Anaerobiosis in the *Rana pipiens* Embryo

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INTRODUCTION

BRACHET (1934, 1950) has proposed that the oxidation source for the anaerobic development of the early amphibian embryo is primarily from a pool of materials such as oxidized glutathione. Inasmuch as an investigation of the defective lactate producing ability of hybrid embryos (Barth, 1946) was contemplated, it seemed desirable to reinvestigate the problem of the source of anaerobic oxidations in this embryo. This was particularly necessary since, as will be discussed later, amphibian embryos in normal development may always be partly anaerobic and reasonable alternate interpretations of Brachet's data were possible.

Brachet's findings may be summarized briefly as showing (1) a production of carbon dioxide during anaerobiosis without diminution and indeed with some increase of tissue bicarbonates, and (2) a short burst of heightened oxygen uptake during return to oxygen with an R.Q. of about 0·3–0·5.

The low R.Q. appeared to Brachet to rule out oxidation of lactate and he attributed this to the regeneration of an oxidizable reserve that had been reduced during anaerobiosis. It is conceivable, however, that in the presence of substantial amounts of lactate the turnover of lactate to pyruvate might be so rapid as to exceed any disposal of the pyruvate to carbon dioxide or other metabolites. This pyruvate formation involves necessarily the reduction of DPN. Accordingly, a good part of the oxygen uptake might be due to the reoxidation of DPN. Since such reoxidation is believed to involve the generation of high-energy phosphate bonds as ATP (Ochoa & Stern, 1952), this reoxidation would constitute a partial payment of the so-called 'oxygen debt' incurred during the utilization of fermentative degradation of glycogen. Since this reoxidation of DPN does not involve the production of carbon dioxide, a low R.Q. is to be expected.

The gross outlines of the glycolytic system of *Rana pipiens* embryo have been described (Cohen, 1954) and lactic dehydrogenase activity is present. Accordingly the above hypothesis has a real basis and can be tested by concurrently following respiration and lactate disappearance during recovery from anaero-

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biosis. If the CO_2 required for complete oxidation of the disappearing lactate is in excess of correspondence to the appearing CO_2, then the low R.Q. would follow of necessity, since the conversion of lactate to pyruvate is, as far as is known, the first step leading to any disposal of this material.

The question of whether the amphibian embryo, raised in air, can meet the oxygen needs of metabolism in its deeper tissues is an important one. If the deeper tissue layers are forced to utilize anaerobic metabolism to any extent, then intracellular changes will ensue which could conceivably 'trigger' cell differentiations. On the other hand, this metabolic gradient may have no effect whatsoever on differentiation. In such a case the search for 'triggers' may be directed elsewhere. The question of the extent of tissue anaerobiosis during aerobic amphibian embryo development will be discussed, particularly with reference to the observations (Heatley & Lindahl, 1937; Jaeger, 1945) of glycogen disappearance in the material invaginated during amphibian gastrulation.

MATERIAL AND METHODS

Rana pipiens eggs were obtained after pituitary injection and, after fertilization, the jelly was removed with forceps and the embryos subsequently kept under conditions designed to minimize the effects of crowding. These consisted of keeping small numbers of embryos in finger-bowls and also providing gentle aeration by means of an aquarium pump. All embryos were raised at 20° C. and experiments also performed at this temperature.

For the measurement of lactic acid the procedure of Barker & Summerson (1941) was used and the embryos handled as described in our previous paper (Cohen, 1954).

Respiration measurements and respiratory quotients were obtained by use of Warburg respirometers and, in general, the procedure of Barth (1946) was followed. Nitrogen was purified by passage over hot copper and was then washed and cooled. Embryos were rendered anaerobic by flushing the Warburg vessels containing them with nitrogen for 15 minutes. In these experiments either 15-ml. Warburg vessels (with wells and sidearms) or similar 10-ml. vessels were employed. In the larger vessels there were 90 embryos in 3 ml. of 1/10 Ringer's, in the smaller 60 embryos in 2 ml. Similarly, either 0·13 ml. or 0·08 ml. of 20 per cent. KOH was used in the well for trapping carbon dioxide, and either 0·13 ml. or 0·08 ml. of 4·8 M sulphuric acid in the sidearm was used for discharging bicarbonate.

When switching from nitrogen to air, as in the first experiments to be reported, flasks were removed from manometers and reattached after 15 minutes. In the later experiments flasks remained on the manometers and were flushed with oxygen for 2 minutes.

All stages referred to are those of Shumway (1940).

EXPERIMENTAL RESULTS

I. *General picture of recovery from anaerobiosis*

Embryos of late blastula or gastrula stages were exposed to anaerobic conditions for 8–12 hours and then transferred to air for 15 minutes. After reattachment of the flasks, recovery respiration was followed for 5 hours. The three-manometer method of Barth (1946) was used for measuring recovery respiration and R.Q. levels were calculated. Lactate was measured initially, at the termination of anaerobiosis (transition time), and at the end of the five recovery hours. Results are given in Table 1.

TABLE 1

Recovery from anaerobiosis

Stage of embryos	*8*	*9*	*10*	*12*
Recovery* lactate disappearance (micromoles/90 embryos/5 hours)	1·50	1·47	1·14	1·9
Equivalent O_2 or CO_2 for complete oxidation of above lactate (mm.^{3})	100·1	100·8	76·8	129·9
Actual O_2 consumed (5 hrs., mm.^{3})	38·7	33·1	29·6	78·2
Actual CO_2 evolved (5 hrs., mm.^{3})	23·3	14·0	15·0	65·4
Recovery R.Q.	0·6	0·4	0·5	0·8

* 12 hours of anaerobiosis.

These results show that more lactate disappears than is accounted for by either the oxygen uptake or evolved CO_2 during the recovery period. This tends to support the hypothesis that the oxidative reserve being oxidized during recovery is the DPN reduced via pyruvate formation. The R.Q. is low, confirming Brachet's results (1934), but the levels were somewhat above his levels (*circa* 0·3). It was decided to repeat the experiment in detail with greater resolution of the changes at transition and to switch from anaerobiosis by flushing the attached flasks with oxygen for 2 minutes.

II. *Detailed picture of recovery from anaerobiosis*

In detail, the method involved the use of 10 groups of 60 embryos, employed as follows:

Group A gave initial bound CO_2 by tipping in acid.

Group B was used for an initial lactate level.

Groups C and D were observed for gas exchange during 7 hours of anaerobiosis. At the end of this time acid was tipped into C while D was used for a lactate determination.

Groups E and F were anaerobic for 7 hours and gas exchange noted. They were flushed with oxygen and resealed. After 20 minutes acid was tipped into E, while F was used for a lactate level.

Groups G and H were treated as were E and F, save for being sampled after 90 minutes of recovery.

Group I had an air atmosphere and KOH in its flask well. After 7 hours, during which gas exchange was noted, its flask was flushed with oxygen and resealed. Oxygen uptake was followed for 90 minutes. The embryos were then sampled for lactate.

Group J had KOH in its flask well and was anaerobic for 7 hours. Gas exchange was noted. After flushing its flask with oxygen, gas exchange was followed for 90 minutes and the embryos were then sampled for lactate.

Thus, under anaerobic conditions, the sum of the final bound CO_2 and the CO_2 evolved during the run, less the initial bound CO_2, gives the 'new' CO_2 appearing during anaerobiosis. Under aerobic conditions, after correcting for bound CO_2 changes, CO_2 is the measured difference in gas exchange between flasks having KOH in their centre wells and those lacking this CO_2 absorbing material.

TABLE 2

Typical protocol of anaerobiosis and recovery. Stage 12 (yolkplug) embryos, 60 embryos per group

Nitrogen atmosphere			
Gas exchange (7 hours)	+35·6 mm.³	Lactate present	1·63 μmoles
Transition bound CO_2	+17·1		
	+52·7		
Less initial bound CO_2	36·8	Less initial lactate	0·11
'New' CO_2	+15·9	'New' lactate	1·52
Nitrogen to oxygen			
20 min. bound CO_2	+19·2	20 min. lactate	1·22
'New' bound CO_2	+2·1	Less initial lactate	0·11
20 min. O_2+CO_2	−10·9		1·11
Less 20 min. O_2	−15·4	Lactate loss	0·41
20 min. CO_2	+6·6		
20 min. R.Q.	0·4		
90 min. bound CO_2	+17·1	90 min. lactate	0·72
'New' bound CO_2	0·0	Less initial lactate	0·11
90 min. O_2+CO_2	−19·5		0·61
Less 90 min. O_2	−29·4	Lactate loss	0·91
90 min. CO_2	+9·9		
90 min. R.Q.	0·5		
Air Atmosphere			
O_2 uptake (7 hours)	−66·7	Lactate present	0·20
Air to oxygen			
20 min. O_2	−13·6		
90 min. O_2	−30·6	90 min. lactate	0·17

Table 2 shows the detailed results for one of four experiments giving essentially similar results. Many individual features of these experiments were confirmed in numerous pilot experiments. Table 3 shows the data for the oxygen

uptake on transfer from *either air or nitrogen* to an oxygen atmosphere. It is evident that there is indeed a burst of oxygen uptake on transfer and also that *there is no significant difference between embryos going from air or nitrogen to oxygen.*

TABLE 3

Respiratory response of gastrulae placed in oxygen

	20 minutes		*90 minutes*	
Expt.	*Air*—O_2	N_2—O_2	*Air*—O_2	N_2—O_2
A	−13·1 mm.³	−13·8 mm.³	−24·2 mm.³	−21·8 mm.³
B	−11·3	−10·5	−21·2	−18·5
C	−13·6	−15·4	−30·6	−29·4
D	−16·5	−16·9	−25·0	−26·3

If the burst of oxygen uptake were due to complete oxidation of lactate, the R.Q. should have been about 1·0, but, in confirmation of Brachet (1934), it is about 0·3. An hypothesis of partial oxidation would find the data for the embryos going from nitrogen to oxygen consistent, but would fail to explain an uptake burst of similar magnitude for embryos going from air to oxygen. The R.Q. tends to rise after the burst, but, as Table 1 showed, was still low (normal = 0·87) for some time. Again in confirmation of Brachet is the finding of some CO_2 produced during anaerobiosis but not attributable to bicarbonate discharge. The level of this 'new' CO_2 roughly corresponds to the level of excess oxygen uptake during the burst. This tends to confirm Brachet's conjecture that an oxidizable reserve exists. Moreover, 0·7 of a micromole of 'new' CO_2 reflects an oxidizing potential equal to 2·1 micromoles of lactate and Table 2 shows but 1·6 micromoles of this material. Accordingly this reflects a source of anaerobic oxidations at least equal to lactate production. The fact that the burst on going from air to oxygen is equal in magnitude to that on going from nitrogen to oxygen, suggests that this is a more immediate source of oxidations than lactate production. It is also possible that fermentation involves the cleavage of sugar to lactate, CO_2, and some other fragment, but inasmuch as examples of such heterolactic fermentation are thus far limited to micro-organisms, this hypothesis to explain 'new' CO_2 should await elimination of more conventional hypotheses.

DISCUSSION

Since a burst of oxygen uptake occurs on transferring embryos from air to oxygen, it follows that after the material responsible for this burst is in the reduced state, it can no longer serve as a source of anaerobic oxidations. It will be necessary to precisely locate the time when this reduction takes place for embryos raised in air. Lactate production, of course, still remains a substantial means of anaerobic oxidations and, as Table 1 shows, the payment of the oxygen

debt for lactate is a gradual process, the extent of which will depend on the embryo's energy needs and the efficiency of DPN reoxidations. The rapid disappearance of lactate during recovery from anaerobiosis, however, permits the expectation of a low R.Q. during this process.

Frog embryos, on crowding, accumulate lactic acid. This suggests that the embryos are normally close to partial anaerobiosis. Moreover, glycogen loss first becomes apparent during gastrulation (Brachet & Needham, 1935; Gregg, 1948) and, in fact, disappears from the material being invaginated (Jaeger, 1945). It is of interest, therefore, to consider whether such loss indicates that the metabolism of the invaginated material is oxygen limited.

In the absence of a circulatory system, the oxygen supply to the forming embryonic tissues of the gastrula is dependent on diffusion. Estimation of whether diffusion could supply oxygen to the invaginated material is quite difficult inasmuch as (1) the tissue is not homogenous, and (2) the respiration is not uniform, (3) the oxygen concentration at the gastrula surface will be changing if the medium is static or unchanging if the medium is in motion, and (4) the rate of diffusion of oxygen through the tissue is unknown.

It is possible to show by a few considerations of size that, at the very least, the dimensions of a frog gastrula must place the embryo on the borderline of anaerobiosis at the usual temperatures employed in physiological experiments on amphibian embryos (*circa* 18–20° C.). In the following discussion we will assume a uniform respiration of 0·162 microlitres per mg. per hour (Sze, 1953) or $2{\cdot}7 \times 10^{-3}$ ml. per g. per minute. We shall also assume as the diffusion constant the figure which Krogh (1941) gives for 15 per cent. gelatin at 20° C., $2{\cdot}8 \times 10^{-5}$ ml. per minute (through a volume of 1 ml. at 1 atmosphere). For comparison, water is $3{\cdot}4 \times 10^{-5}$ and muscle $1{\cdot}4 \times 10^{-5}$ ml. per minute.

If we now make the assumption that the gastrula is a sphere of 0·25 cm. in diameter and of uniform composition, then the external oxygen concentration necessary for the sphere centre to have a zero concentration is given by the equation of Harvey (1928). The sphere radius is R, the respiration A, and the diffusion constant D.

$$C_0 = \frac{AR^2}{6D} = \frac{2{\cdot}7 \times 10^{-3} \times 0{\cdot}0156}{2{\cdot}8 \times 10^{-5} \times 6} = 0{\cdot}26 \text{ atmospheres.}$$

This value, of course, exceeds the concentration of oxygen in air.

Another approach involves the use of Warburg's equation for calculating the maximum thickness that a tissue slice may have if diffusion of oxygen is not to limit respiration (in Umbreit *et al.*, 1949). Since a tissue slice receives oxygen from two surfaces while the tissue comprising the archenteron roof to the embryo surface receives oxygen from one surface only, the latter must be compared to a tissue slice of twice its thickness. Thus, if a fair estimate of the supra-archenteron material is 0·05 cm., it must be compared to a tissue slice of 0·1 cm. thickness.

In the Warburg equation

$$d = (8C_O D/A)^{\frac{1}{2}},$$

where d is the limiting thickness, D the diffusion constant for oxygen in ml. per minute, A the respiration in ml. per gramme per minute, and C_0 the external oxygen concentration in atmospheres. Substituting,

$$d = \left(\frac{8 \times 2{\cdot}8 \times 10^{-5} \times 0{\cdot}2}{2{\cdot}7 \times 10^{-3}}\right)^{\frac{1}{2}} = 0{\cdot}044 \text{ cm.}$$

Thus the calculated critical thickness is less than the actual.

Since the Harvey equation is, in fact, a modified version of the Warburg equation, the same assumptions of diffusion theory are involved. A reader of this paper has called our attention to a more modern approach by Linderstrøm-Lang (1947). The latter's equation (17) considers the ratio of oxygen concentration at the centre of a sphere to outside concentration. Since respiration is herein treated as a function of volume rather than mass, for a 1·3-mg. dry-weight gastrula of radius 0·125 cm. the respiration at 20° C. would be 21×10^{-5} cm.^{3} per hour. Substituting gives a ratio of 0·66. This would hardly seem limiting. The disparity between these approaches is obvious, but Linderstrøm-Lang does not state specifically why his approach yields more accurate results or even different results. If he is correct, then either the embryo is not anaerobic or it is so for reasons other than size. If the Warburg type of approach is valid, then the dimensions of a gastrula (developing at 20° C.) are sufficient to make the oxygen supply critical.

By assuming that glycogen utilization would either be expressed as oxygen uptake or lactate production and that the R.Q. of the gastrula was 1·0, we were able to show (Cohen, 1954) that no combination of oxygen uptake or accumulating lactate can account for the amounts of glycogen reported to disappear during gastrulation (Jaeger, 1945). This seemed to suggest that glycogen utilization was not associated with either an increased energy demand or a less efficient use and accordingly greater consumption of glycogen by anaerobic tissue. Since CO_2 production does not exceed the O_2 uptake at this time (i.e. the R.Q. is less than 1·0), the participation of an oxidizable reserve in the oxidation of the glycogen would not appear to alter these interpretations. Recently Gregg & Ornstein (1953) attempted to find glycogen loss in explanted chorda-mesoderm kept for 24 hours under anaerobic conditions (during which time controls went through gastrulation), but failed to do so. From the same authors earlier (Ornstein & Gregg, 1952), data on anaerobic CO_2 evolution (25° C., 24 hours) from explants, one might expect a loss of 7–8 per cent. of the glycogen of the explants. The data seem to support the concept that glycogen does not disappear simply because the invaginated tissue is anaerobic. These anaerobic glycogen loss experiments, however, were performed at a lower temperature.

Since cell differentiations may be 'triggered' by environmental gradients, the

above considerations are of importance to embryologists. There is clearly substantial evidence for regarding oxygen as being limiting for some portions of amphibian gastrulae.

SUMMARY

1. Evidence is presented demonstrating that although a short burst of oxygen uptake follows transfer of *Rana pipiens* embryos from a nitrogen to an oxygen atmosphere, a similar burst follows transfer from air to oxygen. Since the material being oxidized exists in the reduced state in gastrulae raised in air, it cannot constitute a major source of anaerobic oxidations in gastrulae. Data and considerations suggesting that the amphibian embryo is normally partially anaerobic in the usual temperature range employed in physiological experiments are presented.

2. A detailed description of the respiratory and lactate level changes of embryos transferred to an oxygen atmosphere after anaerobiosis is given and the significance of the findings is discussed.

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