LXXVII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

XII. THE ACTION OF PHENYLHYDRAZINE ON THE PASTEUR REACTION AND ON TISSUE RESPIRATION.

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THE most pronounced feature of the metabolism of the cancer cell is its property of aerobic glycolysis. With the possible exception of a few spontaneous mouse tumours, where the metabolism of the cancer cells may be masked by that of the connective tissues, all tumours yet examined continued to form lactic acid under aerobic conditions [Warburg, 1930]. The aerobic glycolysis is an expression of a deficiency in the tumour respiration, characterised by a lowering of the R.Q. of the tumour tissue to a level below the carbohydrate value of R.Q. found in normal tissues with similar powers of glycolysis [Dickens and Šimer, 1930, 2; 1931, 2] whether or not the total respiration is diminished in extent. The appearance of aerobic glycolysis shows that in such tissues the mechanism, called by Warburg the Pasteur reaction, which normally correlates respiration and glycolysis, is defective; for normally in a wide variety of aerobic animal and vegetable cells there is a virtually complete suppression in oxygen of the formation of products of the cleavage type of metabolism.

There are two methods by which this problem can be more closely investigated; namely either by the study of methods of artificial production of the property of aerobic glycolysis or by attempts to restore the damaged metabolism to normal. The second of these possibilities is the more difficult to achieve and experiments on this point are in progress.

The most comprehensive study of the conditions under which aerobic glycolysis may be artificially produced is due to Warburg, whose summarised results are as follows [see Warburg, 1929]. I. Inhibition of the respiration; reversibly by means of HCN, irreversibly by temporary asphyxia by keeping the tissue in nitrogen. Spontaneous loss of respiration associated with loss of nuclear material (red blood cell) and accompanying a change of cellular type (embryonic lens). II. Inhibition of the Pasteur reaction, reversibly by ethyl isocyanide, irreversibly by heat and, partially, by exposure of certain embryonic and germinal tissues to salt solutions. To these we may now add certain agents which, like ethyl isocyanide, may be classed under the rather comprehensive title of "heavy metal reagents": 1-amino-2-naphthol-6-sulphonic acid [Krah, 1930], and also reduced glutathione which, according to Bumm and Appel [1932] specifically and reversibly inhibits the Pasteur reaction.

Warburg [1926] pointed out that the poisoning action of ethyl isocyanide on the Pasteur reaction favours the theory of a heavy metal catalyst; the metals

in consideration being iron or, less probably, manganese. Since, on this theory ethyl isocyanide reacts with the catalyst of the Pasteur reaction but not with the respiratory catalyst, whilst the same compound is without action on methaemoglobin but reacts with haemoglobin, the further view that the catalyst of the Pasteur reaction may contain iron in the ferrous state was tentatively suggested. Krah concludes from the action of the reagent mentioned that he has demonstrated the ferrous nature of the catalyst.

In the present paper the action of phenylhydrazine on tissue metabolism is investigated. Phenylhydrazine belongs, according to Lipschitz [1924] to the class of "indirect blood poisons" which are able to oxidise blood pigments only in the presence of oxygen, becoming themselves oxidised in the process. Warburg *et al.* [1931] showed clearly that in oxygen, but not in inert gas (CO), erythrocytes treated with phenylhydrazine become brown in colour, without spectroscopically detectable methaemoglobin formation, whilst on haemolysis there is a heavy deposition of denatured globin, whose production is presumably accompanied by liberation of haematin; for on reduction in an atmosphere of CO the amount of gas taken up by the phenylhydrazine-treated cells was equal to their loss of oxygen capacity. On shaking these cells with air the spectrum of methaemoglobin slowly appears owing to the catalytic action of the haematin on part of the free haemoglobin, and by the reduction of this methaemoglobin sugar can be oxidised in the cells. The treated cells thus show an ability to oxidise glucose which is absent from untreated ones, this property being dependent on the high content of haemoglobin in the starting material. Such an increased sugar oxidation could not therefore be expected to occur in other cells. On the other hand it was of importance to test the reaction upon ordinary body cells of this substance which reacts in the presence of oxygen with compounds such as haemoglobin containing ferrous iron, destroying the complex and causing denaturation of the associated protein. A substance possessing these properties might be expected to influence the Pasteur reaction, since reagents which react within the cell with ferrous iron (ethyl isocyanide, heavy metal reagents of Krah) or denaturation of protein (heat, salt solutions) are known to interrupt the connection between respiration and glycolysis.

In the present paper it is shown that phenylhydrazine, in low concentration, acts as what may be termed an "indirect cell poison," in that in the presence of oxygen, but not in its absence, it is capable of bringing about an aerobic glycolysis which can rise to the anaerobic level. Its action is irreversible, and the substance is without appreciable effect on the anaerobic glycolysis. Whilst with some tissues the respiration is little affected, or even slightly increased, with others it is inhibited to a greater or less extent. The significance of this will be discussed after the description of the experiments.

EXPERIMENTAL.

Methods. The tissue, usually of the rat and in thin razor-slices where necessary, was well rinsed with three changes of Ringer solution, to remove as much of the loosely adhering blood as possible, and was suspended in glucose-bicarbonate-Ringer solution at room temperature and containing sufficient of the $M/20$ solution of phenylhydrazine described below to give the final concentration stated (usually $2.5 \times 10^{-3} M$). A control portion of the same tissue was suspended in the untreated Ringer solution. Oxygen containing 5 % CO_2 was allowed to bubble briskly through the suspensions; this pre-treatment usually lasted for 15 minutes. The tissue was then removed, well rinsed first in salt solution,

afterwards for a few minutes in bicarbonate—or phosphate—Ringer while passing the appropriate gas mixture (O_2 , or 5 % CO_2 in O_2). It was then transferred to the manometer-vessels containing bicarbonate—or phosphate—Ringer solution with 0.2 % glucose.

Phenylhydrazine. The colourless, recrystallised hydrochloride (B.D.H. "A.R." 73 mg.) was dissolved in 5 ml. water, and made up to 10 ml. with salt-solution and the addition of 0.5 ml. *N* NaOH. This solution (*M*/20) was prepared freshly for each experiment.

Measurement of metabolism. For short experiments, and where the course of the metabolism with time was to be followed, Warburg's [1924] two vessel method was used. Unfortunately with this method not only must a value of R.Q. be assumed in the calculation of aerobic glycolysis, but with increasing value of aerobic glycolysis relative to respiration, the values of the observed pressure changes in the two vessels become more nearly equal, and it follows that the difficulty of obtaining accurate values of respiration with such tissue becomes increasingly great. The technical difficulty of obtaining an accurate measure of respiration in bicarbonate media presents a serious problem with such tissues. To supplement the respiration figures, where the material available was sufficient, the methods of Dickens and Šimer [1931, 1] and Dickens and Greville [1933, 3] have been used. Both need a fairly prolonged experimental time, but the values of respiration, and with the second method of glycolysis, obtained are free from the objection inherent in the use of two different tissue-slices for measurement. The value of aerobic glycolysis in some extreme examples of phenylhydrazine-treated tissue is so great compared with the respiration that no existing method is adequate for really accurate measure of their R.Q. in bicarbonate, the whole extent of the manometer scale being used up by the glycolysis before a sufficiently great respiration had occurred. Finally, measurements of respiration in phosphate-Ringer were made by the method of Dickens and Šimer [1930, 1], but these, while free from such disadvantages, are in a less physiological medium, in which the respiration may not always be the same as in the bicarbonate medium used for the measurement of glycolysis. In the experiments with yeast, where the cells were suspended in acid phosphate, with or without potash as CO_2 absorbent in the vessels, these difficulties did not of course arise. In spite of the technical difficulties mentioned, it is believed that the precautions taken were adequate to ensure reliable measurement in all cases, though in the experiments made by Warburg's method with these highly glycolysing tissues importance should not be attached to minor variations in respiration.

The measurements are collected in Tables I, II and III, and will be briefly surveyed.

Table I. *Embryonic tissues.*

Exp.	Time (mins.)	Treated phenylhydrazine									Medium
		Control			Pre-treatment						
		Q_{O_2}	$Q_{O_2}^M$	$Q_{N_2}^M$	Conc. $M/lit.$	Time mins.	Exp. time mins.	Q_{O_2}	$Q_{O_2}^M$	$Q_{N_2}^M$	
Mouse outer membrane	—	12.9	5.9	—	3×10^{-3}	13	—	14.3	20.6	26.0	B
Mouse placenta	—	—	—	—	3×10^{-3}	13	—	8.2	5.2	6.5	B
Mouse embryo	—	20.0	14.5	31.0	3×10^{-3}	12	30	16.0	22.5	25.0	B
Guinea-pig outer membrane	—	—	—	—	2.5×10^{-3}	15	—	12.3	16.0	20.6	B
Rat outer membrane	90 { 60	14.1	—	—	3×10^{-3}	15	90 { 60	9.3	—	—	Phosphate B
15.5		6.2	26.0	—	10.2	31.8		28.5			
Rat outer membrane	40	9.8	3.2	18.7	3×10^{-3}	15	40 { 120	7.3	17.3	20.0	B Phosphate
								5.5	—	—	
Rat embryo (3 mg. dry wt.)	40	11.3	8.4	14.2	3×10^{-3}	10	40	14.1	16.2	16.1	B

Table II.

Treated phenylhydrazine											
Exp. no.	Time mins.	Control			Pre-treatment	Time mins.	Exp. time mins.	Q_{O_2}	$Q_{O_2}^{N_2}$	$Q_{N_2}^{N_2}$	Medium
		Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$							
Rat testis											
1	60	10.8	2.5	7.3	2.5×10^{-3} (no glucose)	8	60	9.4	6.3	8.3	B
	60	9.6	3.5	11.0	2.5×10^{-3}	5	60	14.1	6.9	—	B
						15	60	10.6	8.4	9.2	B
3	30	12.0	4.3	12.0	2.5×10^{-3} (tissue not washed)	15	30	9.4	2.9	11.6	B
		10.4	3.9	—	2.5×10^{-3} (tissue slightly rinsed)	15	30	10.3	6.3	—	B
4	30	12.8	4.8	11.0	2.5×10^{-3} (tissue in two changes Ph. NH. NH ₂)	15	30	9.9	11.0	10.1	B
	100	9.9	3.5	—	2.5×10^{-3}		100	6.0	7.0	—	B
5	1st 30	13.3	5.0	10.6	"	15	1st 30	15.5	8.9	8.6	B
	next 60	10.3	3.3	10.6	"		next 60	12.3	6.2	8.6	B
6	—	—	—	—	"	15	120	6.5	4.7	6.6	B†, R.Q. 0.80
7	30	10.3	5.5	9.0	"	15	30	15.1	9.0	9.0	B
8	120	10.6	2.3	—	R.Q. 0.98	15	80	8.2	6.4	—	B
								8.6	7.7	—	B
								9.6	7.1	—	B
							120	8.8	6.2	8.1	B†, R.Q. 0.90
Rat liver											
	120	11.8	0.2	3.0	R.Q. 0.83	15	120	8.2	0.5	2.9	B†, R.Q. 0.88

Abbreviations: B=method of Warburg [1924]; B*=method of Dickens and Šimer [1931, 1]; B†=method of Dickens and Greville [1933, 3]; Phosphate=method of Dickens and Šimer [1930, 1].

Table III. *Jensen rat sarcoma.*

Treated phenylhydrazine											
Exp. time mins.	Control			R.Q.	Pre-treatment	Exp. time mins.	Q_{O_2}	$Q_{O_2}^{N_2}$	$Q_{N_2}^{N_2}$	R.Q.	Method
	Q_{O_2}	$Q_{O_2}^{N_2}$	$Q_{N_2}^{N_2}$								
30	11.2	24.9	37.5	—	2.5 × 10 ⁻³ M	30	{ 13.3	40.5	36.5	—	B
					Ph. NH. NH ₂		{ 12.9	36.2	36.5	—	B
90	6.8	17.4	30.2	—	for 15 mins.	90	5.9	28.6	31.8	—	B†
90	—	—	32.0	—	in all exps.	90	5.6	28.0	34.0	0.7	B†
90	8.4	16.7	27.6	—		90	{ 13.0	27.3	—	—	B
							{ 9.7	24.2	27.6	—	B
30	13.3	20.6	35.7	—		30	15.8	32.5	34.8	—	B
150	13.2	18.0	—	0.73		150	{ 9.0	—	—	0.75	B*
							{ 10.3	(25)	—	0.69	B*
150	9.3	—	—	0.70		150	7.6	—	—	0.77	Phosphate

Abbreviations: B=method of Warburg [1924]; B*=method of Dickens and Šimer [1931, 1]; B†=method of Dickens and Greville [1933, 3]; Phosphate=method of Dickens and Šimer [1930, 1].

Effect of phenylhydrazine on the anaerobic glycolysis. The figures given for $Q_{N_2}^{N_2}$ in the tables show the effect of phenylhydrazine on the anaerobic glycolysis of a number of tissues. In some cases a slight fall of glycolysis is observed, but the variation is not as a rule much greater than may be found with duplicate pieces of control tissue after similar pre-treatment but without phenylhydrazine. The tables show that on the whole the pre-treatment with phenylhydrazine in the concentration used is without appreciable effect on the glycolysis measured under anaerobic conditions.

Effect of phenylhydrazine on the aerobic glycolysis. In all the tissues used (with the exception of liver, where the glycolysis is small under ordinary conditions) the effect of treatment with phenylhydrazine is to increase the aerobic glycolysis ($Q_M^{O_2}$) very markedly. In most cases $Q_M^{O_2}$ rises to a level very near to the anaerobic value. This is best seen with tissues showing high $Q_M^{N_2}$, such as the embryonic tissues (Table I), and tumour tissue (Table III). As is well known, many of the embryonic tissues show an appreciable glycolysis in Ringer solution which is not observed when the measurements are made in serum or in amniotic fluid: very young embryos may also glycolyse even in these media unless the enveloping membranes are intact [Negelein, 1925]. Even in such cases (Table I) the value of $Q_M^{O_2}$ found in Ringer solution is greatly increased by pre-treatment with phenylhydrazine.

Of tissues with an intermediate degree of glycolysis, testis (Table II) provides a suitable example. Unlike the other tissues studied, for some unknown reason apparently connected with the animals used, testicular tissue of the rat suspended in Ringer solution shows a very variable aerobic glycolysis. Thus Warburg *et al.* [1924] give the following figures (based on R.Q. = 1) $Q_{O_2} - 12.3$, $Q_M^{O_2} + 7.2$, $Q_M^{N_2} + 8.5$; and a similar though less pronounced aerobic glycolysis was observed by Dickens and Greville [1933, 1]. On the other hand, in earlier experiments Dickens and Šimer [1931, 2] found much lower values of $Q_M^{O_2}$, and these are similar to the figures given in the present paper. At the other extreme, Barron [1930] reports that though manometrically he observed a small acid formation ($Q_M^{O_2} + 1.2$), chemically (in phosphate) he was unable to detect any aerobic lactic acid formation in salt solutions with the specimens of rat testis used by him. Since in manometric experiments we have invariably observed some aerobic acid production with this tissue, it was considered desirable to compare the manometric readings with chemical estimations of lactic acid formed. As the result (Table IV) of the analysis (Friedemann and Kendall's [1929]

Table IV. *Lactic acid estimations.*

	Rat testis		Manometrically found mg. acid	Chemical estimation mg. lactic acid
	mg. dry wt. tissue	Time mins.		
Control tissue I	13.6	120	0.39	0.45
	14.8		0.42	0.38
Control tissue II	22.9	100	0.69	0.62
	21.1		0.64	0.70
Phenylhydrazine tissue I	13.8	120	0.78	0.70
	15.8		0.68	0.66
Phenylhydrazine tissue II	24.3	100	1.23	1.12
	8.65	180	1.00	1.08
Jensen sarcoma				
Control tissue	12.17	90	1.22	1.07
	11.43		1.15	0.96
Phenylhydrazine tissue	8.05	90	1.32	1.25
	8.72		1.43	1.14
	8.68		1.25	1.32

modification of Clausen's method), some 90 % of the manometrically observed acid appeared as lactic acid. Since this is almost within the limits of error with a tissue of such moderate glycolysis, there does not seem any reason to doubt that this is also a true lactic acid fermentation. Thus there appears to be a real

difference between the behaviour of rat testis as studied by different workers for which no explanation appears sufficient at the present time.

These considerations however do not affect the present experiments, where control and phenylhydrazine portions of the same tissue were always compared side by side. As will be seen from Table II, phenylhydrazine had a similar effect on aerobic glycolysis to that shown by the embryonic tissues; the value of $Q_M^{O_2}$ rises after treatment to a higher level, near or equal to $Q_M^{N_2}$.

A further type was provided by tumour tissue. Well-growing non-necrotic specimens of Jensen rat sarcoma (derived from a stock tumour kindly supplied by Miss Scott, Barnato Joel Research Laboratories, Middlesex Hospital, London) were used in all experiments. All methods of measurement of metabolism showed the same result, the already pronounced aerobic glycolysis shown by the Jensen sarcoma is greatly increased in the phenylhydrazine-treated tissue (Table III) and reaches a value which is, within the limits of error, equal to the glycolysis of the same tissue in absence of oxygen.

Nature of the acid formed. The possibility that the acid formed in the manometric observations might not have been lactic acid has been excluded by analysis. For this purpose rat testis and Jensen sarcoma were used and the manometric readings compared with Clausen estimations of the lactic acid formed in the vessels, the results (Table IV) show that some 90 % of the acid formed is lactic acid.

Conditions affecting the action of phenylhydrazine on the metabolism.

(a) *Washing.* Since phenylhydrazine is known to react with haemoglobin under similar conditions to those in which it reacts with the cell-ferments, it is not surprising that incomplete washing of the tissue reduces the extent of its action. It has been observed that in several experiments where completely unwashed testis, for example, has been used phenylhydrazine ($2.5 \times 10^{-3} M$) had no measurable action on the metabolism. If, however, the same tissue were treated with a second portion of the phenylhydrazine Ringer solution, the usual increase of aerobic glycolysis was produced (Table II). This point has not been further studied, well washed tissues being always used in subsequent experiments.

(b) *Time and temperature.* At room temperature the reaction with phenylhydrazine is not instantaneous but is usually complete in 15 minutes. Exp. 2, Table II, performed with rat testis illustrates this point.

The reaction also occurs at 37° and is not dependent on the presence of glucose in the medium as the following experiments show.

Jensen rat sarcoma. Temperature 37° . Control, $Q_M^{O_2} + 23.5$; the same tissue with $1.7 \times 10^{-3} M$ phenylhydrazine in vessel (result, corrected for autoxidation of phenylhydrazine) $Q_M^{O_2} + 37$.

Rat testis. Pre-treatment without addition of glucose to the Ringer-bicarbonate used: 8 minutes at room temperature:

	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Control	- 10.8	2.5	7.3
With $2.5 \times 10^{-3} M$	{ - 9.9	6.3	8.3
phenylhydrazine	{ - 9.0	6.3	—

(c) *Oxidative nature of the reaction.* The following experiments show that the action of phenylhydrazine occurs only in the presence of oxygen.

The pre-treatment was carried out at room temperature in the closed vessels attached to their manometers; these were filled with either nitrogen + 5 % CO_2 , or oxygen + 5 % CO_2 and were shaken as usual. The phenylhydrazine solution (0.2 ml. $M/20$) was contained in the side-bulb, while the tissue, suspended in

Ringer-bicarbonate-glucose solution (4 ml.) was in the main part. The solutions were first saturated with the appropriate gas mixtures. In the experiments in nitrogen, chromous chloride solution (0.3 ml.) was contained in the central well as oxygen absorbent. The vessels containing solutions and tissue were first shaken for 10 minutes to allow absorption of any residual oxygen in the anaerobic experiments, the phenylhydrazine was tipped in from the bulbs, and the shaking was continued for 15 minutes. The tissue was then quickly removed, rinsed and transferred to the vessels used for measurement of metabolism. It was noted that the colour of small traces of blood present changed to brown in the oxygenated phenylhydrazine portions only.

	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Rat testis			
Control: pre-treatment in $O_2 + 5\% CO_2$	- 10.3	5.5	9.0
Ph.NH.NH ₂ : pre-treatment in $O_2 + 5\% CO_2$	- 15.1	9.0	—
Control: pre-treatment in $N_2 + 5\% CO_2$	- 12.8	5.5	—
Ph.NH.NH ₂ : pre-treatment in $N_2 + 5\% CO_2$	- 12.5	5.8	—
Jensen sarcoma			
Control: pre-treatment in $O_2 + 5\% CO_2$	—	—	32.4
Ph.NH.NH ₂ : pre-treatment in $O_2 + 5\% CO_2$	- 9.5	31.0	—
	- 7.7	27.8	—
Ph.NH.NH ₂ : pre-treatment in $N_2 + 5\% CO_2$	- 8.6	19.1	—

Effect of phenylhydrazine on respiration. If the figures for Q_{O_2} shown in Tables I–III are examined, it will be seen that the rise in glycolysis is not accompanied by a corresponding fall in respiration. Occasionally in short experiments the latter appears to be slightly increased; a similar result was observed by Warburg [1926] in ethyl isocyanide poisoning of surviving tissues, but there does not seem to be any explanation at present for this. On the other hand, and particularly in experiments of greater duration, there is usually a small fall of respiration in the phenylhydrazine tissue; the extent of the fall varies, though not entirely regularly, with different tissues and is fairly uniformly found in extended experiments with testis, sometimes with some of the embryonic tissues, and is less pronounced, or absent, with Jensen sarcoma.

In considering these figures there is a suggestive parallelism with the respiration of the same tissues in the absence of glucose from the medium; this effect of glucose-lack has been fully examined in a previous publication [Dickens and Greville, 1933, 1]. It was shown that, in experiments extending over some hours, the respiration of testis falls slowly, but that of most of the other tissues used in Tables I–III is less affected by withdrawal of glucose; probably even with different samples of any particular tissue the extent of the fall is to some extent dependent on the state of nutrition of the animal. The respiration of the Jensen sarcoma is practically identical in extent whether glucose is present or not.

This suggested that the action of phenylhydrazine in inhibiting the Pasteur reaction might be connected with a loss of ability of the poisoned tissue to oxidise glucose. This question has been attacked in two ways. Firstly, the value of R.Q. in a few examples of the poisoned tissue was examined. Unfortunately little help could be expected from these measurements, for with the tissues available the fall in R.Q. in absence of glucose is by no means marked and may even be lacking entirely, as was shown by Dickens and Greville [1933, 1] in a number of instances. The most favourable example from this point of view was testis, and here the values of R.Q. and respiration both suffered a fall in the longer experiments (Table II) suggesting that carbohydrate oxidation may be reduced.

Similar measurements with Jensen sarcoma showed that neither the R.Q. nor the respiration undergoes any marked change in the poisoned tissue (Table III); here again the result is comparable with the absence of change of these quantities with the untreated tissue in glucose-free Ringer, as has been previously shown.

The most serviceable examples of cells suitable for the demonstration of carbohydrate oxidation are provided by brain grey-matter [Loebel, 1925] and the yeast-cell [Meyerhof, 1925]. In both of these, unlike the tissues already considered, the continuance of the respiration is dependent on the supply of carbohydrate from without.

The following experiments were designed to determine to what extent the oxidation of glucose is influenced in these cells by treatment with phenylhydrazine, since this appeared to be a critical test of how far it is the oxidation of carbohydrate that is specifically interfered with by this reagent.

Table V. *Rat cerebral cortex.*

No.	Exp. time mins.	Treated phenylhydrazine											Method
		Control				Pre-treatment		Q_{O_2}	$Q_{O_2}^M$	$Q_{N_2}^M$	R.Q.		
		Q_{O_2}	$Q_{O_2}^M$	$Q_{N_2}^M$	R.Q.	Conc. M/lit.	Time mins.						
1	30	11.1	3.5	—	—	$\begin{cases} 5 \times 10^{-4} \\ 10^{-2} \end{cases}$	15	12.9	5.6	—	—	B	
							15	3.9	21.4	19.4	—	B	
2	40	13.4	2.5	28.0	—	2.5×10^{-3}	15	10.3	11.3	24.4	—	—	
	90	—	—	—	—	2.5×10^{-3}	15	8.6	8.5	—	—	B	
							15	11.6	10.7	24.0	—	—	B*
							15	4.4	15.7	—	—	—	B*
3	60	—	—	—	—	2.5×10^{-3}	15	12.2	10.0	27.0	—	—	
	120	—	—	—	—	2.5×10^{-3}	15	9.6	8.8	—	—	B	
							15	6.4	11.2	—	0.98	—	B†
							15	8.3	—	—	0.96	—	Phosphate
4	60	12.3	4.3	15.9	—	2.5×10^{-3}	15	5.9	—	—	1.02	—	Phosphate
							15	9.5	7.0	17.8	—	—	B
							15	8.4	—	—	—	—	Phosphate
	120	12.6	2.3	—	0.98	2.5×10^{-3}	15	9.9	7.5	17.0	1.01	—	B†
5	120	—	—	—	—	2.5×10^{-3}	15	$\begin{cases} 5.0 \\ 5.8 \end{cases}$	15.2	18.4	—	—	B†
								11.1	18.4	—	—	B†	

Abbreviations: B=method of Warburg [1924]; B*=method of Dickens and Šimer [1931, 1]; B†=method of Dickens and Greville [1933, 3]; Phosphate=method of Dickens and Šimer [1930, 1].

Rat cerebral cortex. The experiments on rat brain tissue were carried out just as for the other tissues in Tables I–III. As in the other experiments glucose was present throughout in the media, a necessary precaution here since the respiration of brain tissue quickly suffers in absence of sugar. The results are shown in Table V. As before, it will be seen that the phenylhydrazine pre-treatment is without effect on the anaerobic glycolysis, whilst in some experiments the aerobic glycolysis rises considerably. In all of these, however, the respiration sinks, more or less slowly, and in spite of repeated trials it has not been found possible by action of phenylhydrazine with this tissue to raise the glycolysis to the anaerobic level without the occurrence of a serious fall in respiration. The R.Q. does not appear to be affected by phenylhydrazine pre-treatment of brain, and it is often similarly unaffected by lack of glucose [Dickens and Greville, 1933, 1].

Thus with brain tissue poisoned by phenylhydrazine, as the aerobic glycolysis increases there appears to be a progressive change in the respiratory metabolism towards that shown by the same tissue in absence of glucose. The question therefore arises: is the oxidation of other metabolites similarly affected? Loebel [1925] showed that the respiration of normal brain tissue can be supported

by glucose, lactate or pyruvate. In the following experiment the effect of these metabolites on the respiration of normal and poisoned brain was studied.

Rat cerebral cortex. Control brain. As control, slices prepared from two brains were treated with phenylhydrazine ($2.5 \times 10^{-3} M$) for a time insufficient for the metabolism to be affected.

Medium	Substrate	Respiration (Q_{O_2})		R.Q.
		1st hr.	2nd hr.	
Phosphate-Ringer	None added	- 8.0	- 2.6	—
	Glucose 0.2%	- 11.0	- 9.6	1.03
	<i>dl</i> -Lactate 0.2%	- 12.0	- 12.0	1.03
	Pyruvate 0.2%	- 9.5	- 9.3	1.30

Bicarbonate-glucose-Ringer: $Q_{O_2} - 11.2$; $Q_M^{O_2} + 5.3$; $Q_M^{N_2} + 21.5$.

Phenylhydrazine brain. Pre-treatment: oxygenation for 15 minutes at room temperature with $2.5 \times 10^{-3} M$ phenylhydrazine.

Medium	Substrate	Respiration (Q_{O_2})			R.Q.
		1st hr.	2nd hr.	3rd hr.	
Phosphate-Ringer	None added	- 5.5	- 2.1	- 1.0	—
	Glucose 0.2%	- 10.0	- 4.0	- 0.8	0.99
	<i>dl</i> -Lactate 0.2%	- 8.3	- 6.0	- 4.0	1.01
	Pyruvate 0.2%	- 10.7	- 9.6	- 8.6	1.32

Bicarbonate-glucose-Ringer (1st 40 mins.): $Q_{O_2} - 11.8$; $Q_M^{O_2} + 10.3$; $Q_M^{N_2} + 20.0$.

These figures show how the respiration changes with time, and from them it is clear that whilst the ability to oxidise glucose and lactate is severely damaged in the partially poisoned brain, the ability to oxidise pyruvate is in no way affected. This result is particularly noteworthy, since it was shown for tumour tissue [Dickens and Šimer, 1930, 2] that whilst glucose and lactate have little effect on the level of R.Q., pyruvate increases this very markedly to above unity (Jensen sarcoma, Rous sarcoma). Except for the fact that the tumour respiration in absence of added substrate does not fall, a property known to be characteristic of brain tissue, the behaviours towards these metabolites of the partially poisoned normal tissue after treatment with phenylhydrazine and of the tumour tissue appear to be very similar or identical. Pyruvate is the only one of these three substances to be readily oxidised by the two types of tissue, in both of which the respiration is damaged in such a way that it is unable to exercise its normal influence on the aerobic glycolysis.

Baker's yeast. The behaviour towards glucose of the respiration of the yeast cells used (Table VI) was found to be essentially similar to that of brain tissue, but the fall of respiration in absence of glucose (about 90 %) in the washed cells showed that the yeast cells were even more dependent than brain on glucose from the medium for their respiration.

Phenylhydrazine pre-treatment of this yeast inhibited its respiration without appreciably affecting its power of anaerobic fermentation. The aerobic fermentation rose, and calculation of the Meyerhof quotient shows that there was only a slight inhibition of the Pasteur reaction, or in other words the poisoning of the carbohydrate respiration ran almost parallel with the appearance of the carbohydrate fermentation. Finally, with the high concentration of phenylhydrazine, poisoning of the respiration was complete, within the limits of error, whilst the fermentation in air became equal to anaerobic (HCN-poisoned) fermentation of the untreated yeast (Table VI).

As with higher cells, this toxic action of phenylhydrazine on the yeast cell is dependent on the presence of oxygen. With yeast the effect is very marked:

Table VI. *Baker's yeast.*

1. Effect of glucose-lack.

Suspension in $M/20$ NaH_2PO_4 , oxygenated by bubbling the gas. 0.6 mg. dry wt. of yeast per ml.

Control portion washed three times at the centrifuge with solution of NaH_2PO_4 ($M/20$), glucose, $M/18$.

"Glucose-free" portion washed three times at the centrifuge with solution of NaH_2PO_4 ($M/20$), without glucose.

Both portions made up to suitable volume in the same solutions as used for washing.

	Q_{O_2} (Respira- tion)	$Q_{\text{G}}^{\text{O}_2}$ (Aerobic ferm.)	$Q_{\text{G}}^{\text{N}_2}$ (Anaer- obic ferm.)
Result: (temp. 25°) Control portion with glucose	54.5	8.5	111
"Glucose-free" portion in glucose-free phosphate	$\begin{Bmatrix} 4 \\ 7.5 \end{Bmatrix}$	$\begin{Bmatrix} 1.5 \\ 7.5 \end{Bmatrix}$	4.5
"Glucose-free" portion on addition of glucose	55.5	10	123

2. Effect of treatment with phenylhydrazine.

Suspension as above of a different batch of yeast from the same commercial source. 0.3 mg. dry wt. of yeast per ml. in glucose-phosphate solution.

Control portion. Washed three times at centrifuge with glucose-phosphate solution. Made up to original volume.

Phenylhydrazine pre-treatment. To the oxygenated yeast suspension at room temperature, sufficient of a neutralised solution of phenylhydrazine hydrochloride to give the concentration stated was added. After 15 mins. centrifuged, washed three times with glucose-phosphate solution, and made up to the original volume.

Result: (temp. 25°):

Concentration of phenyl-
hydrazine $M/\text{lit.}$

	Q_{O_2}	$Q_{\text{G}}^{\text{O}_2}$	$Q_{\text{G}}^{\text{N}_2}$
0 (control)	80	23	180
10^{-4}	82	16	—
2.5×10^{-4}	68	60	167
10^{-3}	51	91	161
2×10^{-3}	10	145	150
3×10^{-3}	0	163	168
7×10^{-3}	0	175	165

Note. The values of "anaerobic fermentation" were obtained by poisoning with $2 \times 10^{-3} N$ HCN.

mixture of the phenylhydrazine solution with the non-aerated suspension of yeast merely in contact with air but not shaken is not enough to cause the full alteration of metabolism. Saturation with oxygen however quickly leads to the usual result:

Baker's yeast. 1 mg. (dry wt.) per 3 ml. in NaH_2PO_4 $M/20$, glucose $M/18$; 25°. Pre-treatment with concentration of phenylhydrazine stated for 15 minutes.

		Phenylhydrazine pre-treatment							
		Nitrogen (with CrCl_2)		Air (not shaken)			Oxygen (shaken)		
Conc. of phenylhydrazine	Control	7×10^{-3}	10^{-3}	7×10^{-3}	3×10^{-3}	10^{-3}	7×10^{-3}	$3 \times 10^{-3} M.$	
Respiration (Q_{O_2})	— 45	—47.5	—46.5	—29.5	—42.5	—47	0	— 6.5	
Aerobic fermentation ($Q_{\text{G}}^{\text{O}_2}$)	+ 8	+17	+ 6	+39	+24	+ 9	+101	+110	
Anaerobic fermentation ($Q_{\text{G}}^{\text{N}_2}$)	+108	—	—	—	—	—	+ 93	+109	

Other reagents: phenylhydroxylamine and amyl nitrite, both of which lead to methaemoglobin formation in the erythrocyte [Heubner, 1913; Lipschitz, 1924; Warburg *et al.* 1930; 1931] and to increased respiration of the latter in glucose, are without similar effect to that of phenylhydrazine on the Pasteur reaction

Table VII. *Other reagents.*

		Control			Treated tissue				Method
		Q_{O_2}	$Q_{O_2}^{1/2}$ <i>M</i>	$Q_{N_2}^{N_2}$ <i>M</i>	Conc. in Ringer <i>M</i> /lit.	Q_{O_2}	$Q_{O_2}^{O_2}$ <i>M</i>	$Q_{N_2}^{N_2}$ <i>M</i>	
1. Rat testis.	Phenylhydroxylamine†	10.0	3.5	8.8	6×10^{-4}	1.3	0.7	9.2, 7.8	B
					1.2×10^{-4}	7.7	2.6	—	B
	Quinone†	—	—	—	10^{-3}	4.9	1.9	3.1	B
					5×10^{-4}	4.4	1.3	3.3	B
2. Jensen sarcoma.	Phenylhydroxylamine*	16.4	22.8	34.4	10^{-3}	3.9	15.0	30.0	B
	Amyl nitrite†	7.8	19.7	30.4	<i>ca. M</i> /100	4.2	15.2	24.6	B

Abbreviations: B = method of Warburg [1924]; Phosphate = method of Dickens and Šimer [1930, 1].

* = pre-treatment; † = in vessel with the tissue.

(Table VII), their action being to lessen the aerobic metabolism generally; in depressing Q_{O_2} they are more like the indifferent narcotics. Phenylhydroxylamine was the pure colourless recrystallised substance freshly prepared according to Wohl [1894]. It was dissolved in the Ringer solution in the concentration stated immediately before use; the solution however very quickly undergoes decomposition. Amyl nitrite (B.D.H., A.R.) was also added to the Ringer solution directly and the mixture shaken mechanically for half an hour before use. In both cases corrections for spontaneously occurring pressure changes were applied.

Since these reagents, in their reactions with haemoglobin at any rate, are without effect on the globin, whilst phenylhydrazine brings about its denaturation, it seems likely that it is to this property of protein denaturation that phenylhydrazine owes its action on the Pasteur reaction.

The mechanism of the Pasteur reaction. According to Warburg's conception [see Warburg, 1930], the increase of aerobic glycolysis without a corresponding fall in the respiration is the criterion by which we may judge the specific inhibition of the Pasteur reaction. The extent of the inhibition is to be estimated by the fall in the value of the Meyerhof quotient: (aerobic *minus* aerobic glycolysis)/(respiration). From a normal value of about 2, this quotient will sink to zero when inhibition of the Pasteur reaction is complete.

Judged by this standard, phenylhydrazine is a specific, irreversible inhibitor of the Pasteur reaction only in certain tissues; these are the tissues whose continued respiration is not dependent upon their being provided with carbohydrate from without. On the other hand, in tissues whose respiration is dependent on exogenous carbohydrate, phenylhydrazine acts as a specific poison for the respiratory enzymes, where its effect on the respiration may, as with the yeast cell, resemble that of cyanide in its completeness.

A number of questions are at once suggested by this apparently dual rôle of phenylhydrazine as a poisoner on the one hand of the Pasteur reaction and on the other of the oxidation of carbohydrate. In the first place it does not seem probable that this property possessed by phenylhydrazine of interference with glucose oxidation without interfering with the mechanism of glucose cleavage is limited to tissues whose respiration falls in the absence of glucose. It is therefore a probable assumption, supported by the evidence as far as this is available, that the mechanism of phenylhydrazine poisoning is similar in all the tissues studied,

and that the inhibition of the Pasteur reaction by phenylhydrazine in these tissues is bound up with the inhibition of glucose oxidation. The question at once arises as to whether the continuance of the Pasteur reaction is necessarily linked with oxidation of carbohydrate, or whether the oxidation of other foodstuffs may also bring about the disappearance of glycolysis. This question cannot be regarded as settled at the present time. There are very few cases where the relative amount of carbohydrate oxidised can be assessed with anything approaching the certainty with which an estimate can be formed for tissues such as brain and retina whose continued respiration depends on such supplies. Moreover, peripheral frog-nerve [Gerard, 1932] provides an example where the existence of such a relationship seems doubtful on the basis of existing information; it should be remembered however that this tissue presents metabolic peculiarities, such as the delayed appearance of glycolysis in nitrogen [Gerard and Meyerhof, 1927] not found in other cases [see also Chang and Gerard, 1933]. Again, with sugars other than glucose (*e.g.* fructose), oxidation of carbohydrate may proceed without anaerobic lactic acid formation (brain, see Loebel [1925], brain and retina, see Dickens and Greville [1933, 1]); it cannot however be assumed that oxidation of glucose and fructose by tissues necessarily proceeds along similar routes, and it is therefore permissible to limit the present discussion to the rôle of the oxidation of glucose in the Pasteur mechanism.

As far as the glucose metabolism of the mammalian tissues here described is concerned, the evidence for the hypothesis advanced by Dickens and Šimer [1930, 2], that the Meyerhof cycle is connected mainly or wholly with the oxidation of carbohydrate, rests principally on observations of R.Q. [Dickens and Šimer, 1930, 2; 1931, 2; Dickens and Greville, 1933, 1, 3], on the effect of sugar-lack on the ammonia formation [Dickens and Greville, 1933, 2] and on the respiration and R.Q. [Dickens and Greville, 1933, 1].

If the estimate of sugar oxidation derived from these values be accepted, it is found that in all the normal tissues studied glucose oxidation runs parallel with their ability to form lactic acid from glucose. The criticism which may be applied to this conclusion is that the evidence of carbohydrate oxidation is necessarily indirect in most cases. Though there does not appear to be any reason to doubt the correctness of the estimates of carbohydrate oxidation, some more direct demonstration of a link between carbohydrate oxidation and cleavage is certainly desirable. Phenylhydrazine may be regarded as helping to fill this gap, but the question cannot yet be regarded as answered. Do other inhibitors of the Pasteur reaction behave towards carbohydrate oxidation similarly to phenylhydrazine, or is the inhibition of carbohydrate oxidation merely an incidental property possessed by phenylhydrazine? Unfortunately Warburg's [1926] experience with ethyl isocyanide does not help us to decide this question, since he did not make use of any tissue in which the respiration falls appreciably in absence of glucose in short experiments.

With yeast cells Warburg states that "similar though not equally convincing experiments can be carried out with yeast cells. . . Without the respiration of the yeast being obstructed to any considerable extent the aerobic fermentation is intensified in the presence of 10^{-3} *N* ethyl isocyanide. The aerobic fermentation by yeast in 10^{-3} *N* ethyl isocyanide does not however attain the dimension of the anaerobic fermentation." As this experiment would provide a crucial test of the interdependence or otherwise of the fermentation and carbohydrate oxidation in this case, it is proposed to repeat the observations made with phenylhydrazine using ethyl isocyanide and other similar reagents in this and related examples when opportunity occurs.

SUMMARY.

The action of phenylhydrazine and some allied substances on the metabolism of surviving tissues has been investigated. Phenylhydrazine, in low concentration, is without action on the anaerobic glycolysis of animal tissues or on the anaerobic fermentation of yeast. On the other hand, in the presence of oxygen, it causes the aerobic lactic acid formation of a wide range of animal tissues and the aerobic fermentation of baker's yeast to rise to a value near or equal to that of the same cells anaerobically. The effect of the same treatment on the respiration is described, and it is found that the tissues which are dependent on the oxidation of glucose (or lactate) for their continued respiration undergo a progressive fall of respiration after treatment. Baker's yeast also belongs to this class of cell. However, the majority of animal tissues are not so dependent, and their respiration is much less obviously affected by phenylhydrazine. Tumour tissue (Jensen sarcoma) behaves similarly to this group of normal cells, and its already high aerobic glycolysis is caused by this treatment to rise to the anaerobic value. The bearing of these observations on the nature of the Pasteur reaction is discussed.

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