THE ROLE OF CREATINE AND ITS PHOSPHATE IN AMPHIBIAN DEVELOPMENT [1]

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Baldwin (1957) has summarized the chemical events that occur in a normal muscle working under anaerobic conditions:

(1) Adenosine triphosphate (ATP) remains unchanged;
(2) Phosphocreatine (PC) disappears;
(3) Free creatine (C_F) appears;
(4) Free inorganic phosphate (P_I) appears;
(5) Glycogen disappears;
(6) Lactic acid appears.

The classical theory of muscle contraction accounts for these occurrences by means of assumptions schematized in Figure 1. As long as it is active, the muscle undergoes endergonic changes of state, as shown on the right. The necessary input of energy is provided by the dephosphorylation of ATP, converting it into ADP (adenosine diphosphate) and liberating free P_I. Part of the ADP thus formed is immediately phosphorylated by PC, thus regenerating some ATP as fast as it is utilized and elevating somewhat the level of C_F. The remainder, together with free P_I, stimulates glycolysis, which in the absence of oxygen terminates in lactic acid, and which helps to relieve the drain on stored PC by generating further ATP. The imbalance finally reached is a measure of the discrepancy between the rate at which energy is demanded by the contractile process and the rate at which it can be supplied by glycolysis.

An energy transfer and storage system similar to that operative in muscle is believed to occur in amphibian embryos, and in the sequel some of the evidence will be reviewed. However, the role of creatine and phosphocreatine has never been thoroughly investigated, although it has been hinted at by the data of Zielinski (1937) and of Barth and Jaeger (1947). Thus, there has been a considerable hiatus in our knowledge of the major features of the energetics of amphibian development.

The present investigation is, in part, a study of energy transfer and storage in *Rana pipiens* embryos, with special attention to the role of phosphocreatine under both aerobic and anaerobic conditions. Also, advantage has been taken of the opportunity to conduct a parallel study of gastrula-arrested hybrid embryos obtained by fertilizing *R. pipiens* eggs with *R. sylvatica* sperm (Moore, 1941, 1946; Gregg, 1957). Direct estimation of free and total creatine has made possible the calculation of the amounts of phosphocreatine phosphorus (PCP) present at vari-

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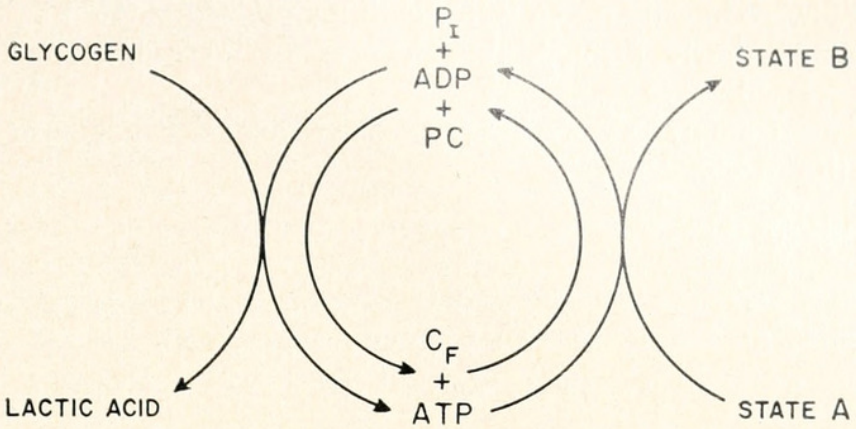

FIGURE 1. Chemical events occurring in muscle contracting anaerobically.

ous stages, aerobically and anaerobically, and this has been compared with directly estimated free inorganic phosphorus. Total phosphorus (P_T) and total acid-soluble phosphorus (P_A) have also been estimated as checks against the possibility of artifacts arising from phosphorus loss to the surrounding medium; and further guarantee that the embryos have been maintained within physiological bounds has been sought in a study of the ability of *R. pipiens* embryos to develop normally after anaerobic treatment.

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METHODS

Embryological

Embryos were obtained by stripping eggs from pituitary-treated *R. pipiens* females into suspensions of *R. pipiens* or *R. sylvatica* sperm. After 30 minutes, the sperm suspensions were poured off and fresh 10% amphibian Ringer's solution (without phosphate or bicarbonate) was added. After an additional 30 minutes, the embryos were separated into small groups and allowed to develop, as convenient, at 10° C., 18° C. or at room temperature (21° C.). Before subjecting the embryos to anaerobiosis, their jelly coats were removed with jewelers' forceps. Aerobic embryos were dejellied just prior to chemical analysis.

Developmental stages, and the corresponding standard ages at 18° C., were ascertained by reference to Shumway (1940).

Anaerobiosis

The apparatus used to provide an anaerobic environment is represented in Figure 2. Dejellied embryos (125) were placed in the bottom of the chamber, along with enough 10% Ringer's solution to immerse the tip of the gas inlet tube. After tipping the chamber so that the inlet tube cleared the surface of the medium, pre-purified nitrogen (oxygen content less than 45 p.p.m.) was flushed rapidly through the chamber for one minute. The chamber was then clamped vertically

in a rack, and lowered partly into a water bath maintained at 24° C. Nitrogen was allowed to bubble slowly through the medium for 10 minutes, after which all stopcocks were closed.

Aerobic control embryos were placed in unstoppered Erlenmeyer flasks in the same water bath.

Protein-free extracts

After a prescribed time in the chamber, the embryos were quickly staged, transferred into chilled graduated centrifuge tubes, washed with ice-cold deionized distilled water, made up to a volume of 5 ml. with water, decanted into a chilled

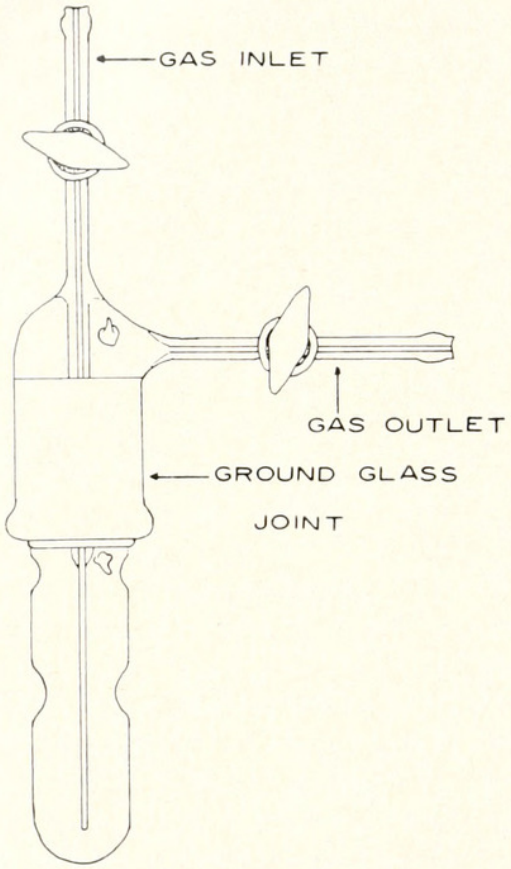


FIGURE 2. Diagram of nitrogen chamber.

glass homogenizer containing 1.25 ml. of 24% trichloroacetic acid and homogenized. After taking an aliquot for the determination of total phosphorus, the homogenate was centrifuged at 0° C. for 10 minutes at 5000 *g*. The clear supernatant was then pipetted into a chilled test tube and kept ice-cold to prevent breakdown of labile phosphates.

Aerobic control embryos were treated simultaneously in exactly the same manner.

Chemical analysis

Creatine moieties were determined by means of the method of Ennor and Rosenberg (1952), slightly modified. In this procedure, creatine is estimated

colorimetrically before (C_F—free creatine) and after (C_T—total creatine) acid hydrolysis, and the difference $C_B = C_T - C_F$ is assumed to be the bound creatine of phosphocreatine.

A portion of the trichloroacetic extract was neutralized with powdered Na_2CO_3 (Kutsky, 1950), and two 0.5-ml. aliquots were transferred into separate test tubes. One aliquot (C_F) was set aside; the other (C_T) was acidified with 0.25 ml. of 0.3 N HCl, hydrolyzed for 30 minutes in a water bath at 56° C., cooled and re-neutralized with 0.25 ml. of 0.3 N NaOH. Two ml. of freshly prepared diacetyl reagent were then added to each tube. The tubes were placed in the dark, to prevent a

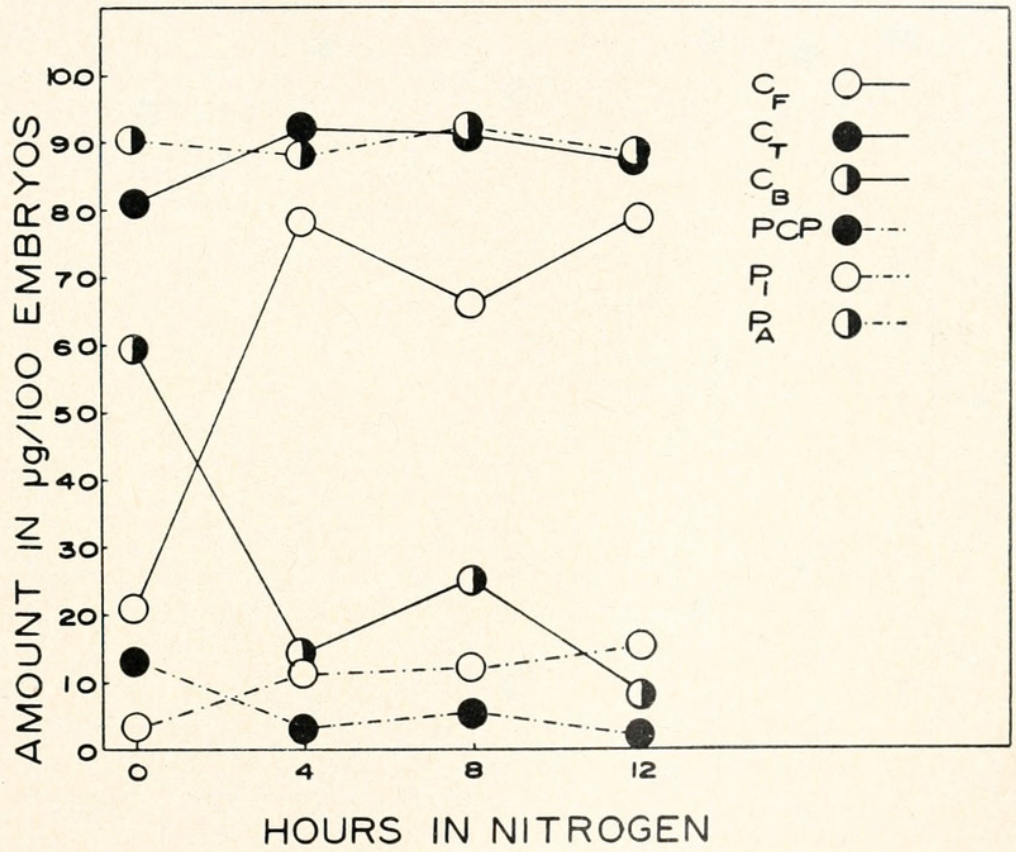


FIGURE 3. Changes in creatine and phosphorus fractions in *Rana pipiens* embryos at Stage 10-, after various anaerobic periods.

photoreaction between trichloroacetate and alpha naphthol, and incubated for 35 minutes at room temperature. Optical densities at 525 mμ were determined with a Bausch and Lomb Spectronic 20 colorimeter. A blank and two standards (creatine hydrate) were run simultaneously.

The amount of phosphocreatine phosphorus was calculated, once the value of C_B was known, from the equation $PCP = 0.2362\ C_B$, where the numerical constant is the value of the ratio of the molecular weight of phosphorus to the molecular weight of creatine.

Inorganic phosphorus was determined by the method of Berenblum and Chain (1938), as modified by Martin and Doty (1949). A 2-ml. aliquot of protein-free extract was placed in a test tube, followed by 5 ml. of 1:1 benzene:isobutanol and 1 ml. of molybdate reagent. The tube was stoppered with a ground-glass stopper

and shaken vigorously for 15 seconds. When it had formed, 2.6 ml. of the upper phase were transferred with a syringe into a centrifuge tube and made up to 5 ml. with acid alcohol. Stannous chloride reagent (0.5 ml.) was then added, and after 20 minutes the optical density at 625 mμ was determined with the colorimeter mentioned above. A blank and two standards (KH_2PO_4) were run simultaneously.

The method of Martin and Doty (1949) was also used for the determination of total phosphorus and total acid-soluble phosphorus, but with the modifications suggested by Ernster *et al.* (1950). Two test tubes were prepared, one (total

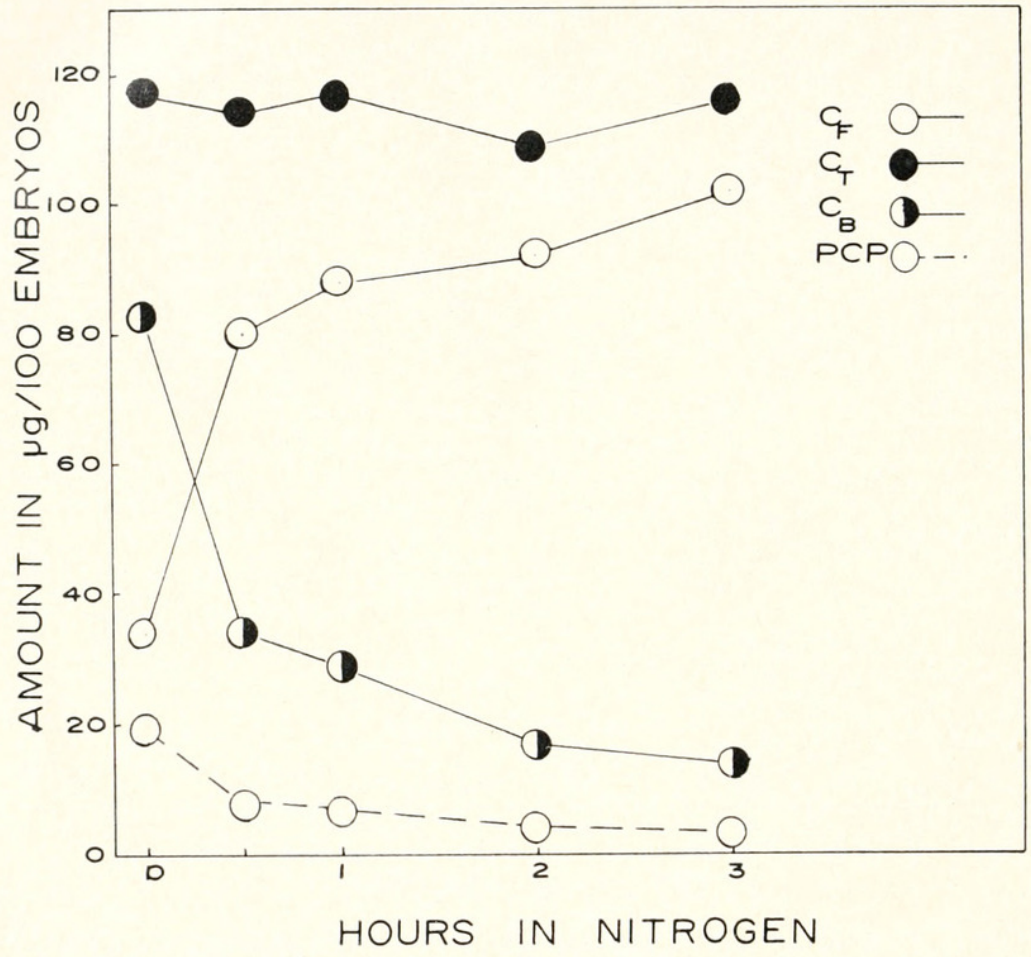


FIGURE 4. Changes in creatine and phosphorus fractions in *Rana pipiens* embryos at Stage 11 after short exposure to anaerobiosis.

phosphorus) containing 0.2 ml. of homogenate, the other (total acid-soluble phosphorus) containing 1 ml. of protein-free extract. After the addition of 1 ml. of 10 N H_2SO_4 to each tube, they were placed in an oven at 130–160° C. After two to three hours, and after partial cooling, one drop of 30% H_2O_2 was added to each tube, which was then heated over a bunsen burner until the contents became colorless or very pale yellow in color. The tubes were then replaced in the oven for 15 minutes. After partial cooling, 1 ml. of water was added to each tube, which was then returned to the oven, now at 100° C., for 10 minutes. The tubes were then cooled, and the contents made up to 7 ml. with water. Inorganic phosphorus present was then determined as described above, with the exception that H_2SO_4 was omitted from the molybdate reagent.

Results and Discussion

Preliminary experiments

In order to avoid working with irreversibly damaged material, an attempt was made to ascertain how long embryos (*R. pipiens*) at various stages could be kept anaerobic at 24° C. and still develop normally when aerobiosis was resumed. It turned out that late-cleavage embryos and gastrulae are less susceptible to permanent damage by anaerobiosis than embryos of other stages, and will survive a sojourn of 12 hours in nitrogen; but two hours of anaerobiosis is the maximum that will permit subsequent normal development of embryos in *all* stages from fertilization to hatching.

Table I

Creatine and phosphorus moieties of R. pipiens and R. pipiens ♀ × R. sylvatica ♂ embryos after 2 hours of anaerobiosis at 24° C., in micrograms per 100 embryos

Treatment	Range of stages	*R. pipiens*					Hybrids					No. expts.
		C_T	PCP	P_I	P_A	P_T	C_T	PCP	P_I	P_A	P_T	
Aerobic	3–4	94.4	14.0	2.2	90.0	2071	90.6	12.6	2.4	89.0	1929	5
Anaerobic		93.8	7.0	5.3	91.2	2068	91.6	6.7	6.1	94.0	1911	
Aerobic	9–11	94.8	14.4	3.7	87.6	1998	91.7	13.8	3.3	88.8	1926	6
Anaerobic		95.8	2.9	11.8	90.0	2081	92.0	2.3	11.3	88.8	1944	
Aerobic	12–14	93.8	12.1	5.1	85.2	2002	93.2	12.0	2.6	88.2	1936	5
Anaerobic		102.4	3.7	15.6	91.0	2062	95.2	2.8	12.1	87.4	2139	
Aerobic	15–16	94.7	13.4	5.1	84.3	2055	86.7	11.4	3.1	80.7	1987	3
Anaerobic		91.7	2.4	18.5	89.3	2029	87.3	3.1	12.4	83.3	1947	
Aerobic	17–18	109.0	15.4	7.4	100.5	1905	94.3	11.4	4.2	93.7	1914	4
Anaerobic		108.0	1.7	21.4	107.0	1931	91.8	1.4	13.8	90.7	2104	
Aerobic	19–20	145.0	19.3	10.2	128.3	1946	86.0	9.9	6.6	96.0	1851	3
Anaerobic		145.0	2.5	35.5	134.3	1922	83.0	2.1	17.5	92.0	1888	

Having established this result, two experiments were made to determine the rapidity with which creatine and phosphorus imbalances are established in *R. pipiens* embryos (gastrulae) under anaerobiosis. The results (Figs. 3 and 4) indicate that the major changes occur during the first hour in nitrogen, although some further change is evident throughout the 12-hour period examined.

As a consequence of the foregoing, two-hour periods of anaerobiosis were adopted as standard for further experimentation; and it is believed that this guarantees comparability of the results obtained with embryos of different ages—and of the results obtained with normal and hybrid embryos, although tests of the nitrogen-tolerance of hybrid embryos were not made.

Results obtained by other investigators indicate that temperature, length of anaerobiosis and embryological type all affect the extent of the phosphorus imbalance induced by oxygen-deprivation. Barth and Jaeger (1947), in a study of

R. pipiens and *R. pipiens* ♀ × *R. sylvatica* ♂ gastrulae, found that free inorganic phosphorus reached a maximum level between 4 to 8 hours after the onset of anaerobiosis and that this level was essentially maintained up to 22 hours (18° C.). Little change in the level of ATP was observed in either type of embryo; and this agrees with the results of Brachet (1954), who examined *Bufo vulgaris* and *B. vulgaris* ♀ × *R. fusca* ♂ embryos subjected to 8-hour periods of anaerobiosis at 12° C. In the case of embryos produced from eggs (*R. fusca*) fertilized with nitrogen-mustard-treated sperm (*R. fusca*), Brachet (1954) observed a 60% decrease of ATP after 8 hours anaerobiosis at 20° C., as compared with a 30% decrease in normal anaerobic controls. In another experiment at the same temperature, embryos of both types lost 62% of their ATP after 17 hours of anaerobiosis.

TABLE II

Comparison of various phosphorus fractions

Species	μg. P_I per 100 embryos	μg. PCP per 100 embryos	μg. C_T per 100 embryos
R. temporaria (Zielinski, stage unspecified)	6.5 (2.9–11.5)	8.2 (3.8–11.4)	104 (85–143)
R. pipiens (Harrison, stage 13)	5.1 (2.8–7.3)	12.1 (11.0–14.8)	94 (78–111)

Creatine and phosphorus moieties

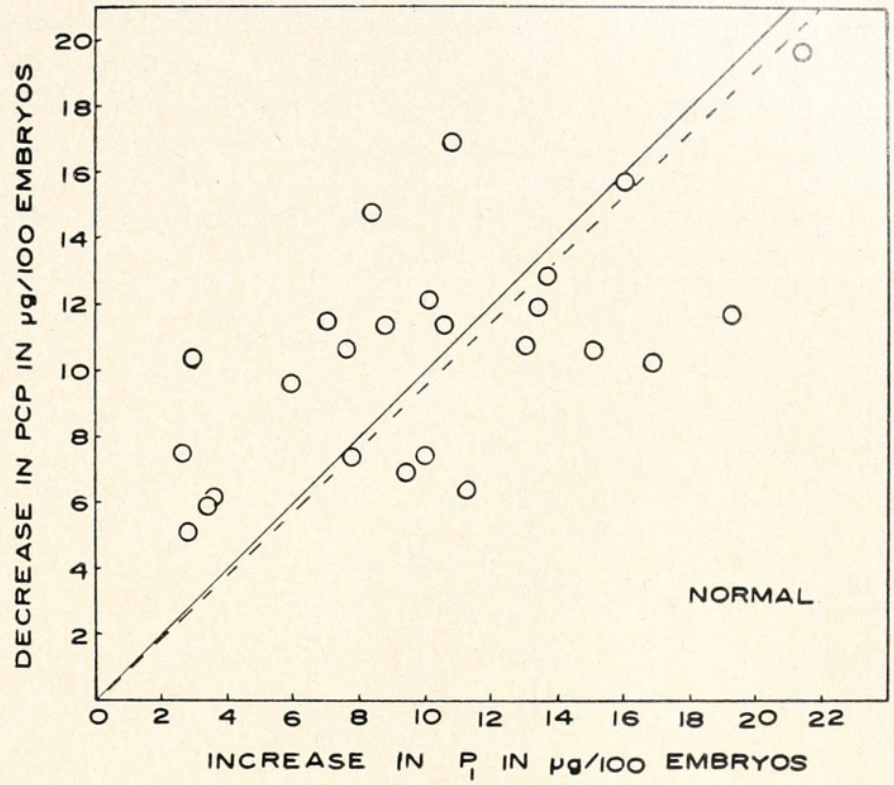
The results of all further chemical analyses are summarized in Table I, whose significance will be elaborated in the following discussion.

As functions of the developmental stage of aerobic *R. pipiens* embryos, total phosphorus is constant, free inorganic phosphorus is steadily increasing, and total acid-soluble phosphorus, phosphocreatine phosphorus and total creatine are approximately constant before stage 16 but increasing thereafter. As regards total phosphorus, free inorganic phosphorus and total acid-soluble phosphorus, aerobic hybrids exhibit roughly the same developmental changes, though to a lesser extent in respect of the latter two fractions; while phosphocreatine phosphorus declines in amount after stage 16, and total creatine is constant throughout.

For *R. pipiens*, it may be noted that the aerobic levels of inorganic phosphorus, phosphocreatine phosphorus, and total creatine approximate those reported for *R. temporaria* by Zielinski (1937), although his methods have been questioned on (unstated) technical grounds by Barth and Jaeger (1947). A comparison of the two groups of results is set out in Table II.

In general, the values for inorganic phosphorus in aerobic *R. pipiens* embryos agree more closely with those obtained by Kutsky (1950) than with the higher values obtained by Barth and Jaeger (1947) and by Gregg and Kahlbrock (1957). Among other things, this may be the result of using a chemical method that reduces the breakdown of labile phosphates by shortening their exposure to acid molybdate reagent. As compared to hybrid embryos, the higher levels of free inorganic phosphorus developed by normal embryos suggested to Gregg, MacIsaac

and Parker (1964) that they operate at a relative energy-deficit; but in view of the fact that the increases are more than balanced by increasing total acid-soluble phosphorus, it seems better to look upon them merely as a reflection of higher steady-



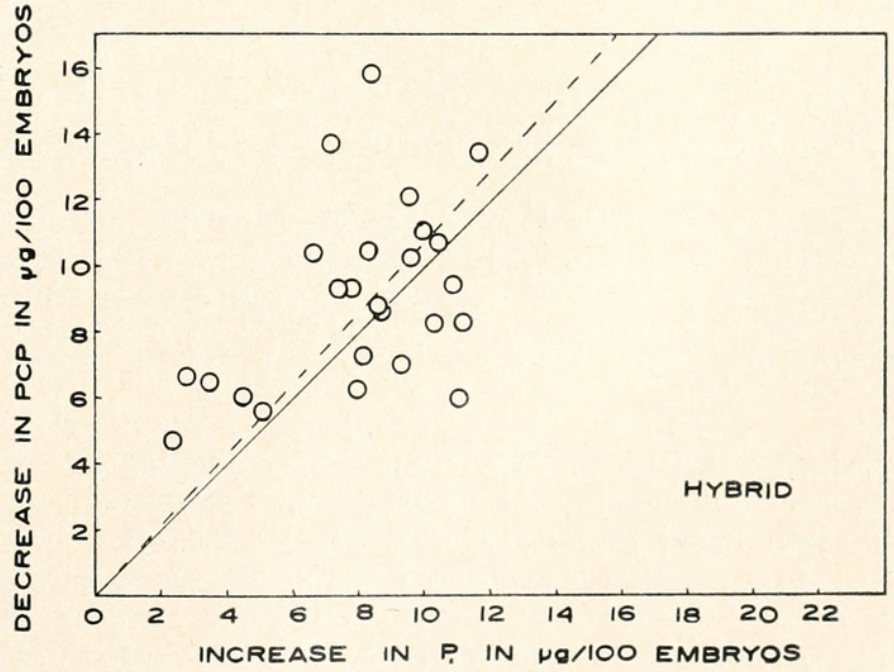


FIGURE 5 (above). Ratio of change in phosphocreatine phosphorus to change in inorganic phosphorus in *Rana pipiens* embryos during a two-hour anaerobic period. The solid line is the theoretical regression line and the dotted line the calculated regression line.

FIGURE 6 (below). Ratio of change in phosphocreatine phosphorus to change in inorganic phosphorus in *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrid embryos during a two-hour anaerobic period. The solid line is the theoretical regression line and the dotted line is the calculated regression line.

state levels. This interpretation is reinforced by the subnormal total creatine levels of post stage 16 hybrids, which may place some limitation on the amount of energy that can be stored as phosphocreatine.

Under anaerobiosis (two hours), total phosphorus, total creatine and total acid-soluble phosphorus remain relatively stable in both types of embryos. Phosphocreatine phosphorus, however, undergoes a marked depletion, which is almost exactly balanced by the appearance of free inorganic phosphorus (Figs. 5 and 6). This may be taken to indicate that no change of ATP-level occurs, as in muscle. Under longer periods of anaerobiosis (12 hours), more free inorganic phosphorus appears than can be accounted for by the disappearance of phosphocreatine

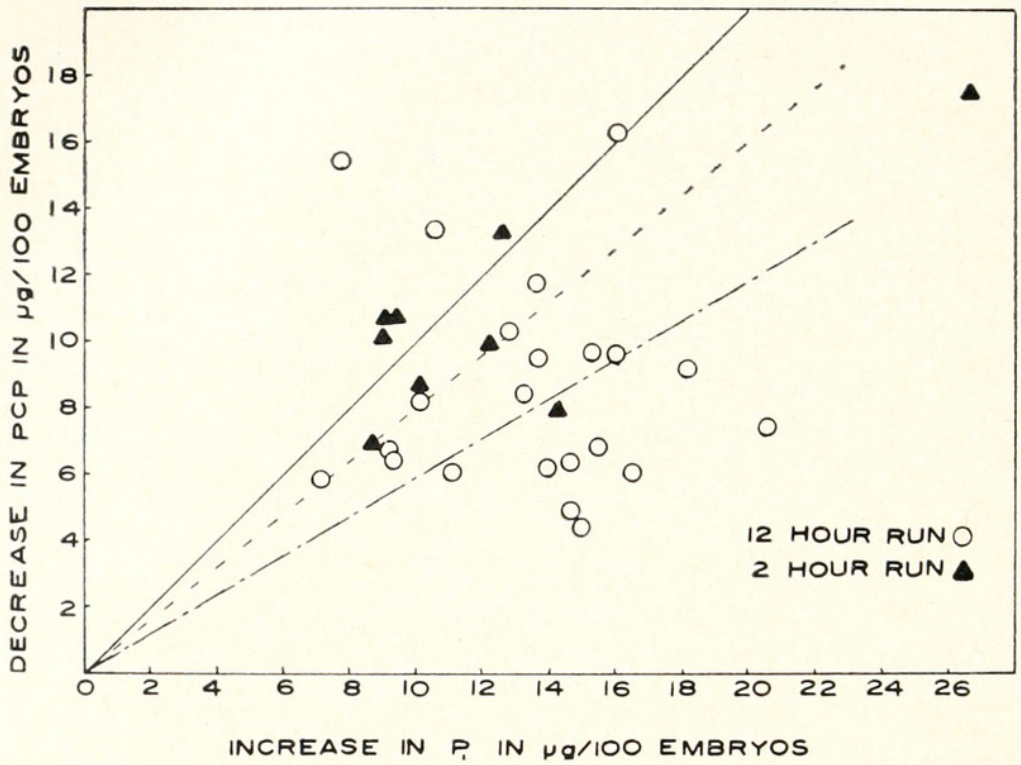


FIGURE 7. Ratio of change in phosphocreatine phosphorus to change in inorganic phosphorus during anaerobiosis in *Rana pipiens* embryos. The solid line represents the theoretical line on which points should fall if the ratio is 1:1. The dotted line is the calculated regression line for the two-hour runs and the dotted-dashed line is the calculated regression line for the 12-hour runs.

phosphorus, at least in normal embryos (Fig. 7), perhaps owing to the breakdown of ATP. These observations are in agreement with the results of Carlson and Siger (1957), who found that net phosphocreatine breakdown in iodoacetate-poisoned frog sartorius muscle is a linear function of work done, and that no net breakdown of ATP occurs until the phosphocreatine concentration drops by 60% or more. They also account for the findings of Barth and Jaeger (1947) that during long periods of anaerobiosis (10–22 hours), more inorganic phosphorus appears than can be attributed to ATP breakdown, in both normal and hybrid embryos.

It should be noted that the phosphocreatine phosphorus-inorganic phosphorus imbalance induced by anaerobiosis is much more pronounced in normal embryos than in hybrids, at least in older embryos. This is readily understandable in terms of the relatively greater energy-demands imposed by the complexity of the de-

velopmental changes occurring in normal embryos, in comparison with those of lesser complexity occurring in blocked hybrids.

Taken all together, the foregoing results suggest that the role of the creatine moieties in the energetics of amphibian development is the same as in that of contracting muscle: the phosphorylation of creatine provides a store of phosphate bond energy that may be drawn upon in periods of anaerobic stress. Indeed, in view of the demonstration of a phosphorylating anaerobic glycolysis in amphibian embryos (Cohen, 1954), the parallel between muscle and embryos appears to be nearly complete.

We may now add a few remarks about the role of energy metabolism in the development of blocked hybrids. Post-blockage hybrids have subnormal respiratory and glycolytic rates (Barth, 1946; Gregg, 1960, 1962), and utilize stored glycogen at a rate lower than normal (Gregg, 1948). Some ultimate limitation upon their capacity to generate and store energy is indicated by their subnormal respiratory response to the uncoupling agent 2,4-dinitrophenol (Gregg, 1960) and by their failure to synthesize creatine after stage 16, as mentioned in the discussion above. Nevertheless, homogenates of hybrid embryos exhibit respiratory and glycolytic rates that are quantitatively similar to those of normal embryos (Gregg and Ray, 1957; Gregg, MacIsaac and Parker, 1964), and the general picture of energy production, transfer and storage that emerges is one of essential normality, in which actually occurring energy-demand is met without undue difficulty. Thus, the lowered rates of energy metabolism may be regarded as a reflection of some independent block, internal or imposed by the embryonic environment, to developmental transformations that would normally require a greater expenditure of energy.

Summary

1. A study has been made of the role of phosphorylated creatine in the energy metabolism of *Rana pipiens* and gastrula-blocked *R. pipiens* ♀ × *R. sylvatica* ♂ hybrid embryos.

2. It is shown that the longest period of anaerobiosis that can be survived by *R. pipiens* embryos of all pre-hatching stages, with subsequent normal aerobic development, is of two hours duration, at 24° C.

3. In hybrid embryos, creatine synthesis is deficient after stage 16, and the levels of phosphocreatine phosphorus and inorganic phosphorus are subnormal.

4. In both types of embryos, two hours of anaerobiosis result in a decrease of phosphocreatine phosphorus, accompanied by a quantitatively similar increase of free inorganic phosphorus. Both changes are greater in normal embryos than in hybrids. In *R. pipiens* embryos, prolonged anaerobiosis (12 hours) results in the appearance of excess inorganic phosphorus, perhaps at the expense of ATP.

5. A comparison is made of energy metabolism in muscle and embryos, and the role of energy metabolism in the development of hybrids is briefly discussed.

LITERATURE CITED

Baldwin, E., 1957. Dynamic Aspects of Biochemistry. The University Press, Cambridge.

Barth, L. G., 1946. Studies on the metabolism of development. *J. Exp. Zool.*, **103**: 463–489.

Barth, L. G., and L. Jaeger, 1947. Phosphorylation in the frog's egg. *Physiol. Zoöl.*, **20**: 133–146.

BRACHET, J., 1954. Constitution anormale du noyau et teneur en acide adénosine-triphosphorique de la cellule. *Experientia,* **10**: 492.

BERENBLUM, I., AND E. CHAIN, 1938. An improved method for the colorimetric determination of phosphate. *Biochem. J.,* **32**: 295–298.

CARLSON, F. D., AND A. SIGER, 1957. The dependence of creatine phosphate and adenosine triphosphate breakdown on work in iodoacetate poisoned muscles. *Biol. Bull.,* **113**: 324.

COHEN, A. I., 1954. Studies on glycolysis during the early development of *Rana pipiens*. *Physiol. Zoöl.,* 27: 128–141.

ENNOR, A. H., AND H. ROSENBERG, 1952. The determination and distribution of phosphocreatine in animal tissues. *Biochem. J.,* **51**: 606–618.

ERNSTER, L., R. ZETTERSTRÖM AND O. LINDBERG, 1950. Method for the determination of tracer phosphate in biological material. *Acta Chem. Scand.,* **4**: 942–947.

GREGG, JOHN R., 1948. Carbohydrate metabolism of normal and of hybrid amphibian embryos. *J. Exp. Zool.,* **109**: 119–134.

GREGG, JOHN R., 1957. Morphogenesis and metabolism of gastrula-arrested embryos in the hybrid *Rana pipiens* ♀ × *Rana sylvatica* ♂. *In:* The Beginnings of Embryonic Development, edited by Albert Tyler, R. C. von Borstel and Charles B. Metz, Publication No. 48 of the American Association for the Advancement of Science. Washington, D. C.

GREGG, JOHN R., 1960. Respiratory regulation in amphibian development. *Biol. Bull.,* **119**: 428–439.

GREGG, JOHN R., 1962. Anaerobic glycolysis in amphibian development. *Biol. Bull.,* **123**: 555–561.

GREGG, JOHN R., AND MARGIT KAHLBROCK, 1957. The effects of some developmental inhibitors on the phosphorus balance of amphibian gastrulae. *Biol. Bull.,* **113**: 376–381.

GREGG, JOHN R., AND FRANCES L. RAY, 1957. Respiration of homogenized embryos: *Rana pipiens* and *Rana pipens* ♀ × *Rana sylvatica* ♂. *Biol. Bull.,* **113**: 382–387.

GREGG, JOHN R., JANE J. MACISAAC AND MARY ANN PARKER, 1964. Anaerobic glycolysis in amphibian development. Homogenates. *Biol. Bull.,* **127**: 256–270.

KUTSKY, PHYLLIS B., 1950. Phosphate metabolism in the early development of *Rana pipiens*. *J. Exp. Zool.,* **115**: 424–460.

MARTIN, J. B., AND D. M. DOTY, 1949. Determination of inorganic phosphate—modification of iso-butyl alcohol procedure. *Anal. Chem.,* **21**: 965–967.

MOORE, J. A., 1941. Developmental rate of hybrid frogs. *J. Exp. Zool.,* **86**: 405–422.

MOORE, J. A., 1946. Studies in the development of frog hybrids. I. Embryonic development in the cross *Rana pipiens* ♀ × *Rana sylvatica* ♂. *J. Exp. Zool.,* **101**: 173–220.

SHUMWAY, W., 1940. Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.,* **78**: 139–147.

ZIELINSKI, M. A., 1937. Phosphagen and creatine in frog's eggs. *J. Exp. Biol.,* **14**: 48–55.