XC. THE EFFECT OF CYANIDE ON THE RESPIRATION OF ANIMAL TISSUES.

BY MALCOLM DIXON AND KENNETH ALLAN CALDWELL ELLIOTT.

From the Biochemical Laboratory, Cambridge.

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ACCORDING to Warburg's theory of cell respiration the absorption of oxygen by tissues is due entirely to the reaction between molecular oxygen and a complex intracellular iron compound. This iron compound, which Warburg believes to be a haematin derivative, he has termed "the respiratory enzyme" (das Atmungsferment), and he considers that molecular oxygen brings about oxidations in the tissues only by its action. "Die Primärreaktion der Atmung ist die Reaktion zwischen molekularem Sauerstoff und Eisen, und nur in dieser Reaktion, nicht mit den organischen Molekülen, vermag der molekulare Sauerstoff in der Zelle zu reagieren" [Warburg, 1923, 1]. According to this theory, then, tissue respiration is to be regarded as being due to the action of one catalytic iron-containing system, rather than as the sum-total of a number of oxidations brought about by a number of more or less independent catalysts.

The theory is based principally on measurements of the inhibiting effect of cyanides, carbon monoxide and similar substances on cell respiration. It is well known that such substances act as powerful inhibitors of most iron-catalysed reactions, and the marked inhibition of cell respiration which they produce is considered to be due to a poisoning of the "respiratory enzyme."

It was pointed out by Dixon and Thurlow [1925] that animal tissues contain systems, such as the xanthine oxidase, whose oxygen uptake is not affected by cyanide, carbon monoxide, etc., and they considered that such systems formed exceptions to Warburg's general statement. The essential part of Warburg's reply was as follows [Warburg, 1925]: "Nach der Theorie sind diejenigen Lebensvorgänge, die durch Blausäure spezifisch gehemmt werden, Schwermetallkatalysen, so die Leberatmung, die durch Blausäure spezifisch gehemmt wird. Wenn die Oxydation von Hypoxanthin durch Blausäure *nicht* gehemmt wird, so liegt hier ein System vor, das in der Atmung der Leber keine Rolle spielt, ein System, das *kein* Respirationssystem ist." In other words: cell respiration is inhibited by HCN, xanthine oxidase is not, therefore xanthine oxidase is not a cell respiration system¹.

Such an argument is obviously valid only if cyanide produces a total inhibition of tissue respiration; if the inhibition is only partial, such systems as the xanthine oxidase may well contribute to that part of the respiration which is not inhibited by cyanide.

¹ It is difficult to see why xanthine oxidase should not be classed as a respiration system, since it is present in the tissues in an active form and the oxidation it brings about is known to occur in the tissues.

Warburg [1928] has recently further defined his position as follows: "Xanthinoxydase und Succinoxydase sind Niederschläge aus Zellextrakten¹, die weder wie das Atmungsferment wirken, noch wie das Atmungsferment beeinflusst werden. Vielleicht enthalten sie denaturierte Reste von Atmungsferment. Nennt man diese Niederschläge Atmungsfermente, so hat man zu unterscheiden zwischen Atmungsfermenten, die die Erscheinungen der Atmung hervorrufen, und solchen, die es nicht tun." This again is only valid so long as the whole of the respiration is due to systems inhibited by cyanide. Warburg continues "Beschränken wir uns auf die Fermente erster Art, so ist auf *Dixons* Frage, wieviel Atmungsfermente es gibt, folgendes zu antworten: Jeder weiss, dass sich die Natur zum Sauerstofftransport vorwiegend des Hämoglobins bedient, dass nicht alle Hämoglobine identisch sind und dass auch andere Sauerstofftransportmittel, z.B. Hämocyanin, existieren. Entsprechend gibt es in der Natur nicht nur 1 Atmungsferment, aber der Mechanismus der Atmung in einem bestimmten Falle ist, wie der Mechanismus des Sauerstofftransports, ein bestimmter und einheitlich. Wie zum Transport des Sauerstoffs, so benutzt die Natur zur Verbrennung durch Sauerstoff *vorwiegend einen* Mechanismus. Denn das Atmungsferment in Hefezellen und Chorion, in Leberzellen und Blutplättchen, in Leucocyten und Seeigeleiern verhält sich im Prinzip gleich."

In view therefore of what is based on the assumption that the *whole* of the respiration of animal tissues is inhibited by cyanide it is necessary to enquire upon what experimental evidence it rests. It appears that practically all the measurements of the effect of cyanide on cell respiration upon which Warburg's theory is based were carried out on such cells as yeast cells, sea urchins' eggs, red blood-corpuscles, etc. Very few measurements appear to have been done on the more typical animal tissues, such as liver, muscle, etc. In a fairly recent paper Warburg [1926] states that the respiration of animal tissues is almost completely inhibited by hydrogen cyanide, giving a reference to an earlier paper [Warburg, Posener and Negelein, 1924]. In this earlier paper the same statement is made, but is supported by only a single experiment, which was carried out on embryonic tissue, and which showed an inhibition of 60 % with $M/1000$ HCN. A search of Warburg's papers revealed one other such experiment [Warburg, 1926, p. 438], showing a 90 % inhibition with kidney and $M/1000$ HCN. So far as we are aware, Warburg has not published any other experiments on the effect of cyanide on typical animal tissues. Meyerhof [1923] found that the respiration of frog muscle was completely inhibited by $M/200$ cyanide. Batelli and Stern [1907] performed some experiments on a few animal tissues which usually gave a very incomplete inhibition, but in one or two cases showed an almost complete inhibition

¹ Incidentally we would point out that xanthine oxidase and succinoxidase, far from being mere precipitates of cell extracts, are two of the most sharply defined and specific enzymes known. They can be prepared by a variety of methods, or studied in the tissue itself. Their behaviour is the same in either case.

when a high concentration of cyanide was used. Few experimental details are given, however, and they do not state that the cyanide was neutralised before use. Neglect of this precaution has vitiated experiments carried out by other authors in some cases. Resnitschenko [1928] gives a number of experiments on *Ascaris* eggs, pointing out later that much of the effect was due to the alkalinity of the cyanide rather than to a specific poisoning. We may mention here the experiments of Allen [1919], who found that cyanide was capable of inhibiting 80 % of the respiration of *Planaria*.

The scarcity of data on animal tissues, coupled with the facts mentioned above, made it highly desirable that a systematic study of the degree of inhibition produced by cyanide on the respiration of a number of typical animal tissues should be carried out. The results of such an investigation are described in the present paper.

The technique employed was as follows, except where otherwise stated. Four differential Barcroft micro-respirometers were used in each experiment. Into the right-hand flask of each apparatus was weighed 0.5 g. of the tissue (the preparation of which is described below), and 1.5 cc. of phosphate buffer p_H 7.3 added. To the four flasks were also added 1 cc. of water, $M/1000$ KCN, $M/100$ KCN, and $M/10$ KCN respectively. The four left-hand (compensation) flasks contained the same cyanide solutions as the corresponding right-hand flasks, together with 2 cc. of phosphate buffer. The total volume added to each flask was therefore 3 cc., so that the final concentrations of cyanide in the four respirometers were 0, $M/3000$, $M/300$, $M/30$ respectively. The cyanide solutions were always carefully neutralised to p_H 7.3 before use. The small tubes in all the eight flasks contained rolls of filter-paper moistened with 6 % KOH so as to give efficient absorption of the CO_2 produced in respiration. The respirometers were then placed in the thermostat at 40° and shaken at a speed of about 120 complete oscillations per minute. After allowing about 5 minutes for equilibration, the taps of the apparatus were closed and readings commenced.

The tissues were used as soon as possible after the death of the animal. It should be noted that they were normal tissues, not washed or extracted in any way. The only treatment they received was the slicing or mincing, according to the form in which they were used. We carried out a number of experiments using tissues sliced according to Warburg's [1923, 2] method, *i.e.* cut with a razor moistened with Ringer's solution into slices 0.5 mm. or less in thickness. This method of preparation is recommended by Warburg for work on tissue respiration, as it avoids injury to the tissue as far as possible, while allowing the oxygen free access. We have also used the tissue in the form of a fine suspension, obtained by the use of a special fine mincer. The tissue, on being passed through this machine, without the addition of any liquid, was reduced to a thick homogeneous paste which, when weighed out into the flasks, readily dispersed in the liquid to give a uniform suspension. The suspension, though fine, was not so fine as to involve excessive injury to

the tissue cells. In a series of comparative experiments we found that the two methods of preparation gave identical results. The rate of respiration and the degree of inhibition produced by cyanide were the same in both cases. (Some of these experiments are quoted in Table I below.) We also determined the effect of substituting mammalian Ringer's solution for the phosphate buffer, using both slices and suspensions of tissue, and we were unable to detect any difference. The greater number of our experiments was therefore carried out on tissue suspensions in phosphate buffer solution.

We found that the reproducibility of the results was so satisfactory as to give us considerable confidence in the method. That is to say, duplicate determinations carried out on different samples of the same tissue always agreed very closely. As examples we may refer to the experiments given in Figs. 7 and 12. Comparing different tissues, however, considerable differences in respiration rate were observed, even with corresponding tissues from different

Fig. 1. Effect of cyanide on respiration of rabbit kidney.

individuals of the same species. This is not surprising, however, as differences of 2000 % are observed even by the most physiological method—that of perfusion [Bornstein and Gremels, 1928]. Probably variations in the state of nutrition have a considerable influence.

Considering now the results obtained with cyanide, four completely typical sets of experiments are represented in Figs. 1-4. It will be seen that the curves are practically linear, *i.e.* the oxygen uptake proceeds with a constant velocity, at any rate for the first hour. The cyanide produces a marked, but by no means complete, inhibition of respiration; usually it leaves about one-third of the total respiration unaffected. An important point is that with most animal tissues $M/300$ cyanide already produces the maximal inhibition obtainable, and this inhibition is not increased by increasing the cyanide concentration tenfold (or even a hundredfold, see below). This is characteristic of most of the animal tissues studied. Its significance is discussed later. These experiments clearly show that from one-half to one-quarter of the total respiration is due to systems which are not poisoned by cyanide.

The results obtained are more conveniently represented in the form of curves showing how the rate of respiration varies with the concentration of cyanide. The method of plotting these curves will be clear from Fig. 5, which also shows the typical form of the curve.

Fig. 2. Effect of cyanide on respiration of pig liver.

Fig. 3. Effect of cyanide on respiration of sheep heart.

Fig. 4. Effect of cyanide on respiration of rat liver slices.

The results of a number of experiments on different tissues have been plotted in this form, and are represented in Fig. 6. The rates of respiration are here expressed as percentages of the corresponding rates with no cyanide,

Fig. 5. Effect of cyanide on respiration of rabbit kidney slices, showing method of obtaining inhibition curves. In this experiment (No. 5 of Table I) the tissue slices had been kept in their respective cyanide solutions for 3 hours at 0° , so as to ensure thorough penetration of the cyanide.

Fig. 6. Cyanide inhibition curves of various tissues.

in order to make the curves comparable. The curves are all of essentially the same form, but the concentration of cyanide necessary to produce maximal inhibition varies with different tissues, as also does the degree of inhibition

obtainable. In no animal tissue however is inhibition complete, however much cyanide is added.

In order to check our methods we carried out a number of experiments on yeast cells. Negelein [1925], working in Warburg's laboratory, has shown that cyanide produces a practically complete inhibition of the respiration of yeast, and our experiments entirely confirm his, showing usually a 95-98 % inhibition, as shown in the lowest curve in Fig. 6. It is clear that yeast behaves unlike animal tissues. We have, however, had one or two samples of yeast in which the inhibition was somewhat less.

It is obviously neither possible nor necessary to give the oxygen uptake curves, or even the inhibition curves, of a large number of experiments; and we have condensed the majority of our experiments into the form of a table showing the normal respiration rate and the percentage inhibition produced by various concentrations of cyanide for a number of different tissues. It should be understood that each figure in the table represents a separate oxygen uptake curve.

Table I.

Exp. No.	Tissue	Rate of O ₂ uptake without KCN (mm. ³ per g. per hr.)	Inhibition produced by			Uptake not inhibited by KCN (mm. ³ per g. per hr.)
			M/3000 KCN	M/300 KCN (in % of normal)	M/30 KCN	
1	Ox kidney	320	42	52	50	160
2	Pig kidney	400	—	41	44	224
3	Sheep kidney	1700	75	80	82	310
4	Rabbit kidney	1100	57	79	79	230
*5	"	560	65	73	72	150
6	"	220	—	—	56	96
7	Pig liver	700	57	74	74	180
8	Sheep liver	360	43	62	61	140
9	"	490	—	—	78	110
10	"	660	—	—	83	110
11	"	1800	—	—	91	160
12	"	1500	—	—	89	160
13	"	700	—	—	77	160
*14	"	900	—	—	87	120
	"	800	—	—	84	130
15	Rat liver	540	29	64	65	190
16	"	860	—	—	65	300
*17	"	490	33	51	59	200
*18	"	760	48	69	74	200
*19	"	680	—	—	66	230
*20	"	690	4	42	69	210
*21	"	600	—	42	—	—
*22	"	500	—	48	—	—
*23	"	600	—	—	61	233
	"	580	—	—	56	255
*24	Rabbit liver	660	—	—	75	165
*25	"	340	—	49	70	100
26	"	580	—	—	76	140
27	"	1200	—	—	88	140
*28	"	1460	—	—	82	266
	"	1800	—	—	89	195
*29	Ox liver	310	16	25	54	140
30	"	360	25	—	—	—
31	"	470	—	—	66	160
*32	"	460	—	—	69	142
	"	460	—	—	74	120

Table I (*continued*).

Exp. No.	Tissue	Rate of O ₂ uptake without KCN (mm. ³ per g. per hr.)	Inhibition produced by			Uptake not inhibited by KCN (mm. ³ per g. per hr.)
			$\frac{M}{3000}$ KCN	$\frac{M}{300}$ KCN (in % of normal)	$\frac{M}{30}$ KCN	
33	Sheep heart	235	24	44	44	130
34	Ox heart	1450	36	71	83	245
35	Rabbit muscle	130	39	62	69	40
36	"	220	—	—	74	56
37	Rat muscle	400	41	78	84	65
38	"	270	—	—	63	100
*39	Fish muscle	20	30	50	50	10
40	Ox pancreas	90	33	40	25	54
*41	Sheep thymus	60	17	55	57	26
42	Ox spleen	300	0	0	33	—
43	"	110	0	50	43	55
44	Pig spleen	170	0	62	53	65
45	Sheep spleen	100	0	55	—	—
46	Rabbit brain	700	40	76	91	65
47	Sheep brain, grey matter	600	—	—	83	100
	" white matter	210	—	—	85	32
48	Ox brain, grey matter	300	—	—	73	80
	" white matter	160	—	—	63	60
*49	Yeast	4000	—	—	85	600
*50	"	2500	—	—	98	50
*51	"	4500	—	—	94	290
*52	" "fed"	36000	78	92	92	2800
*53	" "	18000	44	83	83	3000
*54	" "	41000	—	—	94	1500
*55	" "starved"	1600	0	56	72	450

NOTES.

* All experiments were done at 40° except No. 39 (at 18°) and 50 (at 14°).

All experiments were done at p_H 7.3 except No. 51 (at 3.0).

All experiments were done on suspensions except Nos. 5, 17, 19–24 (on slices).

All experiments were done in buffer except Nos. 18, 20 (in Ringer).

All experiments were done on 0.5 g. tissue except Nos. 23, 28 (1st line with 0.3 g., 2nd line with 0.75 g.).

The respiration of the yeast was too rapid to permit of 0.5 g. being used, and the following amounts were taken:

Exps. 49, 51, 53, 55	...	...	...	0.1 g.
" 50	...	...	...	0.2 g.
" 52, 54	...	...	...	0.025 g.

In Exps. 5, 21, 22 the tissue slices were previously allowed to soak in the cyanide at 0° for 3 hrs., 20 mins. and 1.25 hrs. respectively.

In Exps. 25, 29, 41 the tissue had been kept some hours on ice. Exps. 14, 32 were done on the same samples fresh (1st line) and after keeping about 5 hrs. All the other tissues were absolutely fresh.

The yeast used was ordinary baker's yeast. "Fed" yeast denotes yeast which had been made up in 5 % suspension in 1 % glucose and incubated for 3 hrs. at 37°. Appropriate small amounts of such suspensions were then measured out into the respirometer flasks. "Starved" yeast denotes yeast which had been made up in 5 % suspension in buffer solution and aerated for 5 hours before taking samples.

The percentage inhibition is defined as $\frac{R_0 - R}{R_0} \times 100$, where R_0 and R are the rates of respiration in absence and presence of cyanide respectively.

The respiration is always given per g. fresh weight of tissue.

The values found for the respiration of the tissues in the absence of cyanide, given in the third column of Table I, are in good general agreement with those found by Barcroft and Shore [1912] by direct determination of the oxygen used by the organs *in situ* in the animal. For instance, they found

values for liver varying between 300 and 3000 mm.³ per hour per g. tissue (fresh weight), and for the viscera taken together they obtained values between 480 and 1080.

A number of points in Table I deserve special notice.

Excluding yeast, the part of the respiration which is sensitive to cyanide varies between about 50 % and 80 % of the whole. Only in rare cases does it reach 90 %.

The inhibition given by $M/300$ cyanide is usually the same as that given by $M/30$.

The first traces of the cyanide produce a large effect—in many cases $M/3000$ cyanide gives almost maximal inhibition. A striking exception to this statement is given by the spleen of all the species tested. In this case $M/3000$ cyanide never shows the slightest effect, and in one sample of spleen (Exp. 42) even $M/300$ did not affect the respiration rate. This strongly suggests that spleen, unlike all the other tissues tested, contains some substance which has a greater affinity for cyanide than the “respiratory enzyme,” so that the first amounts of cyanide added are taken up by this, and are thus prevented from acting on the respiratory systems. Further work is, however, required on this point.

Another point of interest is that the cyanide-resistant part of the respiration fluctuates less than the total respiration. For instance, in the first 20 experiments the respiration varied between 200 and 2000 mm.³ per hour, while the cyanide-resistant respiration, given in the last column of the table, only varied between 100 and 300. It varies however from tissue to tissue, being low particularly in skeletal muscle and spleen (compare Exps. 2 and 37, for example). It may possibly be connected with some “endogenous” aspect of metabolism, not directly affected by nutritional conditions, etc.

In view of the importance of the results obtained in the above experiments, as limiting the extent to which Warburg's theory of cell respiration can be applied to animal tissues, it is desirable to consider what criticisms may be brought against the experiments, and what possible alternative interpretations of the results can be suggested.

There is first the possibility that the gas phase in the flasks contains HCN vapour given off from the solution at the beginning of the experiment, and that the apparent oxygen absorption in presence of cyanide is in reality due to the absorption of this HCN by the KOH used for absorbing the CO₂. This possibility is excluded in our experiments by the following considerations. (a) The same amounts of cyanide and KOH are present in both flasks of each Barcroft apparatus, and therefore such an uptake, even if it occurred, would not affect the readings of the manometer. (b) A control experiment was carried out without tissue, and with $M/30$ cyanide in the right-hand flask of the apparatus only, the left containing buffer solution. KOH on filter-paper was present as before. No movement of the manometer occurred, either in the direction of evolution or absorption. (c) The rate of uptake in

presence of cyanide is exactly proportional to the amount of any given tissue added; it was found that 0.6 g. of liver gave an absorption rate twice as great as 0.3 g. The rate is, however, different for different tissues. It is therefore clear that the effect is actually an uptake by the tissue.

The second possibility of error, arising out of the first, is that the cyanide has left the solution as HCN and been absorbed by the KOH in much the same way as the CO_2 produced by the tissue. This possibility is disposed of by the fact that at p_{H} 7.5 $M/3000$, $M/300$, $M/30$ and $M/3$ cyanide usually give the same degree of inhibition. The inhibitory effect of cyanide is reversible (see below), so that if the cyanide were being removed it should disappear from the $M/3000$ solution first and there should be no inhibition in this apparatus, and the inhibition should disappear from the others in turn. We have actually observed this effect in two experiments done in acid solutions. When it does take place, the indications are unmistakable. In these experiments the inhibition in the apparatus to which $M/3000$ cyanide was added was negligible. The uptakes with $M/300$ and $M/30$ both started at a rate corresponding to about 60 % inhibition, but while the one with $M/30$ remained at this rate that with $M/300$ gradually accelerated until its rate was the same as that without cyanide. These effects, which are clearly due to the removal of the cyanide from the solutions, only occur in acid solutions, and we have never observed such gradually increasing velocities at p_{H} 7.3, even with $M/3000$ cyanide. It may be mentioned that at the end of our experiments the odour of cyanide is detectable in the flasks with $M/3000$, strong in those with $M/300$, and very strong in those with $M/30$.

The next possibility is that the uptakes observed in presence of cyanide are due not to respiration but to oxidation of the cyanide itself. It has been stated by Ellinger [1924] that this reaction may occur in tissues under certain conditions. This, however, cannot be the case in our experiments, as the amount of cyanide present is quite insufficient to account for the uptakes observed. Consider for instance Exp. 3 in Table I. This experiment was continued for more than 2 hours, during which time the flask containing the tissue in $M/300$ cyanide absorbed over 340 mm.³ Even if the whole of the cyanide were oxidised the amount of oxygen taken up would only be 112 mm.³ Moreover, if the cyanide were being removed by such oxidation, the inhibition would tend to disappear, and, as has just been mentioned, this is never observed. In Exp. 3 the rates, even with only $M/3000$ cyanide, remained unchanged after 2 hours. In a few experiments, however, such as Nos. 40, 43, 44 in Table I, we found that the oxygen uptake was slightly greater in $M/30$ cyanide than in $M/300$. We are inclined to attribute this effect to a slight oxidation of the cyanide. With the majority of tissues however it does not occur.

The form of the curves disposes also of the objection that the cyanide may not penetrate into the tissue sufficiently to exert its full effect. For the first traces of cyanide exert an immediate marked action, and the degree of

the inhibition produced does not increase with time. In order to make quite certain, however, a few experiments were carried out in which the tissue slices were allowed to soak in the cyanide solutions for some time before measuring the respiration rate. These are indicated in Table I, which shows that no effect was observed.

The possibility that the cyanide does not inhibit more completely because it is preferentially combined with substances other than the "respiratory enzyme," or because the complex which it forms with the latter is partially dissociated, is again negated by the fact that the first traces usually produce the maximum inhibition and a further increase in concentration of a hundred times does not increase the inhibition. Such effects, however, are probably playing a part in those cases where maximal inhibition is not reached with a concentration of $M/300$.

A more serious criticism, if justified, is that our measurements of the respiration rate in the absence of cyanide, since this is much higher than that in the presence of cyanide, may in fact have been limited by the rate at which oxygen could diffuse from the gas into the liquid, so that the true respiration rate, and therefore the percentage inhibitions, are in reality greater than those given. The question of diffusion effects will be fully dealt with in a forthcoming paper, where it is shown that the apparatus is capable of dealing with much more rapid uptakes without errors due to this cause. We have, however, carried out one or two further experiments with animal tissues which definitely disprove this criticism in the case of the experiments given above. In the first place we have found that increasing the amount of tissue taken does not affect the degree of inhibition produced by $M/30$ cyanide, as shown in Exps. 23 and 28 in Table I¹. This result would not have been obtained had the rate of uptake been limited by the apparatus; for increasing the amount of tissue would not increase the normal uptake if this were limited by diffusion, while the uptake with cyanide, being much smaller, would not be limited thus and would increase with the amount of tissue. Thus, in such a case the more tissue taken the less would be the degree of inhibition observed. This is not the case.

Another experiment, represented in Fig. 7, was carried out by varying the amount of tissue taken and measuring the oxygen uptake in the absence of cyanide. The results showed that the rate of uptake was exactly proportional to the amount of tissue taken, so that diffusion effects could certainly be neglected, provided the rate was not much greater than the maximum rate represented on the curve, *i.e.* about 800 mm.³ per hour. That is to say, in Table I, where 0.5 g. of tissue was normally employed, the figures give true values in all cases where the uptake does not exceed 1600 mm.³ per g. per hour. As a matter of fact the maximum rate is probably much greater than this. We found also that both the rate of shaking and the partial pressure

¹ The slight differences are within the experimental error, and, in the case of the more rapid uptake, in the opposite direction.

of oxygen in the apparatus could be widely varied without affecting either the respiration rate or the degree of inhibition produced by cyanide (see the results in Table II).

Finally it may be objected that the tissue is not under normal conditions in the experiments. To this we will only say that a slice of perfectly fresh tissue, cut with a razor moistened with Ringer's solution, and suspended in

Fig. 7. Observed rates of oxygen uptake with varying amounts of rat liver in the absence of cyanide.

Fig. 8. Effect of higher concentrations of cyanide on respiration of rabbit liver.

this solution with free access of oxygen, is probably as close an approximation to the normal tissue as it is possible to get in experiments of this type; and the slight variations from these conditions which we have made have been shown to have no effect on the results.

A few other experiments on the effect of cyanide remain to be considered. Fig. 8 shows an experiment in which the cyanide solutions were 10 times the normal strength. Even with $M/3$ cyanide no greater inhibition is produced.

Fig. 9 shows that the cyanide effect is reversible: on removing the cyanide by washing, the inhibition disappears and the respiration returns to its former rate. The reversibility of cyanide inhibition is of course well known.

Fig. 9. Reversibility of cyanide inhibition. Thin slices of rat liver were cut with a razor moistened with Ringer. Three portions of 0.5 g. were taken. *A* was placed in 2.5 cc. buffer solution, *B* and *C* in 2.5 cc. buffer containing *M*/30 cyanide, and all kept at 0° for 1 hour. Then the slices were taken out, quickly rinsed with buffer solution, and lightly dried with filter-paper. *A* and *C* were then immediately placed in 2.5 cc. fresh buffer in respirometers, *B* in 2.5 cc. buffer containing *M*/30 cyanide, and the oxygen uptakes measured.

The effect of increasing and diminishing the partial pressure of oxygen in the flasks is shown in Table II. The flasks were filled with mixtures of oxygen and nitrogen in different proportions, the percentages of oxygen being as indicated in the third column of the table.

Table II. *Effect of oxygen pressure.*

Exp. No.	Tissue	O ₂ %	Rate of oxygen uptake (in mm. ³ per g. per hr.) in presence of			Inhibition %
			No KCN	<i>M</i> /3000 KCN	<i>M</i> /30 KCN	
1	Rabbit liver	20	620	—	170	72
		100	600	—	180	60
2	Sheep liver	20	490	—	110	77
		100	500	—	120	76
3	"	7	660	—	125	81
		20	660	—	140	79
4	Ox liver	7	340	270	—	21
		20	350	270	—	23

As the figures show, wide variations of oxygen pressure in both directions produced no alteration in either rate of oxygen uptake or degree of inhibition. Exp. 4, in which only enough cyanide was added to produce a rather slight inhibition, shows that there is no competition between the oxygen and the cyanide.

A few determinations of the effect of cyanide on the respiratory quotient were made by the following method. Four respirometers were used with the same amount of tissue in all. One pair of these had KOH on filter-paper to absorb the CO₂; in the other pair this was omitted. To one of each pair *M*/30 KCN was added, the other two being without cyanide. The pair without

KOH were provided with small tubes which could be upset into the solution. These contained strong oxalic acid, and were upset at the end of the experiment in order to drive off CO_2 from the buffer solution. The reading of the first pair of respirometers at the end of the experimental period gave of course the oxygen used with and without cyanide. The differences between the readings of the corresponding members of the first and second pair gave the amount of CO_2 evolved in the presence and absence of cyanide. From this, however, the amount of CO_2 already present in the tissue and buffer at the beginning of the experimental period had to be subtracted. This was determined by setting up a third pair of respirometers, exact duplicates of the second pair, in which the oxalic acid was upset at the beginning of the experiment. The amount of CO_2 thus liberated was then subtracted from the gross amount given by the second pair, to give the amount actually produced during the experimental period. Using this technique it was found that the respiratory quotient was always lowered by the addition of cyanide. For instance, the value found for slices of rat liver in the absence of cyanide was 0.8, and with $M/30$ KCN this fell to 0.64.

Fig. 10. Effect of pyrophosphate on respiration of two different samples of rat liver.

In addition to the experiments described above on the effect of cyanide on cell respiration, experiments were also performed using pyrophosphate instead of cyanide. Pyrophosphate acts as an inhibitor in many iron-catalysed reactions, but according to Keilin [1929] does not inhibit the "respiratory enzyme." In all experiments the pyrophosphate was freshly made up and adjusted to p_{H} 7.3 before use. In Fig. 10 are given two inhibition curves showing the effect of neutral pyrophosphate on rat liver. The inhibition is much less than with cyanide, as was expected, and a much higher concentration is required before any inhibition appears. These results are typical of liver. Muscle, however, behaves differently in that pyrophosphate is actually considerably stronger than cyanide as an inhibitor, as shown in Fig. 11. With yeast pyrophosphate has not the slightest effect. It may, however, be unable to pass through the cell membrane into the interior of the yeast cells.

Fig. 11. Effect of pyrophosphate and cyanide on respiration of rabbit muscle.

In Table III are summarised the results of a number of comparative experiments showing the relative effects of $M/30$ pyrophosphate and $M/30$ cyanide on the respiration of a number of tissues. The effect of adding pyrophosphate and cyanide simultaneously is also shown.

Table III.

Exp. No.	Tissue	Rate of O_2 uptake by tissue alone (mm. ³ per g. per hr.)	Percentage inhibition with		
			$M/30$ Pyroph.	$M/30$ KCN	$M/30$ Pyroph. + $M/30$ KCN
1	Rat liver	600	40	—	—
2	"	560	30	—	—
3	Ox liver	470	28	66	74
4	" fresh	460	30	69	80
	" kept	460	30	74	85
5	Sheep liver	1800	75	91	95
6	" fresh	900	64	87	91
	" kept	800	60	84	90
7	"	1500	70	89	93
8	Rabbit muscle	220	87	74	95
9	Rat muscle	270	67	63	82
10	Yeast	4000	0	85	85
11	"	4500	0	94	93

In order to determine whether the brief delay, unavoidable in some cases, between the death of the animal and the beginning of the respiration measurements had any effect, several comparative experiments were made between absolutely fresh tissues and portions of the same tissues which had been kept at room temperature for some hours before slicing or mincing. Fig. 12 and Exps. 4 and 6 of Table III are such experiments, and they show that the effects of a short delay can be neglected.

The inhibiting effect of pyrophosphate, like that of cyanide, is reversible, as the experiment represented in Fig. 13 shows.

The results given in Table III seem to give indications of some degree of parallelism between the cyanide and pyrophosphate inhibitions, at any rate with a given kind of tissue. For instance, in the seven samples of liver for

which both the inhibitions are given, the order of sensitivity of the samples is the same for pyrophosphate as for cyanide. It is not clear why this should be so, since it is commonly assumed that the effect of cyanide is due to its action on the "respiratory enzyme" (indophenol oxidase), whereas pyrophosphate has no action on this, so that they would be acting on quite different

Fig. 12. Effect of pyrophosphate and cyanide on ox liver, fresh and after keeping 5 hours. A, tissue alone; B, tissue + $M/30$ pyrophosphate; C, tissue + $M/30$ cyanide; D, tissue + $M/30$ pyrophosphate + $M/30$ cyanide. Curves numbered 1 on fresh tissue, those numbered 2 on kept tissue.

Fig. 13. Reversibility of pyrophosphate inhibition. The experiment was done on rat liver slices in exactly the same way as that given in Fig. 9, using $M/30$ pyrophosphate instead of $M/30$ cyanide.

systems. It has not, however, been definitely established that the inhibiting action of cyanide on respiration is entirely due to its action on the "respiratory enzyme," for it is known to inhibit other oxidation catalysts in the cell, and it is not unlikely that some of the systems inhibited by pyrophosphate will also be inhibited by cyanide.

It is, however, certain that pyrophosphate acts upon certain systems which are not poisoned by cyanide, at any rate in muscle, where the inhibition it

produces is greater than the maximum cyanide inhibition. One such system is the succinodehydrase, as shown in the experiment represented in Fig. 14. This is an unpublished experiment carried out by Dixon and Thurlow in 1925. While $M/1000$ pyrophosphate had a definite inhibiting action on the reduction of methylene blue by this system, it was found that cyanide, even up to $M/50$, had absolutely no effect on the reaction. On the other hand, even $M/25$ pyrophosphate had no effect on the reduction of methylene blue by the xanthine oxidase system.

Fig. 14. Effect of pyrophosphate on rate of reduction of methylene blue by succinoxidase. Each Thunberg tube contained 2 cc. succinoxidase solution + 0.5 cc. $1/5000$ methylene blue + 0.2 cc. (= 0.4 mg.) succinate + 1.3 cc. phosphate buffer p_H 7.6 containing the necessary amount of pyrophosphate. "Rate of reduction" = $10,000 \times$ reciprocal of reduction-time in minutes.

DISCUSSION.

We consider that the experimental evidence presented in this paper shows quite definitely that the respiration of typical animal tissues is made up of two parts, one of which is inhibited by cyanide while the other is cyanide-stable; and that, while the relative proportion of the two parts varies in different tissues, the latter usually contributes a considerable part, sometimes more than half, to the total respiration. Thus, a considerable part of the respiration must be due to systems which are not poisoned by cyanide. As all Warburg's mechanisms are cyanide-sensitive, his theory can no longer be regarded as able to account for the whole of the respiration of animal tissues; and it can no longer be maintained that all oxidations by molecular oxygen in the cell are brought about by the same type of mechanism.

In the light of this evidence Warburg's remarks quoted in the introduction to this paper are seen to lose much of their force; and in particular this applies to his argument that those tissue oxidases which are unaffected by cyanide cannot be regarded as respiration systems because they behave unlike respiration, since it is now clear that only one part of the respiration behaves in the manner referred to. Such cyanide-stable systems as the xanthine

oxidase may well contribute to the cyanide-stable part of respiration. Of course the xanthine oxidase itself cannot account for the whole of this part (it is altogether absent from muscle [Morgan, 1926]), and it seems likely that there are a number of other systems of this type.

The recent important work of Keilin [1929] has shown that the "respiratory enzyme" is to be identified with the well-known indophenol oxidase of tissues. Cyanide, CO, etc., act upon this, and the inhibition of respiration is due, in part at any rate, to the poisoning of this enzyme. It acts by oxidising cytochrome, which is in turn reduced by the dehydrase systems. Our experiments show that the indophenol-oxidase-cytochrome mechanism likewise cannot account for the whole respiration of animal tissues. They do not of course show how much of the total respiration is actually due to this mechanism, they merely set an upper limit to its action; for it is not yet established that this system is responsible for the whole of the cyanide-sensitive respiration of animal tissues, and the cyanide may act upon other systems as well.

SUMMARY.

1. A study has been made of the effect of various concentrations of cyanide on the rate of respiration of a number of animal tissues.

2. In most cases a cyanide concentration of $M/1000$ is sufficient to produce the maximum degree of inhibition obtainable. Above this point no greater inhibition is produced by increasing the cyanide concentration 100 times.

3. The maximum inhibition varies in different tissues between 40 % and 90 %, the average value being about 60 %.

4. It is concluded that the respiration of animal tissues is made up of two parts. One, accounting for about two-thirds of the total, is due to systems poisoned by cyanide; the other one-third is due to systems which are stable to cyanide.

5. Warburg's "respiratory enzyme" (Keilin's cytochrome-indophenol-oxidase system) can therefore only account at most for about two-thirds of the total respiration of animal tissues.

6. The respiration of spleen, unlike that of all the other tissues tested, is not affected by small amounts ($M/1000$) of cyanide. It appears to contain some substance with a greater affinity for cyanide than the "respiratory enzyme."

7. Negelein's statement that the respiration of yeast is almost completely inhibited by cyanide is confirmed. Some samples of yeast, however, show an incomplete (85 %) inhibition.

8. The effect of pyrophosphate on the respiration rate of liver, muscle and yeast has also been studied. With liver, small concentrations produce a slight acceleration, but larger amounts produce inhibitions which vary between 30 % and 70 % but are always considerably less than the corresponding

cyanide inhibitions. With muscle, on the other hand, pyrophosphate produces a greater inhibition than cyanide. Pyrophosphate produces no effect on yeast: possibly the cell-membranes are not permeable to it.

9. Since the "respiratory enzyme" is not inhibited by pyrophosphate, the inhibition of respiration must be due to its action on some other system. It is shown that pyrophosphate inhibits the reduction of methylene blue by the succinoxidase system, though this reaction is unaffected by cyanide.

10. The actions of both cyanide and pyrophosphate on respiration are reversible.

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