-compound will be established from within the tissue towards the surface (and perhaps oxidisable substances produced by the dehydrogenation of the tissue). Here the leuco-compound, diffusing out of the tissue will immediately be oxidised by the molecular oxygen and thus the oxygen consumption actually increased. It follows that in tissue sections which exceed the thickness in which molecular oxygen would be available, the addition of reducible dyestuffs induces an increase in respiration which is purely mechanical without involvement of the fundamental chemistry of the cell.

Table IV. *Influence of thickness of section on the respiration of rat kidney.*

Ringer solution				2.5/1000 M NaHCO ₃		
Glucose				0.2 %		
Final pyocyanine concentration				M/500		
Atmosphere				air		
Absorption of CO ₂ by NaOH						
Temperature				37.6°		
Thickness of section 0.2 mm.				Thickness of section 3.0 mm.		
mm. ³ O ₂ consumed in 30 mins.				mm. ³ O ₂ consumed in 30 mins.		
Experi- ment No.	Without pyo- cyanine	With pyo- cyanine	Difference %	Without pyo- cyanine	With pyo- cyanine	Difference %
1	46	44.5	-3.2	108	132	+13.3
2	18	16.5	-8.3	55	80	+45.5

This effect is illustrated by Table IV. As has been shown in Table I, the respiration of rat kidney cut in sections not thicker than the layer accessible to molecular oxygen (0.2 mm. in an atmosphere of oxygen) is not increased on the addition of pyocyanine; in sections 3.0 mm. thick pyocyanine enhances the respiration as much as 45 %.

The catalytic action. As has been shown in the case of bacteria [Friedheim, 1931] in thin sections of tissue the mechanism of the respiration brought about by pyocyanine may be explained as a true catalysis in that the dye is reduced by

the tissue and reoxidised by the molecular oxygen. The significance of the increase in respiration for the cell may be judged by the influence of pyocyanine on the aerobic glycolysis.

Table V. *Aerobic glycolysis of various tumours, expressed as $Q_{CO_2}^{O_2}$, i.e. mm.³ CO₂ driven out of a bicarbonate solution by lactic acid formed per hour per mg., dry weight of tissue.*

		Ringer solution			0.025 M NaHCO ₃		
		Glucose			0.2 %		
		Atmosphere			5 % CO ₂ in O ₂		
		Temperature			37.5°		
		Final pyocyanine concentration			M/500		
		Thickness of tissue section 0.2 mm.					
Experiment No.	Tumour	$Q_{CO_2}^{O_2}$		Difference %	Without pyocyanine		
		Without pyocyanine	With pyocyanine		I Respiration Q_{O_2}	II Anaerobic glycolysis	III U^1
1	Cori rat sarcoma	20.1	13.1	-34.7	12.5	26.1	+ 1.1
2	Walker rat carcinoma 256	11.4	9.0	-21	6.36	22.1	+ 8.4
3	Walker rat sarcoma S 20	18.5	7.88	-87.6	13.3	68.2	+41.6
4	"	40.4	25.9	-35.8	17.94	62.5	+32.5
5	"	8.51	0.0	-100			
6	Mouse sarcoma	13.0	5.46	-58			
7	"	15.3	6.0	-60.8			
8	Mouse sarcoma S 27	19.9	9.76	-50.9			
9	Human breast carcinoma	5.2	3.4	-34.6			
10	"	6.8	4.4	-22.2			
11	Human spindle-cell sarcoma	5.28	3.1	-41.3			
12	Cori rat sarcoma	13.0	13.0	0.0	-13.9	24.3	- 3.5
13	Walker rat sarcoma	23.7	22.2	0.0	-16.25	28.6	- 4.4

Table V shows that in a series of tumours the aerobic glycolysis is actually decreased on the addition of pyocyanine. The two exceptions in Table VII (Exps. 12 and 13) happen to be tumours with a negative U value¹, i.e. tumours in which theoretically the respiration would be sufficient to suppress aerobic glycolysis. Attention has recently been drawn by several authors to the existence of tumours which show at the same time high respiration and low glycolysis. As suggested by Warburg the respiration in tumours may be not only quantitatively insufficient to dominate glycolysis, but as in the case of ethyl carbamate poisoning, by the coupling of these two processes the mechanism of the Pasteur-Meyerhof reaction might be broken.

¹ U stands for the anaerobic glycolysis *minus* twice the value of the respiration. This concept of U was introduced by Warburg as a measure of the efficiency of respiration since two molecules of oxygen are sufficient to cause the disappearance of one molecule of lactic acid during the course of the Pasteur-Meyerhof reaction. Where $U < 1$, the respiration is "sufficient" and there is no aerobic glycolysis.

Also in our positive experiments in which pyocyanine decreases aerobic glycolysis without eliminating it entirely it must be remembered that the measurements were carried out in Ringer's solution, which is liable in itself to damage the Pasteur-Meyerhof reaction.

In view of the difficulties in interpretation it might seem that the biological significance of the increased respiration brought about experimentally by accessory catalysts, especially in its bearing on the question of malignancy, might best be brought out not with surviving tissue sections but with living cells in animal experiment, or in tissue cultures, as suggested by Barron¹. For such experiments it is important to have a catalyst available which combines the properties of minimum toxicity and maximum respiratory catalysis. Pyocyanine appears to fulfil these requirements.

SUMMARY.

1. Pyocyanine increases the respiration of tissues with aerobic glycolysis such as testicular tissue and a series of animal and human tumours.

2. The increase in respiration is dependent on the presence of glucose.

3. In this action one must differentiate between an apparent increase in respiration, brought about in thick tissue sections through an increase in the depth of the respiring layer, and a true catalysis.

4. While in carbonate-Ringer the respiration of normal rat kidney is not increased by pyocyanine, a considerable increase is brought about in phosphate-Ringer. In the presence of phosphate the oxidation mechanism of the cell seems to be fundamentally altered.

5. The toxicity of pyocyanine is compared with that of methylene blue. Pyocyanine shows minimum toxicity and enormous action in increasing respiration.

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¹ These experiments are under way.

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