*THE EFFECT OF CONTRACTION AND OF EPINEPHRINE ON THE PHOSPHORYLASE ACTIVITY OF FROG SARTORIUS MUSCLE**

By William H. Danforth,† Ernst Helmreich, and Carl F. Cori

DEPARTMENT OF BIOLOGICAL CHEMISTRY, WASHINGTON UNIVERSITY SCHOOL OF MEDICINE, ST. LOUIS

Communicated May 25, 1962

Glycogen phosphorylase in skeletal muscle exists as phosphorylase *a*, fully active, and phosphorylase *b*, active only in the presence of 5′-AMP.[1, 2] Phosphorylase *b* is phosphorylated at the expense of ATP by phosphorylase *b* kinase[3] and dimerizes to form phosphorylase *a*. The reverse reaction is catalyzed by phosphorylase phosphatase, formerly called PR enzyme.[4, 5]

In 1956, C. F. Cori[6] emphasized the speed with which glycogen breakdown occurs in muscle during contraction and suggested that the speed of the $b \rightleftharpoons a$ interconversion was of sufficient magnitude to be the pacemaker of the concomitant changes in the rates of glycolytic reactions. Recently, Rulon *et al.*[7] reported a statistically significant increase in phosphorylase *a* content of rat anterior tibial muscle during tetanic contraction. The high and variable baseline levels of phosphorylase *a* in resting muscle obtained with previous methods have precluded a quantitative analysis of changes in the phosphorylase system.

Improvements in techniques for the arrest of enzymatic reactions make it now possible to study the time course of the interconversion of phosphorylase *a* and *b* in the thin frog sartorius muscle during and following contraction. The present studies show that phosphorylase *a* rises from less than 5 per cent to nearly 100 per cent of the total phosphorylase (phosphorylase *a* and *b* combined) within three seconds after the onset of tetanic contraction at 30°. On relaxation, phosphorylase *a* activity decreases exponentially to baseline values. Kinetic analysis suggests that changes in the phosphorylase *b* kinase rather than phosphorylase phosphatase activity are responsible for the increase and decrease in phosphorylase *a*.

Variations in muscle phosphorylase *a* in response to epinephrine, as previously studied by G. T. Cori and B. Illingworth,[8] have been reinvestigated with the use of the improved fixation technique. The increase and decrease of phosphorylase *a* in muscle as a consequence of the addition and removal of epinephrine is slow when compared with the effect of contraction, requiring minutes rather than seconds. Moreover, the response to epinephrine but not that to muscular work can be inhibited by an inactive analogue of epinephrine.

Experimental Procedure.—Female *Rana pipiens* weighing approximately 50 to 70 gm were obtained from Minnesota between November and March. The animals were kept in water at about 3° prior to use. After low spinal transection, the sartorii were carefully dissected and transferred to ice-cold Krebs-Ringer's-bicarbonate solution[9] adapted for frog muscle[10] and equilibrated with 5 per cent CO_2 in argon (pH $\sim$ 6.8). The muscles were allowed to recover for about 30 min before the experiment was initiated.

Muscle stimulation: Muscles to be stimulated were left attached to the pubic bone. The proximal end of the muscle was mounted in a platinum holder, which served as an electrode. The distal tendon was anchored to a small chain. A second platinum electrode surrounded the distal end of the muscle. Once in place, the muscle was immersed in anaerobic Ringer's solution at the temperature desired for the experiment. Five to ten min before stimulation, the fluid was removed and the muscle surrounded by a constant temperature chamber equilibrated with moist 5 per cent CO_2-95 per cent argon. Stimuli were supplied by a Grass model S-4-B stimulator.[11] The

muscle contracted isometrically. Details are given in the legend to Figure 1. Tension was monitored by a strain gauge and a Sanborn strain gauge amplifier and recorded by a Sanborn single-channel direct writer.[12] Immediately before termination of the experiment, the constant temperature chamber was removed. The muscle was then suddenly immersed in isopentane or dichlorodifluoromethane chilled to near its freezing point (−160°) in liquid nitrogen. The freezing left a mark on the tension record which allowed the duration of the contraction to be estimated to the nearest 0.1 second.

Incubation with epinephrine: Sartorii were incubated in closed 25 ml Erlenmeyer flasks containing 4 ml of Ringer's solution; 0.1 mg of ascorbate per ml was added to prevent the oxidation of epinephrine. The system was made anaerobic by flushing with a stream of 95 per cent argon-5 per cent CO_2. Experiments were terminated by lifting the muscles by the tendon and quickly freezing them in isopentane.

Fixation: Phosphorylase *b* kinase and phosphorylase phosphatase are inhibited by EDTA[1] and fluoride respectively. These agents do not affect phosphorylase *a* or *b* activity. The selective action of these inhibitors was the basis for previous techniques for the study of phosphorylase *a* and *b* content of living muscle. Muscles with or without prior freezing were homogenized in an ice-cold solution of these inhibitors.[8] In the present study, it has been established that neither technique is adequate to preserve the true resting baseline, mainly because the kinase is able to act with such great speed.

In preliminary experiments, the phosphorylase *a* content of resting frog sartorius muscle, although lower than previously reported, was still quite variable despite rapid freezing by a method similar to that of Seraydarian *et al.*[13] The frozen muscle was placed in a precooled cartridge in which it was pulverized by shaking with a steel ball. NaF and EDTA were then added in aqueous solution to the cold cartridge, so that ice formed in intimate contact with the frozen muscle powder. On further shaking, heat generated by friction of the steel ball caused thawing. It was soon realized that the rate of penetration of the inactivating solution into the muscle particles was in most cases too slow to prevent significant activity of phosphorylase *b* kinase (Table 1).

In the method finally adopted, the inhibitors were added in a glycerol-water solution which remained liquid at −35°. This mixture allowed the inhibitors to penetrate the muscle powder at a considerably lower temperature and acted as a lubricant to facilitate rapid and smooth mixing of the muscle powder in the inactivating solution. Heat resulting from friction between the ball and the cartridge was also kept at a minimum. Use of glycerol for this purpose was possible only because it did not inhibit phosphorylase at the final concentrations present in the test system.

The procedure was carried out as follows. Each frozen muscle, weighing 100–150 mg, was transferred to liquid nitrogen and then to a stainless steel cartridge of about 20 ml capacity precooled in liquid nitrogen. One stainless steel ball large enough to slide easily but with minimal clearance into the cartridge was added and the cartridge closed. The muscle was pulverized by shaking for 8 sec at about 100 oscillations per sec in a Nossal shaker.[14] The cartridge was then removed and warmed to −35° in an alcohol bath. 2 ml of a precooled 60 per cent glycerol solution containing 2×10^{-2} *M* NaF and 1×10^{-3} *M* EDTA (pH 6.7) were added to the powder. The cartridge was again closed and shaken for an additional 6 sec. 8 ml of an aqueous solution of 2×10^{-2} *M* NaF, 1×10^{-3} *M* EDTA, 1.9×10^{-2} *M* Na glycerophosphate, and 1.9×10^{-2} *M* cysteine (pH 6.7) at 3° were then added and the cartridge was again shaken for 4 sec. The importance of introducing the inactivating substances at such low temperatures is shown in Table 1. No other method gave such a consistently low baseline of phosphorylase *a* content of resting muscle.

Larger particles were removed by centrifugation at $1,400 \times g$ for 10 min at close to 2°. The supernatant fluid was analyzed for phosphorylase activity.

Phosphorylase assay: The reaction mixture for assay of total phosphorylase activity at 30° at pH 6.7 contained 7.5×10^{-2} *M* glucose-1-phosphate, 5×10^{-4} *M* 5′-AMP, 1 per cent glycogen, 7.5×10^{-3} *M* Na-glycerophosphate buffer, 7.5×10^{-3} *M* cysteine, 1×10^{-2} *M* NaF and 5×10^{-4} *M* EDTA. The total volume was 1 ml and contained 0.5 ml of the centrifuged muscle extract (final dilution of the muscle approximately 1:200). For measurements of phosphorylase *a* activity, 5′-AMP was omitted from the reaction mixture. At two or more suitably chosen time intervals, 0.2 ml aliquots were removed and inactivated in 8 ml 0.125 *M* Na-acetate buffer pH 4. Inorganic phosphate released by phosphorylase was measured according to Lowry and Lopez.[15]

In this method, hydrolysis of the acid-labile glucose-1-phosphate is held to a minimum. It was thus possible to measure phosphorylase activity in the presence of high concentrations of glucose-1-phosphate (i.e., 15 times the K_m value of glucose-1-phosphate for phosphorylase). At these concentrations, the phosphorylase reaction proceeds essentially linearly until about 12 per cent of the added glucose-1-phosphate have been used up. Phosphoglucomutase activity should not be significant in the presence of EDTA (5×10^{-4} *M*) in the reaction mixture.

Phosphorylase *a* activity is defined as the activity demonstrable in the absence of 5'-AMP and is expressed as per cent of total phosphorylase activity (i.e., phosphorylase *a* + *b* combined, measured in the presence of 5'-AMP).

In order to determine whether the muscle extract contributed significant amounts of 5'-AMP to the final reaction mixture, it was treated in a 1:100 dilution with acid-washed Norit-A (4 mg per ml at 0° for 10 min). Such treatment resulted in variable losses of total phosphorylase activity but did not significantly change the per cent of phosphorylase *a* in control muscles or in muscles stimulated electrically or incubated with epinephrine. It was shown in control procedures that 5'-AMP added to the muscle extract was effectively removed by treatment with Norit.

Since phosphorylase had been exposed to 60 per cent glycerol during fixation of muscle and 6 per cent glycerol was present in the final phosphorylase reaction mixture, possible effects of glycerol on phosphorylase *a* and *b* activity were investigated. Table 1 shows that the total phosphorylase activity of frog muscle extracted with water or with glycerol was essentially the same. When 15 per cent glycerol was present in the final reaction mixture, there was an 18 per cent inhibition of both crystalline rabbit muscle phosphorylase *a* and *b*. At the concentration used in the assay, little or no inhibition could be detected.

Materials.—EDTA, glucose-1-phosphate, and 5'-AMP were products of the Sigma Chemical Company. Norit-A was purchased from the Pfanstiehl Laboratories, Inc. Epinephrine hydrochloride was obtained from Parke Davis and Company and norepinephrine bitartrate from Winthrop Laboratories. 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol hydrochloride, referred to as dichloroisoproterenol (DCI), was a gift of Dr. I. H. Slater, Eli Lilly Co. Five times recrystallized rabbit muscle phosphorylase *a*, prepared as described by B. Illingworth and G. T. Cori,[16] was used to standardize the phosphorylase assay. Three times recrystallized rabbit muscle phosphorylase *b* was prepared by the method of Fischer and Krebs.[17]

Results.—Phosphorylase a content of resting muscle: The average phosphorylase *a* content of resting frog sartorius muscle, determined as described under *Methods*, was 2.8 per cent of total phosphorylase with a range of values from 1 to 5 per cent (Table 1). These values are much lower and more uniform than any previously

TABLE 1

THE INFLUENCE OF THE METHOD OF FIXATION ON THE PHOSPHORYLASE *a* ACTIVITY OF RESTING FROG MUSCLE

Frog sartorii were frozen in isopentane or dichlorodifluoromethane at −160° and were powdered in the frozen state. In one series of experiments, the muscle powder was extracted at 0° with an aqueous solution containing 2×10^{-2} *M* NaF and 1×10^{-3} *M* EDTA, and in the other series at −35° with a solution containing 60 per cent glycerol in addition to 2×10^{-2} *M* NaF and 1×10^{-3} *M* EDTA.

No. of experiments	Conditions for Fixation			Phosphorylase *a* in per cent of total		Total phosphorylase activity per gm muscle
	Solution	Volume (ml)	Temperature (°C)	Mean	Range	μmoles Pi/min
25	EDTA-NaF in H_2O	10	0	10.1	(3–32)	71.4
10	EDTA-NaF in glycerol	2	−35	2.8	(1–5)	72.4

reported. Extensive experience with various methods of fixation and extraction of muscle lead us to conclude that higher values for the phosphorylase *a* content of resting frog sartorius muscle are artifacts. In this context, it should be emphasized that the phosphorylase activity found in resting muscle is more than enough to

account for the rate of anaerobic production of lactate in an isolated frog sartorius at 30°, i.e., approximately 3.4 μmoles lactate per gm fresh weight of muscle per hr.

Effect of stimulation: Although the ratio of phosphorylase *a* to *b* was altered as the result of stimulation (see below), the total phosphorylase activity was not significantly changed. In ten resting sartorii, the phosphorylase activity assayed in the presence of 5'-AMP corresponded to the formation of 7.2 (S.D. ±1.4) × 10^{-5} moles of inorganic phosphate per gm muscle per min. The mate of each of these muscles was inactivated during tetanic contraction at 10°. The comparable value for the stimulated muscles was 7.7 (S.D. ±1.3) × 10^{-5} moles of inorganic phosphate per gm muscle per min.

In Figure 1, curve (A), is shown the effect of a sustained isometric contraction at

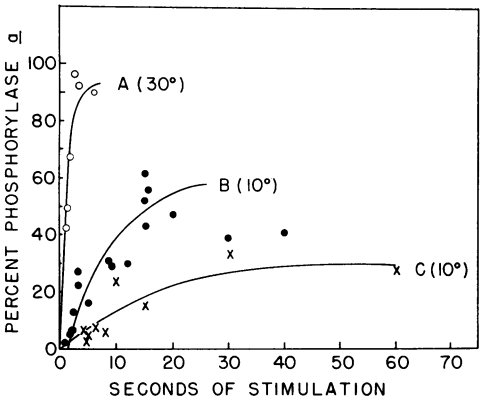

FIG. 1.—Increase in phosphorylase *a* content of frog sartorius during isometric contraction. Each point represents a muscle from a different frog. Stimulation was by 12-volt shocks, 1.5 msec in duration. Curve (A), (○), muscles tetanized with 35 shocks per sec at 30°; Curve (B), (●), muscles tetanized with 15 shocks per sec at 10°; Curve (C), (×), single twitches at 1 per sec at 10°. Theoretical curves are drawn according to equation (1) (see text).

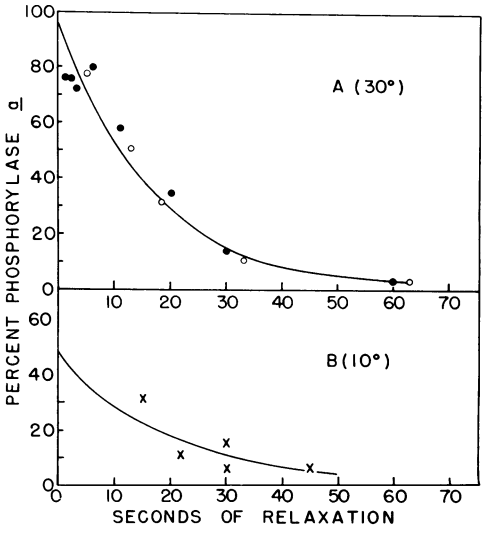

FIG. 2.—Decrease in phosphorylase *a* content of frog sartorius following cessation of isometric contraction. The conditions of stimulation were identical to those in Figure 1. (○) preceding tetanus was 2 sec; (●) preceding tetanus was 4 sec; (×) preceding tetanus was 15 sec. Theoretical curves are drawn according to equation (2) (see text).

30° on the phosphorylase *a* content of muscle; values of 50 per cent *a* are reached after 1 sec and greater than 80 per cent after 2.5 sec of stimulation. Tetanic stimulation at 10°, curve (B), results in a slower rise and lower steady-state level of phosphorylase *a* than stimulation at 30°. With single twitches at 10°, curve (C), the rate of increase and the steady-state level of phosphorylase *a* are still less. Curves (A) and (B) in Figure 2 show the rate of decrease in phosphorylase *a* after a stimulation of sufficient duration to produce maximum phosphorylase *a* activity for the respective temperatures. After about 60 sec, the phosphorylase *a* content has returned to the basal level of five per cent or less of the total phosphorylase content.

The kinetics of the rise and fall of phosphorylase *a* can be treated as follows: phos-

phorylase $b \underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ phosphorylase a. The velocity constants, k_1 and k_2, during stimulation can be calculated by means of the equation:

$$k_1 b_i / a_{st.st.} = t^{-1} \ln a_{st.st.} / (a_{st.st.} - a_t), \quad (1)$$

where $k_1 b_i / a_{st.st.} = k_1 + k_2$. Here, b_i is the initial activity of phosphorylase b, a_t the activity of phosphorylase a at time t, and $a_{st.st.}$ the final activity, which was 94, 60, and 30 per cent for curves A, B, and C in Figure 1 respectively. During relaxation $k_2 \gg k_1$, as shown by the almost complete disappearance of phosphorylase a (Fig. 2). Therefore, equation (1) becomes:

$$k_2 = t^{-1} \ln a_t / a_t. \quad (2)$$

The kinetic treatment is based on the assumption that the two opposing reactions follow the first-order reaction rate equation. This has been shown to be the case for the phosphorylase phosphatase reaction *in vitro*[4] and is reasonably well supported by the curve in Figure 2*A* for *in vivo* conditions. On the other hand, the assumption that k_1 represents a first-order velocity constant may be an oversimplification. During stimulation, for example, it is not known how fast the activation of the kinase is relative to its rate of action on phosphorylase *b*. Furthermore, fatigue during more prolonged stimulation may result in a slowing down of kinase activity. (See the points at 30 and 40 sec in curve (B), Fig. 1.) The chief factor to be considered, however, is the unavoidable variability of the enzyme content of different muscles. It should be emphasized that each point in the curves in Figures 1, 2, and 3 represents a different muscle so that any variation in the

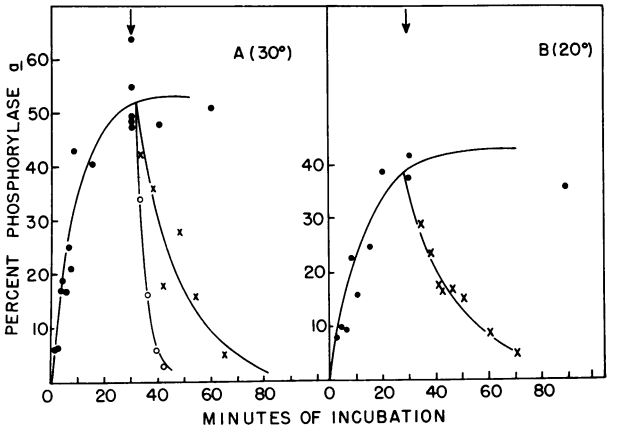

FIG. 3.—Effect of epinephrine on phosphorylase *a* content of frog sartorius. Individual sartorii were incubated anaerobically in Ringer's solution containing 1.1×10^{-6} *M* epinephrine and 0.1 mg per ml ascorbate. After 30 min incubation (see arrow) some muscles were rinsed and transferred to a similar medium but without epinephrine. (●) Epinephrine present; (×) epinephrine removed; (○) epinephrine removed and 1.1×10^{-4} *M* dichloroisoproterenol added. Theoretical curves are drawn according to equations (1) and (2) (see text).

kinase and phosphatase content will be reflected in a scatter of results and in a different steady-state.

All curves in Figures 1, 2, and 3 were drawn according to equation (1) or (2). The apparent steady-state values of the ascending curves were obtained from preliminary plots of the data. The points were then plotted on a semilogarithmic graph. The best fit straight line was determined by the method of least squares. Values for $k_1 + k_2$ and for k_2 were calculated from the slope of this line.

TABLE 2

EFFECT OF STIMULATION ON THE INTERCONVERSION OF PHOSPHORYLASE *a* AND *b* IN FROG SARTORIUS

First-order velocity constants for the conversion of phosphorylase *b* to *a* (k_1) and *a* to *b* (k_2) have been calculated from the data of Figures 1 and 2. k_2 has been calculated from the data obtained during contraction as well as during relaxation. At 30°, the high ratio of phosphorylase *a* to *b* make estimation of k_2 during stimulation inexact

Temperature (°C)	Type of stimulation	Stimulation (sec^{-1}) k_1	Stimulation (sec^{-1}) k_2	Relaxation (sec^{-1}) k_2	
30	Tetanus	0.97	(0.063)	0.057*	0.058†
10	Tetanus	0.080	0.054	0.050	
10	Single twitches at 1 per sec	0.021	0.048	—	

* After 2-sec tetanus.
† After 4-sec tetanus.

The values of k_1 and k_2 are shown in Table 2. Apart from temperature-dependent changes, the value for k_2 remained relatively constant. Thus, at 30° the decline in phosphorylase *a* activity during rest was the same whether preceded by a two- or a four-sec tetanus. At 10°, k_2 calculated from the rising curve of phosphorylase *a* activity during tetanus was not significantly different from k_2 calculated from the decline in phosphorylase *a* activity after cessation of stimulation. Also at 10°, the type of stimulation (single twitches at 1 per sec or a sustained contraction in the form of a tetanus) had no marked effect on k_2.

By contrast, k_1, indicative of the $b \rightarrow a$ transformation, changed markedly with the type of stimulation and with temperature and fell to low values immediately after cessation of stimulation. No exact estimate can be made for the value of k_1 during relaxation except that since the equilibrium is 95 per cent or more to the side of phosphorylase *b*, k_1 would be maximally $^1/_{20}\ k_2$. The rapidity with which phosphorylase *b* kinase activity decreases during rest would explain why repetitive single twitches give a lower k_1 value than a tetanus.

Temperature coefficients for the two enzymes involved in the $b \rightleftharpoons a$ transformation are remarkably different. From k_1 in Table 2, one calculates a Q_{10} of 3.5 for the kinase activity and from k_2 a Q_{10} of 1.1 for the phosphatase activity. As a consequence, the steady-state level of phosphorylase *a* attained during stimulation changes markedly with temperature.

Effect of epinephrine: Incubation with epinephrine or norepinephrine increased the phosphorylase *a* content of frog sartorius muscle (Table 3). Epinephrine was equally and presumably maximally effective at a concentration of 10^{-5} and 10^{-6} *M*, whereas norepinephrine had a pronounced effect at the higher concentration

TABLE 3

DIFFERENTIAL EFFECT OF DICHLOROISOPROTERENOL ON EPINEPHRINE ACTION AND STIMULATION

Paired muscles from a single frog were placed in Ringer's solution containing 0.1 mg per ml ascorbate and gassed with 95 per cent argon-5 per cent CO_2. Both muscles were incubated at 30° for 30 min, one in the presence and one in the absence of 1.1×10^{-4} *M* dichloroisoproterenol (DCI). The flasks were then opened and epinephrine or norepinephrine was added. The media were again equilibrated with the gas phase and reincubation was carried out for an additional 30 min. The muscles to be stimulated were incubated in DCI alone and then mounted in a moist chamber at 30° and gassed with 95 per cent argon-5 per cent CO_2. They were fixed after a 2- or 4-sec tetanus.

Additions	Phosphorylase *a* in per cent of total: No DCI	+ DCI
Norepinephrine 1.1×10^{-6} *M*	10	4
Norepinephrine 1.1×10^{-5} *M*	46	6
Epinephrine 1.1×10^{-6} *M*	64	3
Epinephrine 1.1×10^{-5} *M*	62	8
Muscle contraction	84*	72

* See Figure 1.

only. Dichloroisoproterenol,[18] an inactive analogue, which probably acts by competitive inhibition, completely blocked epinephrine and norepinephrine action at a concentration 10 times greater than that of the sympathomimetic amines. The effect of stimulation on phosphorylase *a* could not be blocked by this antagonist.

In Figure 3 is shown a time curve for the action of epinephrine on phosphorylase *a*. The time scale is in minutes as compared to seconds in the case of stimulation. In fact, on comparing the rise of phosphorylase *a* in the two cases, there is an approximately 500-fold difference in rate (t 1/2 at 30° is 0.7 sec for stimulation and 370 sec for the action of epinephrine, Table 4). The rate-limiting step in the

TABLE 4

COMPARISON OF EFFECT OF STIMULATION AND EPINEPHRINE ON RATE OF CHANGE IN PHOSPHORYLASE *a* IN FROG SARTORIUS

The half-time of rise and fall in phosphorylase *a* was calculated from the data given in Figures 1, 2, and 3.

	Rise in Phosphorylase *a*		Fall in Phosphorylase *a*	
Temperature (°C)	Experimental conditions	Half-time (sec)	Experimental conditions	Half-time (sec)
30	Stimulation	0.7	Relaxation	12
30	Incubated with epinephrine	370	Incubated with epinephrine, then washed	660
30	—	—	Incubated with epinephrine, then washed with DCI*	150
20	Incubated with epinephrine	520	Incubated with epinephrine, then washed	870

* Dichloroisoproterenol.

action of epinephrine on the $b \rightarrow a$ conversion could be a preceding enzymatic step concerned with formation of cyclic 3′,5′-AMP or penetration to and fixation of epinephrine at the binding sites.

Figure 3 shows that the action of epinephrine can be reversed under anaerobic conditions with a return of phosphorylase *a* to the resting level of five per cent or less. The muscles were first incubated with epinephrine until a maximum effect was produced; they were then quickly rinsed with large quantities of Ringer's solution and transferred to fresh medium without epinephrine but containing in some cases 1.1×10^{-4} *M* dichloroisoproterenol. It is interesting to note that the presence of this antagonist hastened the reversal, presumably by displacing epinephrine from its binding sites. The half-time for reversal at 30° was 660 sec in the absence of dichloroisoproterenol and 150 sec in its presence (Table 4).

During incubation with epinephrine at 20°, the final level of phosphorylase *a* was somewhat lower than at 30°, but as can be seen from Figure 3 and Table 4, the effect of temperature on the rate of either the increase or the decrease in phosphorylase *a* was not great. At 10° (not illustrated), the response to epinephrine was slowed down very markedly.

Discussion.—The principal energy-yielding reaction during anaerobic work in an isolated muscle is the conversion of glycogen to lactic acid. During a short tetanus, the rate of glycogenolysis may increase several hundred times and then on relaxation return to the resting rate.[6] These changes in rate are paralleled by a corresponding rise and fall in the phosphorylase *a* content of muscle. From the kinetic analysis presented in this paper, it seems likely that the actual regulatory control over glycogenolysis is exerted by the phosphorylase *b* kinase system which

is rapidly activated and deactivated during and following contraction. Calcium ions have been implicated in this mechanism because they are liberated during contraction and activate the kinase *in vitro*.[19] However, it has not so far been possible to deactivate the kinase *in vitro* by removal of calcium ions.[19]

The differential inhibition of the action of epinephrine by dichloroisoproterenol suggests that muscle work and epinephrine activate the kinase by different mechanisms. The much slower rise and fall in phosphorylase *a* during and after epinephrine action may be mediated by the enzymatic formation and subsequent removal of cyclic 3′,5′-AMP. In fact, it has been shown recently that dichloroisoproterenol inhibits the effect of epinephrine on the formation of cyclic 3′,5′-AMP in a particulate enzyme preparation from dog heart.[20]

Summary.—Phosphorylases *a* and *b* have been determined in frog sartorii fixed by rapid freezing. Extraction was at −35° with a glycerol-fluoride-EDTA solution. In resting muscle, less than five per cent of the total phosphorylase was present as the active or *a* form. Starting with this low and uniform baseline, it was possible to determine the kinetics of the $b \underset{k_2}{\overset{k_1}{\rightleftharpoons}} a$ interconversion, mediated by phosphorylase *b* kinase (k_1) and phosphorylase phosphatase (k_2). It was found that during contraction $k_1 \gg k_2$ and during relaxation $k_2 \gg k_1$ as the result of changes exclusively in k_1.

Increase in phosphorylase *a* during incubation with epinephrine at 30° had a half-time of 370 seconds as compared to 0.7 sec, during an isometric contraction. Dichloroisoproterenol inhibited the effect of epinephrine and norepinephrine and also hastened the reversal of epinephrine action but had no effect on the increase in phosphorylase *a* produced by muscle work. Two different mechanisms appear to be involved in the activation of the phosphorylase kinase by muscle work and by epinephrine.

* This work was supported in part by research grants E-3765 and A-1984 from the National Institutes of Health, U.S. Public Health Service.

† Special postdoctoral fellow of the National Heart Institute, U.S. Public Health Service.

[1] Abbreviations are as follows: AMP, adenosine monophosphate; EDTA, ethylenediaminetetraacetate.

[2] Brown, D. H. and C. F. Cori, in *The Enzymes*, ed. P. D. Boyer, H. A. Lardy, and K. Myrbäck (2d ed.; New York: Academic Press, Inc., 1961), vol. 5, pp. 207–228.

[3] Krebs, E. G., and E. H. Fischer, *J. Biol. Chem.*, **216**, 113 (1955).

[4] Cori, G. T., and C. F. Cori, *J. Biol. Chem.*, **158**, 321 (1945).

[5] Keller, P. J., and G. T. Cori, *Biochim. Biophys. Acta*, **12**, 235 (1953), *J. Biol. Chem.*, **214**, 127 (1955).

[6] Cori, C. F., in *Enzymes, Units of Biological Structure and Function*, ed. O. H. Gaebler (New York: Academic Press, Inc., 1956), pp. 573–583.

[7] Rulon, R. R., D. D. Schottelius, and B. A. Schottelius, *Am. J. Physiol.*, **200**, 1236 (1961).

[8] Cori, G. T., and B. Illingworth, *Biochim. Biophys. Acta*, **21**, 105 (1956).

[9] Krebs, H. A., and K. Henseleit, *Hoppe Seyler's Z. physiol. Chem.*, **210**, 33 (1932).

[10] Narahara, H. T., P. Özand, and C. F. Cori, *J. Biol. Chem.*, **235**, 3370 (1960).

[11] Grass Instrument Company, Quincy, Mass.

[12] Sanborn Company, Waltham, Mass.

[13] Seraydarian, K., W. F. H. M. Mommaerts, A. Wallner, and R. J. Guillory, *J. Biol. Chem.*, **236**, 2071 (1961).

[14] McDonald Engineering Company, Bay Village, Ohio.

[15] Lowry, O. H., and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).

[16] Illingworth, B., and G. T. Cori, *Biochem. Preparations*, **3**, (1953).
[17] Fischer, E. H., and E. G. Krebs, *J. Biol. Chem.*, **231**, 65 (1958).
[18] Powell, C. E., and I. H. Slater, *J. Pharmacol. and Exper. Therap.*, **122**, 480 (1958).
[19] Krebs, E. G., D. J. Graves, and E. H. Fischer, *J. Biol. Chem.*, **234**, 2867 (1959).
[20] Murad, F., Y.-M. Chi, T. W. Rall, and E. W. Sutherland, *J. Biol. Chem.*, **237**, 1233 (1962).

---

*A NEW ENZYME OF RNA SYNTHESIS: RNA METHYLASE**

BY ERWIN FLEISSNER† AND ERNEST BOREK

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY

Communicated by David Rittenberg, May 24, 1962

Soluble RNA is characterized by the presence in small amounts of a variety of methylated purines and pyrimidines. The occurrence of these minor components appears to be universal: the relative amounts, however, are variable. It has been recently demonstrated in our laboratory that the methyl groups of all of these bases in bacterial RNA originate from methionine via the same methyl pool.[1] Biswas, Edmonds, and Abrams[2] have extended these observations to ascites cells and showed methionine to be the methyl donor for the methylated guanines in those cells as well.

The synthesis of 5-methyl uracil (thymine) of RNA in this manner is particularly significant, since the same base intended for incorporation into DNA is produced by an entirely different pathway, from deoxyuridine monophosphate and N^5, N^{10}-methylene tetrahydrofolate.[3]

It was observed several years ago that the methionine auxotroph *E. coli* $K_{12}W6$, unlike other amino acid auxotrophs known at that time, continues the synthesis of RNA during starvation of its essential amino acid.[4] The accumulation is not unique for methionine, for Stent and Brenner have recently shown that double amino acid auxotrophs derived from *E. coli* $K_{12}W6$ accumulate RNA almost as well on deprivation of other amino acids.[5]

Analysis of the RNA accumulating on methionine starvation revealed that even though most of the RNA has sedimentation characteristics of the soluble species,[6] the methylated minor components were lacking from it.[7] The origin of all the methyl groups of the soluble RNA from the same methyl pool and the observed synthesis of soluble RNA lacking the methylated bases on methionine starvation suggested to us the possibility that methylation occurs not at the level of some monomer but at the level of the polynucleotide.[8] According to this view, the accumulation of nonmethylated RNA must be the sequential result of two separate deficiencies: first, the lack of control and continued synthesis of RNA in the absence of an amino acid, and, second, the failure of methylation of the accumulating RNA due to the absence of the methyl source.

We wish to report here evidence from experiments both *in vivo* and with cell-free extracts for the existence of an enzyme which can achieve methylation of S-RNA at the polynucleotide level.

In Vivo Experiments.—In these experiments, *E. coli* $K_{12}W6$ were allowed to accumulate RNA as a result of methionine starvation.[4] The cells enriched with RNA were removed from the starvation medium, washed, and incubated in fresh