The Creatine Phosphoryltransfer Reaction in Iodoacetate-Poisoned Muscle

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ABSTRACT The iodoacetate-nitrogen–poisoned muscle offers the possibility of studying the stoichiometry of the single muscle twitch since metabolic resynthesis by glycolysis and oxidative phosphorylation are blocked, and there remains as an energy source only the creatine phosphoryltransfer system, creatine phosphate reacting with adenosinediphosphate to give the triphosphate and creatine. It is shown, preparatory to a determination of the amount of phosphocreatine split in a single twitch, that iodoacetate does not inhibit creatine phosphoryltransferase at concentrations which block glycolysis. An analysis is developed which assumes that the transferase maintains the creatine phosphoryl transfer reaction in equilibrium following contraction, and further that the creatine phosporyltransfer reaction and the myokinase reaction are isolated in muscle. On the basis of this analysis and the data obtained, an estimate of the equilibrium constant of the creatine phosphoryl reaction in muscle is obtained which agrees with values determined *in vitro*. Using the estimated equilibrium constant, and the concentrations of creatine, creatine phosphate, and adenosinetriphosphate found, a value for the concentration of free adenosinediphosphate is obtained which is considerably less than that found by direct chemical analysis.

The characteristic function of muscle is the performance of work at the expense of chemical energy. At present there is a good deal of evidence to support the view that the muscle is a macromolecular machine of actin and myosin filaments powered by adenosinetriphosphate. It is generally presumed that adenosinetriphosphate is dephosphorylated to adenosinediphosphate during the contraction cycle. The thermal and mechanical behavior of the single twitch has been thoroughly studied. The *in vivo* stoichiometry of energy-yielding reactions which are closely coupled to the contractile event of the twitch remains to be explored.

It is the purpose of the studies reported in this series of papers to examine the stoichiometry of the single twitch and to determine its dependence on mechanical parameters. The basis for a study of the stoichiometry of the

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single twitch is found in the work of Lundsgaard (1931) who established the dependence of phosphocreatine splitting on the integrated peak twitch tension under conditions in which both the glycolytic and oxidative pathways for adenosinetriphosphate resynthesis were blocked by combined iodoacetate and nitrogen poisoning. Lohman (1934) and others subsequently established that adenosinediphosphate reacted with phosphocreatine, in the presence of muscle extracts, to yield the triphosphate and creatine. Accordingly, Lohman suggested that the sequence of events occurring in contraction is: (1) Dephosphorylation of ATP[1] by the contractile mechanisms, followed by (2) resynthesis of ATP by conversion of PC to C. The splitting of PC by the reaction,

$$PC + ADP \rightleftharpoons ATP + C \qquad (1)$$

should therefore parallel the splitting of ATP by the contraction process.

If reaction (1) can be shown to be enzymatically catalyzed in muscle, under conditions in which neither ATP nor PC can be resynthesized, then a study of the dependence of PC splitting on total tension developed, or work done, in a series of twitches can be expected to lead to an estimate of the PC split in a single twitch by extrapolation. The details of the extrapolation procedure will be presented in a subsequent paper. In this paper evidence is presented to show that creatine phosphoryltransferase, the enzyme which catalyzes reaction (1) is active *in vivo* following iodacetate poisoning which is sufficient to block glycolysis, and second an estimate is obtained for the *in vivo* equilibrium constant of reaction (1) that agrees roughly with the *in vitro* value. These results support the contention that the CPT system is operative in IAA-poisoned muscle and hence may be used to study ATP splitting during contraction.

It might be argued on the basis of the results of Mommaerts (1954, 1955) and Fleckenstein *et al.* (1954) which indicate no ATP dephosphorylation during contraction, that ATP is not the primary source of energy for muscular contraction and hence the study of PC splitting is of little interest. The results of Mommaerts and Fleckenstein do not invalidate Lundsgaard's finding that PC splitting depends on the total tension developed. In any case, the splitting of PC remains the major, if not the only chemical event known to correlate with a mechanical parameter of the contractile cycle of intact muscle. A determination of the amount of PC split during a twitch, under conditions which have been shown to leave creatine phosphoryltransferase functional,

[1] The following nomenclature and abbreviations, introduced by Ennor and Morrison (1958) will be used: *N*-phosphoryl creatine, PC; creatine, C; adenosinetriphosphate, ATP; adenosinediphosphate, ADP; adenylic acid, AMP; iodoacetic acid, IAA; creatine phosphoryltransferase, CPT. The reaction ADP + PC $\rightleftharpoons$ ATP + C will be referred to as the creatine phosphoryltransfer reaction, or system.

would enable estimation of first, the amount of ATP split, in a twitch; second, the chemical energy released in a single twitch; and third the number of PC molecules split per myosin, or actin molecule, in a single twitch.

General Procedure

It is possible to demonstrate the *in vivo* activity of CPT in muscle poisoned with IAA by comparing the CPT activity of extracts of poisoned muscle, with extracts from normal muscle.

It is also possible to obtain evidence that the creatine phosphoryltransfer system equilibrium holds *in vivo*, from an analysis which first assumes that the poisoned muscle is a closed system consisting of the coupled equilibria of the creatine-phosphoryltransferase and myokinase reactions,

$$CP + ADP \rightleftharpoons ATP + C \tag{1}$$

$$ATP + AMP \rightleftharpoons 2ADP, \tag{2}$$

and that stimulating the muscle to contract results in changes in the ATP and PC concentrations. Interpretation of experimental data on the basis of this analysis leads to an estimate of the "*in vivo*" equilibrium constant of the creatine phosphoryltransfer reaction which agrees with that found under *in vitro* conditions.

Materials and Methods

PREPARATION OF MUSCLES Paired sartorius muscles, carefully dissected from hibernating frogs (*R. pipiens*), were used throughout. The frogs were killed by decapitation, the hind legs removed, and the dissection carefully performed without damaging the muscle, or causing it to contract. Damaged, spontaneously active, or parasitized muscles were discarded. The muscles were stored overnight, in aerated Ringer solution, at 4°C. before use. In general, overnight storage resulted in more reproducible results, higher PC values, with no change in total creatine (C + PC), and no change in mechanical behavior. Low PC and ATP values, and high C values in resting muscle were invariably associated with damage.

Combined IAA poisoning and anoxia were produced by treating the muscle first with 0.5 mM IAA in phosphate-buffered Ringer solution (pH 7.1) at 20°C. for 25 to 30 minutes. The muscle was then mounted in a sealed moist chamber, thermostated at 1–2°C., and continuously washed with IAA-Ringer equilibrated with pure nitrogen (better than 99.97 per cent) for another 20 to 25 minutes before beginning the experiment. These muscles show the classical IAA rigor when stimulated at room temperature and they show no significant lactate production as is shown by the results in Table I. This is a confirmation of the work of Meyerhof and Boyland (1931) showing that lactic acid production is completely inhibited in frog muscle

treated with 0.25 mM IAA for 1 hour at 1°C., for one-half hour at 15 to 18°C. Wet N_2 gas was flushed through the chamber to prevent room air from entering through small leaks. PC, C, and ATP levels in muscles treated in this way were not significantly different from untreated controls. According to Chance (personal communication) the 20 minute treatment with nitrogen completely inhibits oxidative phosphorylation.

As noted by Lundsgaard (1930), shown by Godeaux (1949), and confirmed by Carlson and Siger (1957), muscles so treated contract 150 to 200 times at 0°C. without developing rigor. The absence of rigor is particularly important for the

TABLE I

A COMPARISON OF LACTATE CONTENTS OF STIMULATED AND UNSTIMULATED MCSCLES AFTER POISONING WITH 0.5 mM IAA

Experiment No.	No. of twitches	Lactate	Δ Lactate (Experimental-control)
		μM/gm.	μM/gm.
111C	0	13.8	
111E	142	10.5	−3.3
115C	0	6.3	
115E	114	7.3	+1.0
136C	0	8.5	
136E	56	7.8	−0.7
137C	0	5.6	
137E	41	6.9	+1.3
166C	0	5.8	
166E	175	8.3	+2.5
173C	0	8.0	
173E	33	4.7	−3.3
174C	0	10.2	
174E	25	12.5	+2.3
175C	0	11.2	
175E	34	6.8	−4.4

Average Δ Lactate μM/gm. −0.57 ± .97 S.E.M.

purposes of this study since it insures that the chemical changes which occur during contraction are not obscured by the changes which accompany rigor. It should be noted, that if warmed to room temperature an IAA-poisoned muscle which has been stimulated to exhaustion at 0°C. will develop full rigor.

The pelvic attachment of the muscle was maintained intact, and the split pelvic bone was used to mount the muscle in an isometric lever by means of a small clamp. The distal end of the muscle was attached to an isotonic lever. This arrangement described by Carlson (1957) permitted the recording of the tension and/or the isotonic shortening in a single contraction, series of twitches, or tetanus. Although not required for the purposes of the present paper, the records of the isometric and/or isotonic twitches were necessary for the studies to be reported later in this series.

The muscles were caused to contract by direct electrical stimulation with supramaximal, 0.5 msec. duration shocks applied through platinum electrodes placed near the ends of the muscle. The frequency of stimulation was 0.5 per sec.

Upon completion of a series of twitches under a given load, the muscle was cut free at its tendons, rapidly dried on an ice cold cellulose tissue, and plunged into a dry ice–petroleum ether bath (at −75°C.). The frozen muscle was then rapidly weighed, pulverized in a mortar cooled to −75°C., triturated with 1 ml. of frozen 8 per cent perchloric acid, allowed to thaw, and stand for 10 to 15 minutes at 0°C. The extract was then filtered cold, neutralized to pH 7 with 1 N KOH, and made up to 25 ml. final volume with cold distilled water. If aliquots were not analyzed immediately, the extract was stored at −20°C. Control muscles were treated and extracted in a like manner, but they were not stimulated to contract.

Analytical Procedures

Creatine and phosphocreatine were determined by the method of Ennor (1957). ATP was determined by the method of Strehler and McElroy (1957). ATP (Na salt) and creatine (hydrate) used for standards, were obtained from Nutritional Biochemicals Corporation and from California Biochemical Corporation. The standards were checked and shown to be better than 98 per cent pure. Lactate was determined by the method of Barker and Summerson as described in Colowick and Kaplan (1957).

DETERMINATION OF ENZYME ACTIVITY Creatine phosphoryltransferase activities were determined according to a procedure used by Colowick *et al.* (1943) based on the production of PC from ATP and C according to the reverse of reaction (1). Muscles were extracted by grinding with sand and centrifuging after adding 25 volumes of 0.07 M KCl. To 0.5 cc. of extract, 1.3 cc. of the following was added:

0.4 cc. 0.1 M borate-KCl buffer, pH 8.8
0.3 cc. 0.1 M creatine in borate-KCl buffer
0.1 cc. 0.1 M $MgCl_2$
0.1 cc. 1.0 M NaF
0.4 cc. 0.15 M cysteine, freshly neutralized to pH 8.8.

After 15 minutes' incubation at 30°C., to convert the enzyme to the reduced form, 0.2 cc. of 0.03 M sodium ATP was added to initiate the reaction. Following 30 minutes at 30°C., 1 cc. of 100 per cent cold trichloracetic acid was added, and the samples were made up to 10 cc. with cold distilled water. *In vivo* poisoning was achieved by soaking muscles in the appropriate IAA solution for 45 to 50 minutes at 20°C. and then washing for 1 hour in IAA-free Ringer. *In vitro* poisoning with 0.5 mM IAA was achieved by adding 0.1 cc. of 50 mM neutral IAA to 10 ml. of crude extract prior to the incubation at 30°C. Phosphocreatine was determined by the method of Fiske and SubbaRow (1929). The amount of phosphocreatine so determined was compared with that found in a sample incubated without any added creatine, and hence unable to form creatine phosphate. The unit of enzyme activity was taken as the amount of enzyme, per gram wet weight of the muscle, required to transform 1 μg. of P per minute from ATP to PC.

RESULTS

CREATINE-PHOSPHORYLTRANSFERASE ACTIVITY Table II summarizes the results of CPT activity determinations done on normal muscles and muscles treated with IAA in the various ways indicated. These results show an average of 10 per cent inhibition *in vivo* of CPT following treatment with 0.5 mM IAA. There is 87 per cent inhibition when treated with 0.5 mM IAA for 30 minutes at 30°C. under *in vitro* conditions, and a 90 per cent inhibition under *in vivo* conditions when treated with 4.5 mM IAA at 20°C.

TABLE II

IN VIVO AND *IN VITRO* INHIBITION OF CREATINE PHOSPHORYLTRANSFERASE BY IODOACETATE

Experiment	IAA concentration	Activity units	Inhibition
			per cent
1	None	17.9	—
	0.5 mM *in vivo*	15.5	13
2	None	17.9	—
	0.5 mM *in vivo*	18.3	0
3	None	17.7	—
	0.5 mM *in vivo*	14.1	20
	4.5 mM *in vivo*	2.0	88
	0.5 mM *in vitro*	1.1	94
4	None	10.6	—
	0.5 mM *in vivo*	9.5	9
	4.5 mM *in vivo*	1.1	90
	0.5 mM *in vitro*	2.2	79

The finding of little or no *in vivo* inhibition by 0.5 mM IAA might be explained on the grounds that *in vivo* IAA poisoning actually occurred, but was reversed by dilution during extraction. There are two arguments against such an explanation. First, it is contrary to the known chemistry of IAA-protein interactions. In their reviews on the chemical modifications of proteins Olcott and Fraenkel-Conrat (1947) and Putnam (1953) point out that IAA is an alkylating reagent that reacts *irreversibly* with —SH groups under physiological conditions. Second, the fact that *in vivo* poisoning was produced by 4.5 mM argues against reversal by dilution. It is possible, but highly unlikely, that the *in vivo* poisoning was actually *in vitro* poisoning by traces of IAA not removed by the washing procedure. The muscles were washed for 1 hour in 200 to 250 volumes of IAA-free Ringer, changed every 20 minutes Based on the 20 minute equilibration time for IAA found by Meyerhof and Boyland (1931) this procedure should have reduced the IAA concentration

to less than 4×10^{-7} mM for the case of treatment with 4.5 mM. There is little chance that any poisoning would occur at this concentration, since Ennor and Rosenberg (1954) report only 12 per cent *in vitro* inhibition of the enzyme at 10^{-3} mM IAA.

We conclude, that incubation of intact frog sartorius muscle with 0.5 mM IAA for one-half hour at 20°C., a treatment sufficient to block glycolysis, has no effect on *in vivo* CPT activity.

EQUILIBRIUM OF CREATINE-PHOSPHORYLTRANSFER REACTION If it is assumed that the IAA-nitrogen–poisoned muscle is a closed system with respect to C, PC, ATP, ADP, and AMP, then it follows from an analysis of the coupled equilibria for the creatine phosphoryltransferase and the myokinase reactions that the ATP and PC content of stimulated muscles should follow the relation (see Appendix):

$$\frac{C_t}{ATP} = \frac{1}{K_2}\left(\frac{C_t}{A_t}\right)\left[(K_2 - 1) + \left(\frac{C_t}{PC}\right)\right] \tag{3}$$

in which C_t/ATP) = ratio of total creatine content, (PC + C), to total ATP content of the muscle.

(C_t/PC) = ratio of total creatine content to PC.

A_t = total adenylic acid content (ATP + ADP + AMP).

K_2 = equilibrium constant of the creatine phosphoryltransferase reaction.

Equation (3) states that, if ATP, or PC is split in a muscle unable to resynthesize either, then for varying amounts of contractile activity the various final concentrations of PC and ATP that result will yield a straight line when plotting (C_t/ATP) against (C_t/PC). The value of the intercept

$$\left(\frac{C_t}{A_t}\right)\frac{(K_2 - 1)}{K_2},$$

and the slope,

$$\left(\frac{C_t}{A_t}\right)\frac{1}{K_2},$$

permit the evaluation of K_2 and (C_t/A_t).

Fig. 1 is a plot of all the experimentally determined values of (C_t/ATP) *versus* (C_t/PC) for 68 different muscles each of which was caused to contract a moderate amount by electrical stimulation. The average value of (C_t/PC), and (C_t/ATP) determined for *128 unstimulated control muscles* is designated by the solid rectangle. This point has 128 times the weight of any of the other points shown.

The muscles used in the plot of Fig. 1 were, as stated above, stimulated moderate amounts. As previously stated, when an IAA-poisoned muscle is stimulated to exhaustion at 0–2°C. it fails in a relaxed state. If such an exhausted muscle is warmed, rigor develops immediately. Furthermore, as shown by Bate-Smith and Bendall (1947) and Bendall (1951, 1957) the development of rigor is accompanied by the rapid splitting of ATP, and the formation of AMP and inosinic acid. These reactions if allowed to occur in the muscles used in the experiments reported here would result in a substantial reduction in the ATP concentration over and above that expected from contractile activity and operation of creatine phosphoryltransferase reaction. The possibility of rigor development due to inadvertent warming with the resulting splitting of ATP, constituted a complication which we

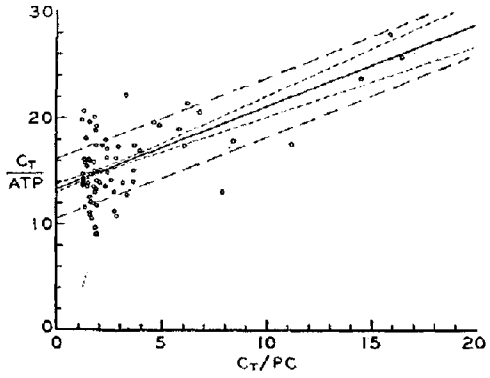

FIGURE 1. Plot of all data obtained on muscles that were stimulated in moderate amounts but not to the point of exhaustion and incipient rigor. These data have been interpreted in terms of a linear relationship between the total creatine to ATP ratio and the total creatine to PC ratio in accord with the analysis presented in the Appendix. The best least squares fit of the data was done under the condition that all the error is in the ordinate. The solid line is the best least squares fit to these data. It has an intercept of 13.25 with a standard error of ± 0.28, and a slope of 0.736 with a standard error of ± 0.095. The parallel dashed lines indicate the standard deviation of the curve. The dotted curves are the 95% confidence limits of the calculated straight line. That is, a repeat of this set of experiments would yield calculated straight lines falling within these limits 95 per cent of the time. Thus the estimated *in vivo* value of the equilibrium constant for the creatine phosphoryltransferase reaction, given by $K_2 = 1 +$ (intercept/slope), is 19 with a standard error of ± 3.

avoided by restricting the range of observations to muscles which were stimulated moderately and not to exhaustion and by conducting all experiments at 0°C. The ATP content of these muscles remained above one-half the level found in resting muscle.

The straight line drawn through the experimental points in Fig. 1 is the

best least squares fit to the points. The slope and intercept, together with their standard errors for this line are: 0.736 ± 0.095 and 13.25 ± 0.28 respectively. This gives a value of 19 ± 3 for K_2, and 14.00 ± 0.29 for (C_i/A_i).

The appreciable scatter of the data, which limits the accuracy with which K_2 can be estimated, requires a comment. From a statistical analysis of the 128 determinations on the unstimulated control muscles it is clear that virtually all the scatter arises from the variability of the ATP values. A possible source of this variability is the quick freezing process. If the quick freezing induces the muscle to twitch and there is a splitting of ATP, then, because of the freezing, it is unlikely that there would be any resynthesis of ATP by PC. The amount of ATP split during quick freezing would vary from muscle to muscle depending on the rapidity of freezing, a process which is subject to some variation. Of course inadvertent thawing of the frozen muscle would also result in thaw rigor and ATP splitting. Both of these sources of variability of ATP values would result in lowered values of ATP (higher values of C_i/ATP). In any case, the scatter is not so great as to prevent a rough estimate of K_2, sufficient for our purposes.

DISCUSSION

It is clear from the results shown in Table II that 0.5 mM IAA causes little if any inhibition of CPT under *in vivo* conditions. There is therefore, no doubt that this enzyme can function in IAA-poisoned muscle. This makes untenable the recent suggestion of Ennor and Morrison (1958) that the conversion of PC to creatine, in the IAA-poisoned muscle, could not involve CPT.

A linear function in Fig. 1 would be a strong indication that the muscle can be considered an isolated system with respect to reactions (1) and (2). The scatter of the points makes the assertion of linearity unreliable. If however, the equilibrium is assumed to be attained in the muscle and the best straight line is drawn for the points in Fig. 1, it is possible to calculate the equilibrium constant from the values of the slope and intercept. The value so obtained is 19 with a standard error of ± 3.

An equilibrium constant of 19 ± 3 for the creatine phosphoryl kinase reaction is at the lower limit of the range of values reported by Kuby, Noda, and Lardy (1954). These authors report at pH 7.4, 30°C., values of the equilibrium constant ranging from 20 to 83 for 2 mM Mg^{++} concentration, and a value of 100 for a 10 to 20 mM Mg^{++} concentration. The Mg^{++} content of frog muscle is reported in Dubisson (1954) to be 12 mM/liter of free muscle water. How much is free and how much is bound to structural proteins and organic phosphates are not known. It is entirely possible that

the Mg^{++} concentration of muscle is low thus favoring a lower value of the equilibrium constant of the creatine phosphoryltransferase reaction.

If the creatine phosphoryltransferase system is operative in IAA-poisoned muscle as the results reported here suggest, then any ADP formed as a result of contraction would be converted to ATP at the expense of PC. The net reaction would be the conversion of PC to C and inorganic phosphate. If the conversion were rapid enough to keep abreast of the ATP formed, the quantities of interest from the point of view of the energetics of the single twitch would be the free energy and heat of hydrolysis of PC.

In Table III the values found for (C_i/PC), (C_i/C), (C_i/ATP), and (C_i/wet weight) for resting muscle are tabulated. Also given are the values of the concentration per unit wet weight of muscle of PC, C, and ATP.

TABLE III

	C_i/PC	C_i/C	C_i/ATP	C_i/weight	PC/weight	C/weight	ATP/weight
Average	1.279	4.49	14.08	32.01 $\frac{\mu M}{gm.}$	24.97 $\frac{\mu M}{gm.}$	6.78 $\frac{\mu M}{gm.}$	2.24 $\frac{\mu M}{gm.}$
Standard error	±0.021	±0.34	±0.27	±0.30	±0.46	±0.39	±0.08
No. of observations	128	128	128	234	141	141	128

ADP CONCENTRATION Using the calculated values of the PC, C, and ATP concentrations and the value of 19 ± 3 found for the equilibrium constant of the creatine phosphoryltransfer reaction a value of 0.035 ± 0.012 $\frac{\mu M}{gm.}$ is obtained for the concentration of ADP in resting muscle. This value is substantially less than the 0.5 to 0.9 μM/gm. concentration reported by Munch-Peterson (1953). In fact an ADP concentration of 0.5 would require that K_2 be equal to 1.2, a value far too low to account for the data shown in Fig. 1 for it would require an intercept of 1.9 and a slope of 9.5. Such a low value for free ADP in muscle is consistent with the finding of Chance and Connelly (1957) that oxidative phosphorylation in muscle behaves as if it were acceptor (ADP) limited. An active creatine phosphoryltransfer system would account for Chance and Connelly's finding of only slight increases in ADP following contraction. A low free ADP level in resting muscle together with high ADP levels found in extracts suggests a bound form of the nucleotide, possibly with actin, or else that ADP is produced by the extraction procedure.

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APPENDIX I

It is assumed that the only reactions occurring in the IAA-poisoned muscle are the splitting of ATP by contraction and the rephosphorylation of ADP by the creatine phosphoryltransfer reaction and the myokinase reaction. We are concerned therefore with the analysis of the closed, coupled equilibrium

$$\mathrm{ATP} + \mathrm{AMP} \rightleftharpoons 2\mathrm{ADP};\ [\mathrm{ADP}]^2/[\mathrm{AMP}][\mathrm{ATP}] = K_1 \tag{2.1}$$

$$2\mathrm{ADP} + 2\mathrm{PC} \rightleftharpoons 2\mathrm{ATP} + 2\mathrm{C};\ [\mathrm{C}][\mathrm{ATP}]/[\mathrm{ADP}][\mathrm{PC}] = K_2 \tag{2.2}$$

$$\mathrm{AMP} + 2\mathrm{PC} \rightleftharpoons \mathrm{ATP} + 2\mathrm{C};\ [\mathrm{C}]^2[\mathrm{ATP}]/[\mathrm{PC}]^2[\mathrm{AMP}] = K_2 \cdot K_1 \tag{2.3}$$

The assumption that the system is closed implies:

$$[\mathrm{C}] + [\mathrm{PC}] = [\mathrm{C}_t], \qquad \text{a constant} \tag{2.4}$$

$$[\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}] = [\mathrm{A}_t], \qquad \text{a constant} \tag{2.5}$$

Elimination of [C] from (2.3) with the aid of (2.4) and inverting yields:

$$\frac{[\mathrm{PC}]^2[\mathrm{AMP}]}{([\mathrm{C}_t] - [\mathrm{PC}])^2[\mathrm{ATP}]} = \frac{1}{K_2^2 \cdot K_1} \tag{2.6}$$

Solution of (2.5) for [AMP]/[ATP] and substitution in (2.6) yields after rearranging and eliminating [ADP] and [C] with the aid of (2.2) and (2.4):

$$\frac{[\mathrm{C}_t]}{[\mathrm{ATP}]} = \frac{[\mathrm{C}_t]}{[\mathrm{A}_t]}\left\{\frac{1}{K_2^2 \cdot K_1}\left(\frac{[\mathrm{C}_t]}{[\mathrm{PC}]}\right)^2 + \left(\frac{1}{K_2} - \frac{2}{K_2^2 \cdot K_1}\right)\left(\frac{[\mathrm{C}_t]}{[\mathrm{PC}]}\right) + \frac{1}{K_2^2 \cdot K_1} + \frac{K_2 - 1}{K_2}\right\} \tag{2.7}$$

Let $[\mathrm{C}_t]/[\mathrm{ATP}] = y$, and $[\mathrm{C}_t]/[\mathrm{PC}] = x$

then,

$$y = \frac{[\mathrm{C}_t]}{[\mathrm{A}_t]}\left\{\frac{x^2}{K_2^2 \cdot K_1} + \left(\frac{1}{K_2} - \frac{2}{K_2^2 \cdot K_1}\right)x + \frac{1}{K_2^2 \cdot K_1} + \frac{K_2 - 1}{K_2}\right\} \tag{2.8}$$

If $K_2^2 \cdot K_1 \ll 1$, then equation (2.8) can be approximated by:

$$y = \frac{[\mathrm{C}_t]}{[\mathrm{A}_t]}\left\{\frac{(K_2 - 1)}{K_2} + \frac{x}{K_2}\right\} \qquad \text{or,} \tag{2.9}$$

$$\frac{[\mathrm{C}_t]}{[\mathrm{ATP}]} = \frac{[\mathrm{C}_t]}{K_2[\mathrm{A}_t]}\left\{(K_2 - 1) + \frac{[\mathrm{C}_t]}{[\mathrm{PC}]}\right\}$$

Kuby, Noda, and Lardy (1954) have shown that K_2 is never less than 20 and $K_1 = 2.3$ according to Eggleston and Hems (1952). Consequently $K_2^2 \cdot K_1 = 920$ and equation (2.9) is a fair approximation (better than 13 per cent) for x less than 15.

Since for a closed system the ratio of the concentrations is equal to the ratio of the total quantities we can write,

$$\left(\frac{C_t}{ATP}\right) = \frac{1}{K_2}\left(\frac{C_t}{A_t}\right)\left[(K_2 - 1) + \left(\frac{C_t}{PC}\right)\right] \tag{2.10}$$

Equation (2.10) is a straight line with intercept $C_t(K_2 - 1)/A_tK_2$ and slope C_t/A_tK_2. A plot of (C_t/ATP) against (C_t/PC) should give a straight line the slope and intercept of which will permit an evaluation of K_2 and (C_t/A_t). If a is the intercept and b the slope determined from the reciprocal plot, K_2 is given by:

$$K_2 = 1 + a/b \tag{2.11}$$

If C_t is known, A_t can be evaluated from the value obtained for the slope b.

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