Anaerobiosis in the segmenting eggs of *Bufo arenarum*

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In the last few years our interest has been devoted to the energy metabolism of the eggs of the common toad *Bufo arenarum* Hensel which, like some other amphibian eggs, can cleave at a normal rate in the absence of oxygen or in the presence of cyanide (Barbieri & Legname, 1957). Under anaerobic conditions a rapid accumulation of lactic acid gives evidence of an intense glycolytic activity, which is inhibited when the eggs are returned to oxygen (Pasteur effect) (Barbieri & Salomón, 1963). Furthermore, an increase in oxygen uptake during the first 2 h of recovery has been observed (payment of the oxygen debt) (Legname, 1966). Taking into account the low value of the respiratory quotient (R.Q. = 0.5–0.7) during this period it can be assumed that most of the oxygen is not involved in the oxidation of lactate (Legname, 1966). A similar respiratory response has already been described in *Rana fusca* (Brachet, 1934) and *R. pipiens* eggs (Cohen, 1955), and has served to support the idea of an ‘oxidative reserve’ in the eggs of these vertebrates (Brachet, 1934).

An alternate interpretation to explain the use of the oxygen absorbed during the payment of the respiratory debt has been proposed by Cohen (1955). Considering from a kinetic point of view the substantial amounts of lactate produced at the end of anaerobiosis, the reaction at the onset of re-oxygenation might be so accelerated that the synthesis of pyruvate exceeds its utilization. As this reaction involves the reduction of nicotinamide-adenine dinucleotide (NAD+) by lactic dehydrogenase, the heightened oxygen uptake might be due, principally, to the re-oxidation of the coenzyme. Since in this oxidation process no CO₂ is produced, a low R.Q. is to be expected. The experimental evidence presented by Cohen in the same paper is only indirect, being restricted to showing that the amounts of O₂ absorbed and CO₂ evolved are too low to be the result of the oxidation of lactate.

According to this hypothesis, some accumulation of pyruvate must follow the disappearance of lactate, unless it has been utilized as fast as it is synthesized through a process not leading to its complete oxidation. Some pilot experiments

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on *B. arenarum* eggs seem to support the first possibility. In fact, by a semi-quantitative isolation of pyruvic acid, through a chromatographic separation of its 2,4-dinitrophenylhydrazone (Barbieri & Pascual, 1962), we have observed an abrupt rise when the eggs are reoxygenated after a period of anaerobiosis (Barbieri & Pascual, unpublished results). This observation compelled us to attempt a kinetic study of pyruvate and lactate levels during the respiratory recovery using more accurate methods. We made use of a sensitive technique successfully proved on this biological material, which permitted us to show that the level of pyruvate varies significantly during normal development (Barbieri & Salomón, 1962) and that it decreases in the eggs subjected to anaerobiosis or to fluoride action (Salomón & Barbieri, 1964). Moreover, the same method allows us to determine simultaneously the level of α-ketoglutaric acid, one of the intermediates of the tricarboxylic acids cycle.

**MATERIAL AND METHODS**

*B. arenarum* eggs were obtained by treating adult females with homologous hypophysis preserved in glycerine according to the method of Pisanó (1956) and fertilization was accomplished *in vitro*.

In order to ensure good anaerobiosis, the egg jelly was removed by ultraviolet irradiation as described elsewhere (Legname, 1966). Preliminary tests allowed us to exclude the possibility that this treatment could affect the level of the metabolites studied here.

Every experiment, started after the first or second mitotic division, included two steps: anaerobiosis and the re-oxygenation of the eggs. Anaerobiosis was achieved by a flow of nitrogen through a series of Warburg vessels (shaking at eighty complete strokes per minute) each of which contained 200 eggs suspended in 2-5 ml of 10% amphibian Ringer's solution without bicarbonate. The gas was previously washed through pyrogalate, vanadous sulfate-amalgamated zinc (Meites & Meites, 1948) and water. After 20 min of flow, the system was closed and kept for 3 h without further shaking. In the second step, an air flow was established through the shaking vessels and maintained until each experiment was finished, in order to ensure a quick and uniform re-oxygenation. The bath was thermostatically maintained at 25 °C.

Samples were taken out every 15 min, homogenized by repeated freezing and deproteinized with 10% metaphosphoric acid. The precipitated protein was removed by refrigerated centrifugation and aliquots of the clear supernatant were collected for the analysis. Lactic acid was estimated by the method of Barker & Summerson (1941) according to the technique of Ishida & Taguchi (1957). Pyruvic and α-ketoglutaric acids were simultaneously measured with the spectrophotometric method of Goodwin & Williams (1952). A Hilger (Uvispek) spectrophotometer was used.
RESULTS

Several pilot experiments in which lactic acid and the two ketoacids were separately determined confirmed our previous results (Salomón & Barbieri, 1964; Barbieri & Salomón, 1964): *B. arenarum* eggs under anaerobiosis accumulate lactate and lose pyruvate and $\alpha$-ketoglutarate; when switching from nitrogen to air, the reverse process is observed, i.e. a fall of lactate and a rise of both ketoacids.

![Graph showing the levels of lactate, pyruvate, and $\alpha$-ketoglutarate during respiratory recovery.](image)

In experiments performed with eggs obtained from four different females, the three metabolites were simultaneously determined with consistent results. The numerical data of one of these experiments are given in the graph of Fig. 1. It can be seen that the pool of lactate decreases in a biphasic curve. The first phase, which lasts about 1 h, tends to have a uniform rate of fall of about 0.01 $\mu$M for 100 eggs per minute; the second phase also shows a uniform rate, but about four times slower (0.0026 $\mu$M/100 eggs/min). The level of pyruvate rises abruptly when the eggs are re-oxygenated until the end of the first hour; thereafter, it decreases at a lower rate. Hence, the pyruvate content also varies in a biphasic curve, with an inflexion point that coincides with that found in the curve of lactate. $\alpha$-Ketoglutaric acid increases also during the re-establishment of respiration, until it reaches its maximum value 2 h after the beginning of re-oxygenation.
DISCUSSION

The kinetics of the three metabolites analysed here are in agreement with the classical metabolic schemes.

The biphasic character of the lactate curve cannot be explained by our experimental data, since it does not depend, as far as we know, only upon the current concentrations of lactate and pyruvate. Among the many possible factors which may operate *in vivo*, we may remember the influence of the relative concentrations of oxidized and reduced NAD, as well as the influence of hydrogen-ion concentration (Tobin, 1964).

The immediate accumulation of pyruvic acid fits well into the hypothesis advanced by Cohen (1955) by showing that the turnover of lactate to pyruvate is so rapid that it may exceed its rate of utilization. In any case, the increments measured remained insufficient to compensate for the lack of balance between the oxygen taken up and the lactate consumed (Legname, 1966). Even taking into account the rise of \( \alpha \)-ketoglutarate, this problem remains unsolved. Two alternative explanations are possible: (1) the probable accumulation of other intermediates of the tricarboxylic acids cycle before the oxidation of pyruvate reaches its completion; (2) the participation of lactate as a precursor in some anabolic process. We may remember in this connexion that when *B. arenarum* eggs are transferred to air after anaerobiosis they seem capable of synthesizing glycogen (Barbieri & Salomón, 1963).

It is worth while to compare our experiments with those performed by Duspiva (1961, 1962), who studied the changes of lactic and pyruvic acids levels in eggs treated with cyanide. When *B. bufo* and *Triturus alpestris* eggs are submitted to the action of this respiratory inhibitor, their lactate level increases continuously, while their pyruvate content, after an initial rise during the first hours, decreases progressively. If these eggs are transferred to a normal medium without HCN, a fall of lactate and a rise of pyruvate take place. These data agree, therefore, with our observations reported here on *B. arenarum* eggs. We notice that Duspiva's graph for *T. alpestris* is the only one which shows a good correlation between the fall of lactate and the rise of pyruvate. This may be due either to the difficulty of getting a rapid elimination of cyanide at the end of the treatment, or to the fact that this inhibitor may interfere not only with the respiratory enzymes but also with other enzymic systems.

SUMMARY

1. A description of the lactate, pyruvate and \( \alpha \)-ketoglutarate level changes of *Bufo arenarum* eggs transferred to air after anaerobiosis is presented.
2. The immediate changes after the onset of the respiratory recovery are a fall
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of lactate content and a simultaneous increase of the level of pyruvate. Subsequently, an accumulation of α-ketoglutarate occurs.

3. The increase of the pool of these ketoacids is not enough to compensate the imbalance between the lactate consumed and the oxygen taken up.

RÉSUMÉ

Anaérobiose chez les œufs de Bufo arenarum

1. On présente une description des changements des teneurs en lactate, pyruvate et α-cétoglutarate lorsque des œufs de Bufo arenarum sont transférés à l’air après une période d’anaérobiose.

2. Les changements immédiats après le début de la reprise de la respiration sont une chute de la teneur en lactate et une augmentation simultanée de celle en pyruvate. Ulteriormente, il se produit une accumulation d’α-cétoglutarate.

3. L’augmentation du pool de ces acides cétoniques est insuffisante pour compenser le déséquilibre entre la consommation de lactate et celle d’oxygène.

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REFERENCES


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