The Mechanochemistry of Muscular Contraction

I. *The isometric twitch*

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ABSTRACT The dependence of PC[1] and ATP[1] dephosphorylation on the number of isometric twitches in the iodoacetate-nitrogen–poisoned muscle has been examined. There is no net dephosphorylation of adenosinetriphosphate. PC dephosphorylation varies linearly with the number of twitches and produces equivalent amounts of C[1] and P_i.[1] Iodoacetate concentrations which block the enzyme, creatine phosphokinase, render the muscle non-contractile. A value of 0.286 μmole/gm. for the amount of PC split per twitch is obtained which gives a value of −9.62 kcal./mole for the "physiological" heat of hydrolysis of PC in agreement with expectations based on thermochemical data. In a single maximal isometric twitch it is estimated that 2 to 3 PC molecules are dephosphorylated per myosin molecule, or 1 per actin molecule. The results support the view that under the conditions of these experiments PC dephosphorylation is the *net* energy yielding reaction. The *in vivo* stoichiometry of the mechanochemistry of contraction revealed by these studies on the one hand, and the known stoichiometry of actin polymerization and its coupling to the creatine phosphokinase system on the other are strikingly similar and strongly suggest that the reversible polymerization of actin is involved in a major way in the contraction-relaxation-recovery cycle of muscle.

The work of Lundsgaard (1930*a*, *b*), Lohman (1934), Engelhardt and Ljubimova (1939) laid the foundation for the now classical view that adenosinetriphosphate and phosphocreatine are the primary and secondary energy sources, respectively, for muscular contraction. Studies on actomyosin threads and glycerinated models by A. Szent-Gyorgyi (1949) and his colleagues, and

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[1] The following abbreviations will be used here: ATP for adenosinetriphosphate, ADP for adenosinediphosphate, PC for *N*-phosphoryl creatine, C for creatine, CPT for creatine phosphoryltranferase, P_i for orthophosphate, IAA for iodoacetic acid.

H. H. Weber (1951) and others have in general corroborated this view. However, recent studies by Mommaerts (1955), Fleckenstein, Janke, Davies, and Krebs (1954), Davies, Cain, and Delluva (1959), and Chance and Connelly (1957) on *living muscle* have failed to demonstrate the expected changes in phosphocreatine and adenosinetriphosphate concentrations during contraction.

In the work of Mommaerts and Fleckenstein *et al.* no appreciable change was found in creatine, phosphocreatine, or adenosinetriphosphate content accompanying the contraction cycle. Chance and Connelly on the other hand, found an increase in adenosinediphosphate accompanying a single contraction which was far less than the expected value, and too small to account for the energy of contraction. The conditions of these latter experiments did not preclude the possibility that ADP was rapidly rephosphorylated by the creatine phosphokinase system and indeed the authors cite the chemical results of Mommaerts and Fleckenstein *et al.* as evidence against this. It appears therefore, that a major argument that has been put forth against the classical picture of the chemical energetics of muscle is based on the failure to find PC dephosphorylation, in the expected amount, during the contraction cycle of living muscle. The details of the dephosphorylation of PC which accompanies a single twitch are therefore essential facts, which must be established unequivocally if cogent theories of the mechanochemistry of muscle are to be formulated.

In an earlier paper by Carlson and Siger (1959), also Padieu and Mommaerts (1960), it was shown that the enzyme, CPT, is not inhibited under *in vivo* conditions by iodoacetate concentrations which are sufficient to block completely glycolysis. In addition, we obtained an estimate of the *in vivo* equilibrium constant of the creatine–phosphoryl–ADP–transfer reaction which agreed roughly with values found under *in vitro* conditions, and which favors ATP formation under *in vivo* conditions. On the basis of these results it can be concluded that if ATP is the primary or essential source of energy for muscular contraction, and is indeed split during contraction, it can be resynthesized at the expense of PC, in the poisoned muscle, in accord with the classical view. It follows that in the IAA-nitrogen–poisoned muscle, since there can be no resynthesis of ATP, or PC, any ATP that might be split during contraction should be reflected ultimately in a drop in PC concentration. Because of the magnitude of the equilibrium constant of the CPT reaction it also follows that if PC were split directly by contraction the ATP concentration would remain almost constant until low levels of PC were reached and then there would be a drop. The determination of the average amount of PC split per twitch under conditions which leave the CPT system functional, and at the same time prevent all other processes leading to ATP resynthesis,

would be equivalent to a determination of the amount of ATP split during the primary contractile event, if indeed it is split. In this paper we report in order, the methods, results, and discussion of a study of the dependence of PC and ATP breakdown, in the IAA-nitrogen-poisoned muscle, on the number of isometric twitches in a series of twitches. Preliminary reports of this work were given by Carlson and Siger (1957, 1958), and Carlson (1959). The results obtained permit an estimate of the amount of PC split on the average during a single isometric twitch contraction cycle, and imply that the *net* energy yielding reaction is the splitting of PC. They form the basis of a critical reevaluation of the current concepts of the chemical energetics and molecular mechanisms of muscular contraction.

GENERAL PROCEDURE

In the absence of a direct method for following intracellular ATP, or PC turnover, a simple procedure for determining the PC split in a single isometric twitch would be to compare the PC content of identical, paired, IAA-poisoned muscles one of which, the experimental muscle, was caused to perform a single twitch contraction, while the other served as a control and remained at rest. Because in frog sartorius muscle the expected amount of PC split in a twitch is small, between 0.25 and 0.5 μmole/gm., and as the results below show, there is an appreciable variability in the PC and C contents of paired muscles, it has not been feasible, in our experience at least, to obtain significant results for the single twitch experiment.

An alternative procedure, which we have pursued, is to determine how the PC content depends on the number of twitches in a series of 10 or more twitches. From the slope of a plot of the difference in PC content between experimental and control muscles versus number of twitches one can estimate the average PC split per twitch as a function of the number of twitches performed. If the plot is linear and extrapolates through the origin, the slope gives the PC split per twitch directly, and this is the same for all twitches. A non-linear plot would indicate a dependence of PC splitting per twitch on the number of twitches performed and a non-equivalence of twitches in a series. There are two technical difficulties with this procedure that require further consideration.

It is well known from Lundsgaard's original work that IAA-poisoned muscle passes into rigor if caused to perform a series of contractions. From the work of Bate-Smith and Bendall (1947) and Bendall (1951, 1957) it is clear that rigor development itself is associated with ATP and PC splitting. If, therefore, rigor develops during the series of twitches there will be an additional amount of ATP and PC split over and above that due to the twitch contractions. The complications due to rigor such as occurred in some of Lundsgaard's experiments can be avoided by performing the contractions at 1–2°C., where no rigor occurs, as was noted by Lundsgaard (1930), and confirmed by Godeaux (1949) and Carlson and Siger (1957).

The second difficulty stems from the fact that the IAA-nitrogen–poisoned muscle

performs 20 to 30 maximal or near maximal twitches at the most; succeeding twitches gradually diminish in magnitude until after 200 or so, the muscle fails to develop any isometric tension at all. All twitches in a series are not equivalent. Mommaerts (1950) obtained a rough estimate of the amount of PC split per isometric twitch by assuming on the basis of Lundsgaard's results, that the 150 to 200 actual twitches were equivalent to 50 "full" twitches, and therefore 50 "full" twitches would completely exhaust the PC content of the muscle. The figure he obtained in this way was 0.5 μmole of PC split per gram of muscle in a single isometric twitch. It should be noted that this estimate only applies to frog sartorius muscle.

A more objective method for determining the number of equivalent maximal isometric twitches performed in a series of twitches of ever decreasing magnitude is as follows. Let the peak twitch tension of the i^{th} twitch in a series of n twitches be T_i, and let T_m be the peak tension developed in the maximum twitch of the series. The *equivalent number of maximal twitches*, N, performed in the series is defined as,

$$N = \frac{T_1 + T_2 + \cdots T_n}{T_m} = (1/T_m) \sum_{i=1}^{n} T_i.$$

The quantity N introduced here serves as a measure of the total amount of contractile activity experienced by the muscle. It is independent of the weight of the muscle and enables the direct comparison of muscles of different weights. This follows from a consideration of what appears to be happening to individual muscle fibers during failure due to IAA poisoning. The single twitch myogram decreases in amplitude without any pronounced change in shape as failure develops. This suggests that failure is due primarily to the falling out of single fibers either in an all-or-none fashion, or with a gradually diminishing maximal tension per fiber, but not as a result of the development of asynchronous contractions of individual fibers. Such a failure is likely to be the result of a failure to activate all, or part of the contractile material of the single muscle fibers. The peak twitch tension becomes then a measure of the amount of contractile substance activated in a twitch. The summated peak twitch tensions of a series divided by the maximum peak twitch tension is then the number of times that *all* the contractile material of the muscle is activated. This is precisely the quantity N. The linear dependence of PC splitting on N follows directly if it is further assumed that PC splitting is directly proportional to the total amount of contractile substance activated.

MATERIALS AND METHODS

Inasmuch as these have been described in an earlier paper (Carlson and Siger (1959)), they will be repeated here in brief merely for convenience.

Preparation of Muscles Paired frog sartorius muscles were carefully dissected following decapitation and removal of the hind legs *without pithing*. The pelvic bone was split in such a way as to insure that the tendonous attachment of one of the pair, the experimental muscle, was not damaged in anyway. The muscles were stored over-

night at 4°C. in 30 to 40 ml. of aerated Ringer's containing; 115 mM NaCl, 4.8 mM KCl, 1.81 mM $CaCl_2$, 1.25 mM Na_2HPO_4, 5.00 mM NaH_2PO_4, (pH 7.1). Unless otherwise noted combined IAA poisoning and anoxia were produced by treatment with 0.5 mM IAA at 20°C. for 25 to 30 minutes, followed by mounting in a 1–2°C. moist chamber and continuously flushing with cold IAA-Ringer and nitrogen (99.97 per cent) for another 15 to 20 minutes.

The muscles were stimulated directly with 0.5 msec. electric shocks every 3 seconds, unless otherwise noted. One stimulating electrode was located at the distal end, the other about 1 to 1.5 cm. from the pelvic bone. All muscles were held fixed at the rest length obtained under a 5 gm. load. The pelvic bone was clamped in an isometric lever and tension was displayed on a cathode ray oscilloscope and recorded on a Sanborn Industrial Recorder.

After completing a series of twitches the muscle was cut free, rapidly dried on a cold cellulose tissue, and plunged into a dry ice-petroleum ether bath at −75°C. Ordinarily 20 to 40 seconds elapsed between the last twitch and quick freezing. The frozen muscle was weighed rapidly, pulverized in a mortar cooled to −75°C., triturated with 1 ml. of frozen 8 per cent perchloric acid, thawed, and allowed to stand at 0°C. for 10 to 15 minutes. The extract was filtered cold, neutralized to pH 7 with 1 N KOH, and made up to 25 ml. volume with cold water. Extracts prepared in this way were either analyzed immediately or frozen and stored at −20°C. for analysis on the following day.

Analytical Procedures Creatine and creatine phosphate were determined each in duplicate by the method of Ennor (1957). This method combines analysis for the creatine moiety by the α-napthol, diacetyl reaction with an analysis for the P_i moiety of PC by the acid molybdate hydrolysis method of Fiske and SubbaRow (1929). ATP was determined in duplicate by the firefly luciferin-luciferase method of Strehler and McElroy (1957), and phosphate in duplicate by the method of Fiske and SubbaRow (1929). Internal standard runs showed no interfering substances present for any of the analyses nor did the perchloric acid extraction procedure result in the loss of any PC, C, or ATP. Although wet weights were determined, concentrations were expressed in terms of total creatine content, C_t, which is equal to (PC + C). This practice is justified by the data, Table II, which show no significant difference (less than 1 per cent on the average) between the total creatine content of the stimulated and unstimulated members of a pair. The constancy of the total creatine content under the conditions of these experiments is thereby established. Expressing creatine and phosphocreatine concentrations in terms of total creatine, C_t, provides an intensive variable which is independent of muscle weight and has the added advantage of reducing the variance of the data. This is due to the appearance of fewer experimentally determined quantities in the expression for concentration and a smaller sensitivity to error then is the case for concentrations expressed in terms of wet weight.

Rejection of Data In all there were forty experiments successfully completed. Since the near identity of paired muscles was an essential prerequisite for procuring meaningful results from these studies, it was necessary to establish criteria that would eliminate widely dissimilar paired muscles. Accordingly experiments were rejected

on the following grounds: (*a*) An experiment was rejected if the control muscle of a pair showed a value of PC/C_t of 0.75 or less, corresponding to a deviation below the mean of two or more standard deviations. This criterion was dictated by previous experience which showed that damaged muscles invariably showed low values of PC/C_t. On this basis, Experiments 161, 183, and 184 were rejected. (*b*) An experiment was rejected if either the wet weights or total creatine contents of the pair of muscles differed from one another by more than 24 or 30 per cent of their mean respectively. These percentages corresponded to three times the coefficients of variation of the mean differences of the weights and total creatine contents respectively of paired muscles, and are indicative of a genuine difference in size. Experiment 181 was ruled out on these grounds. (*c*) If, for a given equivalent number of twitches, the difference in PC/C_t between experimental and control departed by three standard deviations or more from the best least squares straight line fit to the data, with the point in question omitted, the experiment was rejected. Experiments 33 and 163 were rejected on this basis. In the case of Experiment 33 the muscle had not yet failed completely after 103 equivalent twitches, and the decrease in PC/C_t was considerably less than the expected value. No other fully poisoned muscle has in our experience ever contracted so many times before failure, and the possibility of incomplete poisoning due to an oversight must be considered as an explanation in this case. The rejected experiments are indicated by asterisks in Table I and were omitted from all the averages tabulated.

RESULTS

Table I presents all the data taken on 40 pairs of IAA-nitrogen–poisoned muscles. One member of the pair, the experimental, was made to perform a series of isometric twitches spaced at 3 second intervals while the other, the control, remained at rest. Table II is a statistical summary of the data given in Table I. Differences between the values of PC/C_t for experimental and control muscles are plotted as a function of N in Fig. 1. Unless otherwise stated, the level of statistical significance is taken as 0.05 throughout this paper.

Linear Dependence of PC Splitting on Number of Equivalent Twitches The hypotheses that the data shown in Fig. 1 can be fitted either by a straight line through the origin, or by a straight line with a non-zero intercept on the abscissa, cannot be rejected on statistical grounds according to the F test for linearity given by Mood (1950). It may be reasonably assumed, therefore, that a straight line is the simplest function that describes the data adequately. The best least squares straight line through the origin has a slope of 8.98×10^{-3}, with a standard error of $\pm 0.18 \times 10^{-3}$. This is the line drawn in Fig. 1. The standard deviation of the points about this line is ± 0.049. All the variation is attributed to PC/C_t since the tension was determined to ± 0.3 gm. giving a maximum of ± 1 per cent for the error in N.

TABLE I

DATA ON ISOMETRIC CONTRACTION

Experiment	Wet weight	Peak tension	Total tension	N	PC/C_t	ATP/C_t	C_t
	gm.	*gm.*	*gm.*		*mole/mole*	*mole/mole*	*μmole*
32E	0.1412	41.6	3626	87	0.05	0.018	3.96
32C	0.1349			0	0.84	0.068	4.73
33E*	0.1411	41.6	4286	103	0.12	0.021	4.02
33C*	0.1442			0	0.84	0.059	3.82
34E	0.0744	31.6	2463	78	0.16	0.054	2.35
34C	0.0759			0	0.92	0.084	2.66
36E	0.1014	42.6	2667	63	0.30	0.052	3.50
36C	0.0908			0	0.91	0.076	3.22
37E	0.1118	43.6	2604	60	0.22	0.059	3.81
37C	0.1082			0	0.80	0.070	3.89
38E	0.1019	45.5	3669	81	0.11	0.015	3.35
38C	0.1117			0	0.78	0.075	3.63
39E	0.1058	55.1	4152	75	0.24	0.042	4.20
39C	0.1178			0	0.83	0.015	3.02
40E	0.0940	40.0	2965	74	0.16	0.019	3.96
40C	0.0976			0	0.77	0.053	3.63
41E	0.1376	45.2	759	17	0.69	0.085	5.04
41C	0.1438			0	0.81	0.073	5.22
42E	0.1242	49.3	873	18	0.76	0.056	5.15
42C	0.1166			0	0.83	0.079	4.32
43E	0.0999	41.5	789	19	0.56	0.057	3.74
43C	0.1100			0	0.77	0.058	4.06
161E*	0.1162	40.0	1470	37	0.58	0.067	4.04
161C*	0.1150			0	0.65	0.063	3.62
162E	0.1078	44.6	1613	36	0.50	0.063	4.22
162C	0.1086			0	0.81	0.058	4.06
163E*	0.1084	43.1	1187	28	0.43	0.068	3.59
163C*	0.1224			0	0.91	0.056	4.18
164E	0.1136	46.1	1916	42	0.52	0.089	3.64
164C	0.1064			0	0.94	0.067	3.27
165E	0.1050	38.5	1658	43	0.43	0.068	3.30
165C	0.1120			0	0.80	0.060	3.22
167E	0.1036	48.6	1277	26	0.52	0.084	2.81
167C	0.1040			0	0.79	0.089	2.90
168E	0.1522	64.3	2799	44	0.46	0.059	4.28
168C	0.1494			0	0.86	0.054	4.68
169E	0.0996	25.1	882	35	0.68	—	2.74
169C	0.0798			0	0.94	—	2.76
170E	0.1400	47.1	1724	37	0.57	0.045	4.15
170C	0.1400			0	0.86	0.056	3.70
171E	0.0976	55.5	1497	27	0.60	0.042	2.84
171C	0.0902			0	0.87	0.052	2.93
172E	0.1212	45.7	1701	37	0.62	0.054	3.60
172C	0.1210			0	0.89	0.044	3.56
173E	0.1228	47.0	1400	30	0.60	0.095	3.97
173C	0.1342			0	0.81	0.071	4.26

* These experiments were rejected for reasons given in the text. They are not included in averages.

TABLE I (*concluded*)

Experiment	Wet weight	Peak tension	Total tension	*N*	PC/C_t	ATP/C_t	C_t
	gm.	*gm.*	*gm.*		*mole/mole*	*mole/mole*	*μmole*
174E	0.1074	47.0	1083	23	0.69	0.082	3.32
174C	0.1056			0	0.82	0.094	2.99
175E	0.1446	71.6	2112	30	0.46	0.086	4.65
175C	0.1504			0	0.80	0.093	4.33
176E	0.1392	61.0	2256	37	0.54	0.085	4.13
176C	0.1374			0	0.84	0.094	4.08
177E	0.1395	61.0	2079	34	0.50	0.089	4.48
177C	0.1483			0	0.81	0.073	4.65
178E	0.1482	44.0	2391	46	0.42	0.092	3.04
178C	0.1397			0	0.78	0.088	2.90
179E	0.1328	48.3	473	10	0.75	0.066	3.87
179C	0.1276			0	0.82	0.065	3.90
180E	0.1277	54.4	522	10	0.70	0.069	4.66
180C	0.1273			0	0.76	0.063	4.45
181E*	0.1330	62.0	599	10	0.84	0.077	3.36
181C*	0.1261			0	0.78	0.060	4.77
182E	0.1113	51.2	501	10	0.74	0.067	3.40
182C	0.1390			0	0.87	0.065	3.53
183E*	0.1100	46.4	445	10	0.78	0.074	3.46
183C*	0.1184			0	0.73	0.061	4.25
184E*	0.1224	41.6	394	10	0.78	0.057	4.58
184C*	0.1321			0	0.75	0.069	3.67
242E	0.1072	50.0	3935	79	0.09	0.002	3.56
242C	0.0978			0	0.85	0.103	2.90
243E	0.1032	53.7	514	10	0.78	0.077	3.67
243C	0.1220			0	0.88	0.069	4.34
244E	0.1681	58.2	552	10	0.82	0.082	5.69
244C	0.1796			0	0.87	0.094	5.50
245E	0.1378	59.2	4797	81	0.10	0.030	3.73
245C	0.1283			0	0.89	0.091	3.58
246E	0.1154	57.3	4035	70	0.16	0.028	3.67
246C	0.1267			0	0.85	0.107	4.15
247E	0.1504	50.0	513	10	0.78	0.106	4.87
247C	0.1660			0	0.88	0.109	5.19

TABLE II

STATISTICAL SUMMARY OF DATA OF TABLE I

	PC/C_t (Controls)	ATP/C_t (Controls)	Peak tension/C_t	C_t	ΔC_t (Experimental-control)	C_t/weight
	mole/mole	*mole/mole*	*gm./μmole*	*μmole*	*μmole*	*μmole/gm.*
Mean	0.840	0.075	12.88	3.85	0.025	31.90
Standard error	±0.008	±0.003	±0.42	±0.13	±0.069	±0.54
No. of observations	34	32	34	68	34	68

If it is assumed that the data should be fitted by a straight line with a non-zero intercept, (as might be the case if PC breakdown were spared in the first few twitches by some unknown compound) then the best least squares line has an ordinate intercept of $+0.0208 \pm 0.0160$ (standard error), and a slope of $9.34 \times 10^{-3} \pm 0.33 \times 10^{-3}$ (standard error). The value of $+0.0208$ for the intercept, is not significantly different from zero. It is not possible therefore, to conclude from these data that PC is, or is not spared in the first few twitches by some unknown compound. If, however, it is assumed that the PC is spared in the first few twitches, then the line $\Delta PC/C_t = 2.08 \times 10^{-2} -$

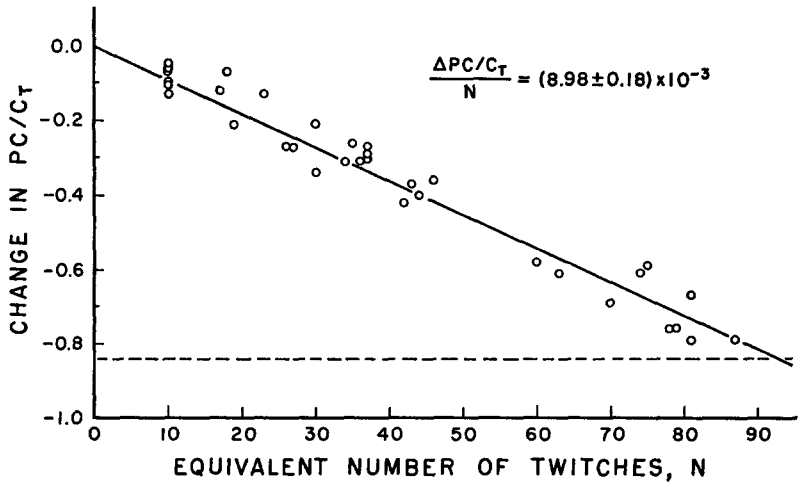


FIGURE 1. Plot of the change in PC/C_t versus number of equivalent twitches, N. The dotted line corresponds to the average maximum change in PC/C_t that would occur if all the PC were split. The solid line is the best least squares fit to the points that passes through the origin, its slope is $8.98 \pm 0.18 \times 10^{-3}$ mole/mole/twitch.

$9.34 \times 10^{-3} N$, intercepts the abscissa at $N = 2.2 \pm 1.7$ twitches. Sparing of PC could, if one allows this interpretation of the data, have occurred for the first 2 to 4 twitches, but it is unlikely that it would have occurred for many more.

Production of Equivalent Amounts of C and P_i It follows from the fact that the total creatine content remains constant that an amount of C is produced equal to the PC split. The plot of Fig. 1 could therefore be regarded as a plot of C increase with number of twitches, the sign of the ordinate being taken as positive.

Lundsgaard (1930*b*) showed that at room temperature the IAA-nitrogen-poisoned muscle, which has passed into rigor, shows little or no increase in P_i. The P_i is esterified mainly as hexosediphosphate and to a lesser extent as the monophosphate. The esterification process is delayed at room tempera-

ture, most of it occurring in the period following contractile activity. At 0°C., however, Lundsgaard found an increase in P_i and little or no esterification. On the basis of these findings it was expected that under the conditions of our experiments (1–2°C.) little or no esterification would occur, and P_i would appear in amounts equivalent to the C produced and the PC split. The presence of P_i in the Ringer solution, and the smallness of the samples available for P_i determinations did not comprise optimal conditions for accurately examining P_i production; it was possible however to confirm the expectation that P_i was produced in amounts equivalent to the C produced, and to the PC split.

In all, P_i analyses were obtained on 26 pairs of muscles, enabling a determination of the mole ratio of orthophosphate produced to creatine produced. These results are summarized in Table III. The data were divided into two

TABLE III

MOLE RATIO OF ORTHOPHOSPHATE PRODUCED TO CREATINE PRODUCED

Equivalent No. of twitches	Mean ($\Delta P_i/\Delta C$)	Standard error of mean	No. of observations
	mole/mole		
$N \leqq 37$ (mean 29)	0.96	±0.09	15
$N \geqq 42$ (mean 70)	0.52	±0.05	11

groups, one in which the muscles performed 37 or fewer equivalent twitches (average of 29) and the other in which they performed 42 or more, (average of 70). In the first case 3 minutes or less elapsed between the beginning of the series of twitches and freezing the muscle for extraction. In the latter case more than 3 minutes, usually 6 to 10 minutes elapsed. Because of the smallness of the samples available for analysis, in most cases we were unable to obtain complete quantitative recovery of P_i from the extracts. Tests for the efficiency of recovery by the method used (forming calcium phosphate with alkaline calcium chloride) showed it to be about 90 per cent on the average. The data of Table III have been corrected accordingly.

For the case of 37 or fewer twitches, the value of 0.96 obtained for the mole ratio of P_i to C is not significantly different from one. For the larger number of twitches the mole ratio of 0.52 is significantly different from one.

We conclude from these results that at 1–2°C., for series of single twitches lasting less than 3 minutes, there is no appreciable esterification of the P_i produced from PC splitting. For longer series there is either esterification or loss of P_i due to leakage. This last possibility was not eliminated in the studies

reported here. The decrease in the ratio of P_i/C with increasing duration of a series of twitches is also in accord with Lundsgaard's finding that esterification is a delayed process. These results support the assertion that, under the conditions of our experiments, the products of the splitting of PC in the intact muscle during the single twitch contraction cycle are equivalent amounts of C and P_i, and that these products enter into no further reactions other than with buffers present in the muscle. Nor is there any other reaction which produces significant amounts of P_i.

PC Dephosphorylation in an Average Maximal Isometric Twitch The trivial differences between the two lines which best fit the data make any choice between them completely arbitrary. However, in the calculations that follow we will accept the simpler of the two hypotheses: namely, that the data are best fitted by a straight line through the origin with a slope of $8.98 \times 10^{-3} \pm$

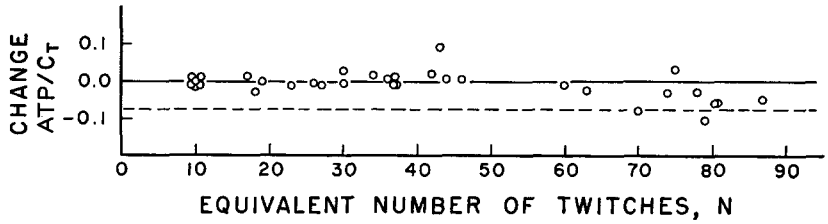


FIGURE 2. Plot of the change in ATP/C_t versus number of equivalent twitches, N. The dotted line corresponds to the average maximum change in ATP/C_t that would occur if all the ATP were split.

0.18×10^{-3} μmolePC/μmoleC_t/twitch. The average C_t per unit wet weight given by the data in Table I is 31.90 ± 0.54 (standard error) μmole/gm.[2] Multiplication of the slope, (the amount of PC split in a maximal twitch per unit of total creatine content) by the average total creatine content per unit wet weight of muscle gives a value of 0.286 ± 0.011 (standard error) μmole/gm. for the average amount of PC split in a single maximal isometric twitch of frog sartorius muscle at 1 to 2°C.

ATP Change during a Series of Twitches The values of the difference between ATP/C_t in experimental and control muscles are plotted as a function of N in Fig. 2. It is evident that for the first 60 to 70 twitches no net change in ATP concentration occurs. Only for larger numbers of twitches, after the PC content has dropped to 25 or 30 per cent of its value in the unstimulated muscle, does the ATP level begin to drop. This is the expected finding if ATP and PC are coupled through the equilibrium of the CPT

[2] This quantity, as well as the concentrations of C, ATP, and P_i, is subject to appreciable seasonal variation which we have reduced somewhat by procuring frogs in the fall and storing them under hibernating conditions.

catalyzed reaction and the equilibrium constant of the reaction *in vivo* is an order of magnitude or so greater than one. Actually a reasonable fit to the points can be obtained by assuming a value of 10 for the equilibrium constant. This is somewhat lower than the value of 19 obtained previously by Carlson and Siger (1959) but in view of the large scatter in the data we do not regard the difference as really significant.

Dependence of PC Level on Time Interval between Twitches This point was not exhaustively explored, but was examined in a preliminary fashion. In order to be able to make statements about the chemical events occurring in a single twitch from a study of a series of twitches it is necessary to have some estimate of the duration of the chemical changes which accompany and follow the twitch. At 1–2°C. the twitch tension decays to zero in 2 seconds or less. This sets a lower bound on the time interval between single twitches if

TABLE IV

DEPENDENCE OF PC/C_t ON TIME BETWEEN TWITCHES

No. of pairs	No. of twitches	Mean difference in $(PC/C_t)_{2.4} - (PC/C_t)_{12}$	Standard deviation	Standard error of mean
		mole/mole		
11	40	−0.0073	±0.054	±0.0137

possible complications due to summation of twitch tension (incomplete tetanus) are to be avoided. While the mechanical response is over in less than 2 seconds it is possible that chemical events are still taking place, which would be altered by initiating another contraction too soon and result in a dependence of PC splitting on frequency of stimulation. It is necessary therefore to establish that the chemical events under study, which accompany each twitch, are both additive and independent of the time between twitches. That the latter was so was shown by comparing the PC contents of paired muscles each of which had been treated with IAA and N_2 and caused to perform 40 isometric twitches, one muscle contracting every 2.4 seconds, and the other every 12 seconds. The results of this experiment are given in Table IV.

The mean difference, −0.0073, is not significantly different from zero. From this we conclude that there is no dependence of the net PC breakdown on frequency of stimulation over the range 0.09 to 0.4 $sec.^{-1}$. The linearity of the plot shown in Fig. 1 establishes the additivity of PC breakdown accompanying a maximal twitch.

Identity of Paired Muscles Data on this point are provided from the results given in Tables I, II, and V. Table V summarizes an experiment de-

signed to determine the amount of variability in C and PC between paired muscles poisoned with 0.5 mM IAA for 45 minutes. In the earlier phases of the studies similar experiments showed standard deviations twice those shown in Table V.

For the data of Table I the standard deviation of the difference in PC/C_t between members of a pair is ± 0.049 μmole/μmole and it is seen from Fig. 1 that there is little or no dependence of the standard deviation on N since the scatter of points is about the same for all N. This figure for the standard deviation of the difference is a measure of the variability of paired muscles under the conditions of our experiments. A similar estimate of this variability comes from the results given in Table IV. Paired muscles one of which was stimulated 40 times (at either of two different frequencies) show a standard

TABLE V

COMPARISON OF IAA-TREATED PAIRED MUSCLES

	C/Weight	Δ(C/weight) (Right-left)	PC/weight	Δ(PC/weight) (Right-left)	PC/C_t	Δ(PC/C_t) (Right-left)
	μmole/gm.	*μmole/gm.*	*μmole/gm.*	*μmole/mg.*	*mole/mole*	*mole/mole*
Mean	5.41	−0.06	30.76	−0.76	0.849	+0.009
Standard deviation	±1.19	±0.65	±4.92	±2.35	±0.031	±0.019
No. of observations	30	15	30	15	30	15

deviation of ± 0.054 in PC/C_t. The experiments to check the identity of right and left members of a pair after IAA treatment but *without* stimulation, summarized in Table IV, gave for the standard deviation of the differences in PC/C_t between paired muscles a value of ± 0.019. The lower values of the standard deviation in unstimulated muscles would suggest that stimulation of one or both members of a pair increases the variability between paired muscles.

The figure of ± 0.049 for the standard deviation of the difference in PC/C_t between members of a pair means that 32 per cent of the time paired muscles will have, in effect, values of PC/C_t that will differ by ± 0.049 or more. This large variability between paired muscles means that to detect, as significant at the $P = 0.05$ level, the change in PC or C accompanying a single twitch would require at least 120 repeat experiments, an impractical number.

Inhibition of the Creatine Phosphokinase Reaction Carlson and Siger (1959) showed that the enzyme CPT, which catalyzes the reaction

$$PC + ADP \rightleftharpoons ATP + C,$$

was inhibited under *in vivo* conditions by treatment with 5 mM IAA. This finding suggested the following, seemingly critical, experiment to decide whether ATP or PC is split first in the contraction cycle. If the classical picture is correct, that ATP is split first and subsequently rephosphorylated by the CPT reaction, a muscle with its CPT, glycolytic, and oxidative systems inhibited should contract normally until all its ATP is gone. It should fail with no change in its PC or C content, but the P_i level should increase by an amount equal to the ATP decrease. Furthermore, since the ATP content is slightly less than one-tenth that of PC, a frog muscle with its CPT system inactivated should, assuming the equivalence of ATP and PC, give about one-tenth as many twitches before failure as does the IAA-nitrogen–poisoned muscle with a functional CPT system. From the plot in Fig. 1 it is found that the muscle with an active CPT system exhausts its store of PC after 92 or 93 maximal twitches. For the case of the CPT inhibited muscle, therefore, 9 to 10 maximal twitches are to be expected before failure.

If, on the other hand, PC is split first by direct interaction with the contractile proteins, then blocking the CPT system should have no effect on contraction, and the muscle should continue to contract, utilizing PC as it does when poisoned with 0.5 mM IAA.

Accordingly, pairs of muscles were treated with 5.0 mM IAA at 20°C. in order to produce complete and nearly complete inhibition of the CPT system. One of the pair was stimulated in the usual manner after equilibration at 0°C. with nitrogenated Ringer's solution. The results of these experiments are given in Table VI. It is clear that muscles treated with 5 mM IAA, for 30 minutes or more, were *unable to produce a single maximal twitch.* Peak tensions were greatly reduced from the normal values of 30 to 50 gm. to only a few grams. The muscles had lost their ability to contract on direct electrical stimulation. Their ATP and PC levels were only slightly less than normal and the total creatine content appeared to be somewhat reduced. Muscles in which contraction block was not complete (Experiments 187, 188, 194) showed a reduced PC level as would be expected if some of the fibers were uninhibited and able to contract while the others were rendered non-contractile.

The experiment does not answer the question of whether ATP or PC is split first during the contraction cycle, since the 5 mM IAA treatment prevents contraction altogether. This latter finding is, however, of considerable interest in itself. It prompts conjecture as to the possible mechanisms by which IAA might cause a loss of contractility. The following testable hypotheses have occurred to us, and are now under investigation: (*a*) High concentrations of IAA alter the membrane system of muscle and result in a loss of excitability. This would be most interesting for in view of the known chemistry of IAA-protein interactions it would very likely implicate SH groups in maintaining

the integrity of the excitability system. It may even be that the CPT system, which is inhibited by high IAA, is necessary for excitability. (*b*) High IAA concentrations result in a loss of myosin ATPase activity *in vivo*, or make myosin otherwise insensitive to the action of ATP, and thereby cause a loss of contractility. On this hypothesis one would expect the excitable system to remain functional, but the contractile system to be irreversibly inactivated.

TABLE VI

CPT INHIBITION WITH 5 mM IAA

Experiment	Wet weight	Treatment		Peak Tension	No. of times stimulated*	PC/C_t	ATP/C_t	C_t
		at 20°C.	at 1–2°C.					
	gm.			*gm.*		*mole/mole*	*mole/mole*	*μmole*
185E	0.1264	30 min.	15 min.	3.5	85	0.701	0.043	2.88
185C	0.1266	"	"		0	0.750	0.037	2.56
186E	0.1235	"	"	6.3	66	0.741	0.061	2.98
186C	0.1180	"	"		0	0.724	0.044	3.05
187E	0.1504	25 min.	5 min.	24.3	103	0.510	0.057	3.76
187C	0.1584	"	"		0	0.800	0.058	4.45
188E	0.1242	15 min.	"	27.8	72	0.428	0.052	3.15
188C	0.1218	"	"		0	0.830	0.064	2.78
190E	0.1300	70 min.	15 min.	0	286	0.630	0.017	2.73
190C	0.1418	"	"		0	0.565	0.021	2.16
191E	0.1138	35 min.	"	1.5	95	0.672	0.059	2.87
191C	0.1148	"	"		0	0.612	0.058	2.81
192E	0.1250	60 min.	"	4.0	88	0.544	0.046	3.79
192C	0.1250	"	"		0	0.626	0.051	3.50
193E	0.1504	40 min.	10 min.	0	88	0.727	0.064	3.30
193C	0.1434	"	"		0	0.679	0.050	3.40
194E	0.0984	30 min.	15 min.	14.5	108	0.362	0.038	2.54
194C	0.1124	"	"		0	0.771	0.072	3.27

* Total tensions and equivalent number of twitches are not tabulated. Since the muscles did not produce any normal maximal twitches the number of equivalent maximal twitches, *N*, cannot be determined. The number of times stimulated is the number of electrical stimuli delivered to the muscle even though no response occurred.

(3) The CPT system is essential for contraction and blocking it with high concentrations of IAA renders the muscle non-contractile. It is expected that further studies on IAA-poisoned muscle and muscle poisoned with other SH agents will clarify this matter.

DISCUSSION

The results reported here provide a precise, quantitative picture of certain net chemical events of the *single twitch contraction relaxation-recovery cycle.* They establish, *in vivo*, the splitting of PC to yield equivalent amounts of P_i and C (and the accompanying buffer reactions) as the major, if not the only, *net*

energy yielding reaction occurring under the conditions of these experiments. The experimental findings and arguments which support this conclusion are: (*a*) direct proportionality between number of twitches or total peak tension and PC breakdown; (*b*) no net breakdown of ATP occurs; (*c*) no glycolytic or oxidative phosphorylating reactions occur; (*d*) the mole ratio of C to P_i is nearly *one*, indicating no esterification of P_i or production by other reactions; and (*e*) the total phosphate content of frog muscle and its quantitative assignment to various organic forms simply does not allow for the existence of an unknown "phosphagen" in anything like the amounts of PC present; see Dubisson (1954), Harris (1956), Ling (1952). Alternative hypotheses, which cannot be ruled out at present, would require either that non-phosphorylated compounds serve as energy sources, or that small amounts of "super high energy phosphate" compounds possessing perhaps 10 to 20 times the free energy of hydrolysis of PC are the major energy sources; and further that the splitting of PC is not essential to the contractile process. In the absence of any evidence in support of such hypotheses we reject them, at least for the time being.

If the splitting of PC is the net reaction occurring, then its free energy change is, in principle, available for conversion to work by the muscle machine. It is of interest therefore to examine those aspects of the results which bear on: (*a*) the thermochemistry of muscular contraction; (*b*) the time of PC splitting in relation to the time course of heat production and contraction; and (*c*) the stoichiometry of the contractile process in terms of the proteins myosin and actin.

Heat of PC Dephosphorylation Since no net work of any kind is done in an isometric twitch, and only heat is produced, it must be concluded that the heat comes from the hydrolysis of PC and the associated buffer reactions, assuming of course that the single isometric twitch is truly a cyclic process. The total initial heat produced in a single isometric twitch of frog sartorius muscle ranges from 2.75 mcal./gm. to 3.3 mcal./gm. according to the results of Hill (1949, 1953, 1958). Assuming this range of values applies to the muscles used here, the figure of 0.286 μmole/gm. for the amount of PC split in a twitch gives for the "physiological heat of hydrolysis" of PC in muscle values ranging from −9.62 to −11.54 kcal./mole.

Unfortunately, a lack of precise quantitative information on the buffer systems present in frog muscle, and their thermochemical properties at 0°C., makes it impossible to calculate the expected "physiological heat" of PC dephosphorylation in muscle. It is possible, and of interest, to calculate the heat of dephosphorylation of PC at 0°C. and at an assumed pH of 6.9 for muscle, exclusive of the "aggregate heat" of the physiological buffer reactions that take place. Gellert and Sturtevant (1960) have shown that for the re-

actions,

$$PC^{-} + H_2O \rightleftharpoons C + HPO_4^{=}; \quad \Delta H = -9.0 \text{ kcal./mole at pH 8.0 and 25°C.} \tag{1}$$

This value can be corrected to pH 6.9 and 0°C. if it is assumed that the heat of the reaction (1), as written, is independent of temperature, and the only heat that need be taken into account is that produced by the reaction:

$$HPO_4^{=} + H^{+} \rightleftharpoons H_2PO_4^{-} \tag{2}$$

which will occur at pH 6.9 and 0°C. From the data and equations given by Bernhard (1956) the number of moles of H^+ produced (consumed) per mole of $HPO_4^{=}$ at pH 6.9 and 0°C. is −0.5; and the heat of ionization of $H_2PO_4^{-}$ at 0°C. and ionic strength 0.70 is +7.6 kcal./mole, giving for the heat due to reaction (2) a value of $-0.5 \times 7.6 = -3.8$ kcal./mole. Addition of this to the −9.0 kcal./mole due to reaction (1) gives a value of −12.8 kcal./mole for the heat of hydrolysis of PC at pH 6.9, ionic strength 0.70, and a temperature of 0°C. Although the ionic strength of frog muscle is 0.25, Dubisson (1954), it is not expected that this difference would introduce much of an error since Bernhard (1956) states that the pK of reaction (2) is invariant over the range of ionic strengths 0.3 to 0.7.

Since the −12.8 kcal./mole calculated for the heat of hydrolysis is less than the estimated *in vivo* value of −9.6 to −11.5 kcal./mole, the difference of +3.2 to +1.3 kcal./mole must be presumed to be the "aggregate heat" of the physiological buffers at 0°C. Gellert (1960) has calculated the "physiological heat of hydrolysis" of PC at 0°C. in frog muscle, under the somewhat questionable assumption that the buffer system given by Bernhard (1956) applies to frog muscle. He obtained a value of −9.65 kcal./mole which is still in agreement with our estimate of −9.62 kcal./mole.

The close correspondence between the values obtained for the "physiological heat of hydrolysis" of PC, as estimated from the heat production and chemical studies on the one hand, and as calculated from thermochemical data on the other hand, corroborates the view that PC hydrolysis and the associated buffer reactions were the net energy yielding reactions occurring in muscle under the conditions of our experiments. From the point of view of the energetics of muscular contraction, PC seems to be capable of assuming the role assigned to ATP before the careful thermochemical studies of Podolsky and Morales (1956) divested the latter of some of its "high energy" properties.

The Time of PC Splitting The present studies, because they entailed a 30 second delay between the cessation of stimulation and quick freezing,

permit us to conclude only that the PC splitting associated with a single twitch is substantially complete in 30 seconds, or less. However, when viewed in the light of the results of Mommaerts (1955), and to some extent of those of Fleckenstein *et al.* (1954) they permit more definite statements about the time of occurrence of the chemical events of contraction.

The conclusion of Mommaerts and Fleckenstein *et al.*, is that there is *no* PC or ATP splitting *during the contraction or relaxation phases*, in the single twitch, or short contraction. We infer from studies on a series of single twitches, that over the entire single twitch contraction-relaxation-recovery cycle there is a net splitting of PC, but not of ATP. These two results imply that PC splitting must have occurred during the recovery phase, and so must have any splitting of ATP that may have occurred. Since, in an isometric contraction virtually *all* the energy of contraction is liberated during the contraction phase, according to Hill (1953), it follows that; (*a*) *if the energy-yielding reaction for contraction is activated during the contractile phase, ATP and PC are not directly involved; or* (*b*) *that the muscle is energized during the relaxation or recovery phases in which case ATP and/or the PC system could be involved and the energy is stored in the contractile machine to be released during contraction as heat and work.* It is important, therefore, that a critical examination be made of the experimental results which lead to these conclusions.

Our results reported here clearly show that PC splitting occurs sometime during the contraction-relaxation-recovery cycle, in direct proportion to the number of contractions, and in amounts that are compatible with the known heat production properties of muscle and the thermochemistry of PC. We have also shown (1959) that the CPT system functions in the IAA-poisoned muscle from which it follows that ATP once split can be resynthesized at the expense of PC. What about the results of Mommaerts and Fleckenstein *et al.* which argue for no PC or ATP splitting during the contraction or relaxation phases?

Mommaerts used turtle sartorius muscle under anaerobic conditions, but did not block glycolysis. The possibility of resynthesis of PC and ATP by glycolysis although unlikely was not, therefore, completely excluded. Further, he did not report how much work the muscles performed per twitch, hence a quantitative comparison with frog muscle is not possible. It is possible that the turtle muscle, unlike frog sartorius, contains an appreciable fraction of slow fibers, and would have a smaller single twitch response with correspondingly reduced chemical changes. Apart from the possibilities that the occurrence of glycolysis, or a species difference between frog and turtle might make the application of Mommaerts' findings on turtle muscle to frog muscle inadvisable, there are, in addition, indications that his criteria for rejecting data may have been too restrictive. From the data given by Mommaerts (1955, p. 591 Fig. 7) on the difference in C concentration between stimulated

and non-stimulated muscles, we have determined the standard deviation of the differences in creatine concentrations to have been 8.5×10^{-2} μmole/gm. This means the standard deviation of the concentration determinations themselves was $\pm 6 \times 10^{-2}$ μmole/gm. giving a coefficient of variation of ± 1 per cent, since the C content was about 6.0 μmole/gm. Such a high precision implies that in these experiments the *total* variation due to biological variability, errors in dissection and extraction procedure, errors in creatine determination, and errors in *wet weight determination*, was only ± 1 per cent of the mean. In our own experience under the best conditions with similar techniques, the standard deviation of the differences in C concentrations obtained was ± 0.655 μmole/gm. (Table V) which gives a coefficient of variation for the concentrations themselves of ± 8.5 per cent. In addition to the biological variability a major source of variation is the determination of the *wet weight*, a procedure which is necessarily crude, because it requires "drying" the muscle with cold cellulose tissues before freezing and weighing. Mommaerts does not state that the muscles used were dried before freezing them; presumably they were. It is doubtful however that the procedure was reproducible to ± 1 per cent or better. How can the extraordinarily low variability obtained by Mommaerts be explained? Possibly turtle muscles are more nearly alike than frog muscles treated with IAA. Possibly the procedure used by Mommaerts to reject data was so restrictive as to select, in effect, only very nearly identical paired muscles. Data were rejected according to Mommaerts if differences ". . . could be correlated with experimental errors (chunks of muscle during extraction) or to obvious inequality of the muscles of a pair." This procedure lacks objectivity and could well lead to the selection of pairs of nearly identical muscles even when genuine differences—due to biological variability, splitting of PC, or inadvertent errors—did exist. Under these circumstances: species differences, glycolysis not inhibited, no data on the magnitude of the twitch response, and an unusually low variability of the data that might have been the result of a questionable data rejection procedure,—we feel that Mommaerts' results *do not* conclusively rule out either ATP or PC splitting during the contraction and relaxation phases, at least for frog sartorius muscle.

Fleckenstein *et al.*, (1954) used frog *rectus abdominis*, a muscle which does not give the classical twitch response. Without blocking glycolysis or respiration, and using brief tetanic contractions, PC splitting was observed at 20°C. in much greater quantities than would have been expected on the basis of the work done by the muscles. At 0°C. the PC content of stimulated muscles increased by 0.32 μmole/gm., but the authors do not state whether or not this increase is significantly different from zero. Presumably it is not and would imply that had a decrease of the same magnitude occurred it would not have been considered significantly different from zero. In order

to obtain a more definite result on a percentage basis, the muscles were poisoned with 2:4-dinitrophenol to drastically reduce the PC content. Stimulation at 0°C. failed to produce the expected decrease of 0.3 μmole/gm. in the PC concentration. This latter experiment is not in accord with our own findings. Muscles with such drastically reduced PC concentrations are, in our experience, *inexcitable* and in a state of incipient rigor. On slight warming they pass into rigor and *do not relax*. This may be the explanation of the statement made by Fleckenstein *et al.*, that ". . . the muscles contracted vigorously even at 0°C. (though they were unable to relax)." These workers could have been dealing, in this instance, with muscles passing into rigor, and not with normal contractions. There is no basis whatever for asserting that the chemical changes which accompany rigor are the same as those occurring during normal contraction.

In summary, the results of Mommaerts and Fleckenstein *et al.*, do not in our opinion provide a conclusive basis for rejecting the hypothesis that ATP and/or PC is broken down during the contraction and relaxation phases of the single twitch. This being the case, the conclusions that (*a*) *a new chemical source, other than ATP or PC, of contractile energy must exist*, or (*b*) *the contractile mechanism is of the stored energy type* are not the only alternatives, for we must admit as a possibility the classical view that (*c*) *ATP is dephosphorylated during contraction and rephosphorylated by PC.*

Heat and Mechanical Studies, a Brief Reevaluation In the preceding section we listed as a possible hypothesis, which cannot yet be rejected, the "stored energy" type of contractile mechanism which views relaxation or recovery as the active or energy transferring state. This implies that we do not consider such a mechanism as definitely ruled out by the heat production and mechanical studies of Fenn (1923), Fenn and Marsh (1935), and Hill (1938, 1949) which favor the view that chemical energy is transferred to the contractile proteins during contraction—the active state theory of contraction. There are five points which form a basis for doubting the validity of the active state theory.

1. The force-velocity studies of Fenn and Marsh (1935) and Hill (1938) do not rule out *non-linear visco-elastic models* (stored energy models) as noted by Wilkie (1954), and experimentally shown by Carlson (1957), and Ritchie and Wilkie (1958) to best fit the force-velocity behavior of frog sartorius muscle.

2. The heat studies of Hill (1938, 1949) are not in agreement with those of Fenn (1923) on the matter of the dependence of total energy produced on load. Fenn finds a maximum in total energy at moderate loads and Hill (1949, 1950) finds a broad maximum near zero load with the value at low loads twice as great as the minimum occurring for the isometric contraction.

In addition Aubert (1956), using Hill's techniques, in an extensive, systematic examination of muscle heat production, has demonstrated a strong dependence of the maintenance heat on time, tension, and initial length. This variable maintenance heat makes it impossible to define operationally shortening heat, for want of a constant base-line.

3. A tacit assumption invariably made in interpreting heat studies is that *all the heat produced during contraction is causally related to the contractile mechanism only*. There is no compelling reason why this should be so, and indeed one can readily conceive of non-essential heat producing phenomena that might occur along with contraction and negate such an assumption.

4. Recently Jewel and Wilkie (1958) were unable to experimentally confirm the time course of the isometric myogram predicted by the active state theory, and the characteristic equation. In the opinion of the authors the observed discrepancy between theory and experiment was genuine and not due to faulty technique.

5. Carlson and Siger (1957) showed that the amount of PC split in a single 5 gm. isotonic twitch was 0.287 μmole/gm., virtually the same as that found here for the isometric twitch and *not twice* as great as would be expected from Hill's (1949, 1950) results. In a preliminary report Carlson and Siger (1958) showed that the dependence of PC splitting on load did not parallel the total heat production according to the data given by Hill (1949). The results of these studies on isotonic contraction will be presented in detail in the next paper in this series.

Jobsis (1959), working in Chance's laboratory, has found that ADP production falls off in a similar manner to PC splitting at low isotonic loads. The little data that exists on the dependence of PC and ATP splitting on load appears to agree roughly with Fenn's (1923) picture of total heat production.

The active state theory and the characteristic equation of Hill have served to provide a simple and clear description of the energetics of contraction. As such they have provoked much fruitful experimentation and thought. Indeed, the very experiments which the characteristic equation has provoked seem to have revealed its incomplete and approximate nature, as indicated above. Under these circumstances, it is difficult to find a firm basis for accepting or rejecting any of the general types of energy transfer mechanisms which have been proposed,—energy transfer during contraction, stored energy mechanisms, or the conception of dynamic energy transfer put forth by Aubert (1956),—and a discussion of the results reported here in terms of any one, or all, of these possible mechanisms hardly seems warranted.

Stoichiometry of Contraction, and Molecular Mechanisms From the point of view of the molecular mechanism of contraction it is important to obtain an estimate of the number of PC molecules split per myosin or actin molecule

during the single twitch. To do so we require a knowledge of the myosin and actin contents of muscle, as well as their molecular weights. Data are not available on the actin or myosin content of frog muscle. It is necessary to assume that frog muscle is similar to rabbit muscle in these respects. Such an assumption is not unreasonable since the frog muscles used here developed 2 to 3 kg./cm.^{2} of tension at 0°C. in a single twitch. At 20°C., in a tetanus, they might be expected to develop tensions of 4 kg./cm.^{2}, or more, which compares with the 5 kg./cm.^{2} given by Weber (1955) for rabbit muscle. In rabbit muscle the percent of the total protein which is myosin has been found to be 38 per cent by Hasselbach and Schneider (1951), 26 per cent by Szent-Gyorgyi and Mazia (1955) and 34 per cent by Huxley and Hanson (1957). For our purposes we will use the average of these figures, 33 per cent for the per cent of total protein which is myosin. Frog muscle contains 18 per cent protein by weight, Dubisson (1954), giving 6 per cent of the wet weight of the muscle as myosin. As for the molecular weight of myosin, Laki and Carroll (1955), Holtzer and Lowey (1958), Gergely (1958), Mommaerts and Aldrich (1958), von Hippel, Schachman, Appel, and Morales (1958) give values ranging from 400,000 to 450,000. Very recently however, Kielly and Harrington (1960) have reported a value of 620,000. For our purposes we will assume that the molecular weight of myosin is no less than 400,000 and no greater than 620,000. Using these figures one obtains for the myosin content of muscle 0.15 μmole/gm. and 0.10 μmole/gm., respectively. Using the value of 0.286 μmole/gm. for the amount of PC split per gram of muscle in a single isometric twitch one obtains for the number of molecules of PC split per myosin molecule the values 1.91 and 2.86. In round numbers, 2 to 3 PC molecules are split per myosin molecule during a single twitch contraction cycle. If ATP interacts with myosin, is dephosphorylated, then rephosphorylated at the expense of PC, it follows from this calculation that no more than 2 to 3 ATP molecules can interact with each myosin molecule in a single isometric twitch.

This is a most interesting result as far as molecular theories of the contractile mechanism are concerned. So far, the only such general theory that has been sufficiently well developed to enable any quantitative predictions is the "entropic-electrostatic" theory originally proposed by Riseman and Kirkwood (1948), later modified by Morales and Botts (1952), and recently revised by Podolsky (1958).

Morales and Botts (1952), and Hill (1952, 1953) calculate, according to their theory, that to obtain contractile forces equal to those found in muscle it is necessary that there be, on the average, a unit of fixed charge for every 10 amino acid residues along the myosin molecule. The neutralization of most, or all of these charges is required for the full development of the force of contraction. Myosin contains 866 amino acid residues per 10^5 gm. according

to Bailey (1954) which gives a total of 3,460 to 5,370 residues depending on the molecular weight. This in turn gives a total of 346 to 537 unit charges per molecule to be neutralized by interaction with ATP. The total of 2 or 3 ATP molecules each bearing 4 negative charges which we find involved in a single twitch would provide hardly enough charge to neutralize the 350 or more charges required to be on the myosin. Indeed, all the ATP in the muscle, about 3 μmole/gm., is only sufficient to supply 20 to 30 molecules of ATP per myosin molecule, still too few. Clearly, our results argue against the "entropic-electrostatic" mechanism as it is detailed by Morales and Botts. It is possible, and even likely, that in some other form the theory can be made to agree with experiment.

Let us now turn to actin and make a similar calculation. In rabbit muscle ratio by weight of myosin to actin is, according to Hasselbach and Schneider (1951), approximately 3:1 from which it follows that muscle contains about 2 per cent, by weight, actin. The molecular weight of G-actin monomer ranges from 57,000 (Laki and Standaert (1960)) to 70,000 (Bailey (1954)) from which one obtains a G-actin concentration of 0.35 to 0.29 μmole/gm. The ratio of PC molecules split in a twitch to G-actin molecules present in the muscle is therefore 1.0 to 0.8.

It might be argued that some form of actin is the contractile substance and that contraction results from a folding due to an unbalance of electrostatic and entropic forces in the actin molecule. If it is assumed that a charge density of the same order of magnitude (1 per 10 residues) as that required for myosin obtains in the case of actin, then, since actin has a total of 637 amino acid residues (Bailey (1954)), a total of 64 charges would be required. Again, 1 ATP molecule bearing 4 negative charges is hardly enough to neutralize this net charge. If actin is the contractile substance it does not operate according to an electrostatic-entropic mechanism of the type assumed.

The finding of one PC or ATP split per G-actin molecule in a single twitch immediately suggests the G-F transformation of actin as being involved in the contractile cycle. This transformation, or polymerization, is believed to occur with the splitting of the single G-actin-bound-ATP molecule to yield F-actin-bound-ADP according to the work of Straub and Feuer (1950), and Mommaerts (1952). Very recently, Strohman (1959) has reported new and interesting properties of actin which include: (1) the phosphorylation of F-actin-bound-ADP by the CPT system during depolymerization to give a G-ATP actin which can polymerize, and (2) the existence of G-actin-H meromyosin complexes which in the presence of CPT can liberate C from PC. These results of Strohman's will be considered further below in connection with possible mechanisms of contraction.

In vivo Reactions Chance and Connelly (1957) originally cited the results of Mommaerts (1954, 1955) and Fleckenstein *et al.* (1954) as evidence against the view that the CPT system transphosphorylates ADP as fast as it is produced. The results reported here clearly show that PC is dephosphorylated sometime during the contraction cycle, and the arguments cited above make it doubtful that one can exclude the possibility of ADP phosphorylation by PC solely on the basis of the results of Mommaerts, and Fleckenstein *et al.* Recently, Chance (1959) has argued that the kinetics of ADP production and dimunition in muscle do not support the view that ADP is phosphorylated with any great rapidity. In addition, he cites the higher affinity of mitochondria for ADP, over the CPT system, and the decreased activity of CPT at reduced temperature, to support the view that ADP phosphorylation is due almost exclusively to increased mitochondrial activity. Let us review briefly Chance's results and those reported here that bear on this point.

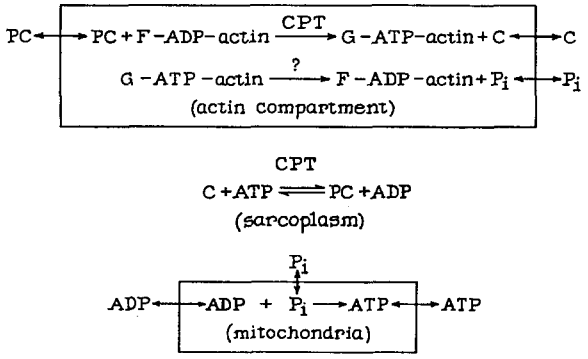
Chance's (1959, Figs. 7 and 8) data show that following a twitch ADP is produced by a process with a short time constant, 5 seconds or so, and is expended by a process with a long time constant, 30 seconds or more. The latter process is according to Chance almost exclusively due to increased mitochondrial activity.

In the section on *the dependence of PC level on the time interval between twitches* above, data is given (Table IV) which allows one to obtain an estimate of the *upper bound* of the time constant of the PC breakdown that was observed in our experiments, by assuming: (*a*) that during contraction ADP is produced rapidly and consumed due to PC phosphorylation; and (*b*) the later reaction is regarded as a pseudo-unimolecular reaction with a 30 second or larger time constant, in approximation to Chance's (1959, Fig. 8) results. On this basis, the expected difference in the value of PC/C_t between paired muscles can be calculated under the conditions that both contract the same total number of times (40); one at short intervals (2.4 seconds) the other at long intervals (12.0 seconds). The delay time between cessation of stimulation and freezing being taken as 30 seconds. Had the time constant been *30 seconds or more*, the difference, $(PC/C_t)_{2.4} - (PC/C_t)_{12}$, should have been $+0.027$ or greater. The value found, -0.0073, is significantly less ($P = 0.02$) than the expected value, hence we must reject the hypothesis that the dephosphorylation of PC occurred with a time constant of 30 seconds or more, and conclude that the time constant is less than 30 seconds. While further study is required on this point, it is highly likely that under the conditions of our experiments, 0°C., most if not all the PC breakdown observed occurs with a time constant of 30 seconds or less.

An Hypothesis One obvious explanation for finding PC breakdown under the conditions of reduced CPT transphosphorylating activity (accepting Chance's argument) is that PC is split directly during the contraction cycle,

without ADP. There is however no evidence that PC splitting without ADP can occur, consequently this hypothesis must be tabled for the present.

An alternative explanation for which strong support can be found particularly in the work of Strohman (1959), is to assume that ADP and ATP present in muscle are located in compartments as follows: (*a*) ADP and ATP bound to actin, and the enzyme, CPT, are located in the "actin compartment", where the ATP and ADP are not free to exchange at any appreciable rate with "sarcoplasmic" ATP and ADP. (*b*) ATP, ADP, P_i, C, PC, and CPT are present in the sarcoplasmic compartment where they diffuse freely. (*c*) PC, C, and P_i are free to diffuse in and out of the "actin compartment" and ADP, ATP, and P_i are free to diffuse "in and out" of the mitochondria. This system of compartments is schematized as follows:—



The ADP "seen" by Chance and Connelly, according to this model, would be that which occurs in the sarcoplasm and reaches the mitochondria by diffusion. The ADP which reacts with PC, *via* CPT, is that bound to F-actin according to the process discovered by Strohman (1959). Because of the equilibrium characteristics of the CPT reaction, and the high PC content of muscle, C produced in the actin compartment will result in only a slight (not equivalent) increase in the sarcoplasmic ADP. This could account for the slight increase in ADP reported by Chance and Connelly, if one rejects the alternative possibility which they mention, that P_i is stimulating increased mitochondrial activity.

At present it is not possible to identify the G-F actin transformation, and its reversal, with any particular phase of contraction for lack of knowledge of the time constants of the various processes shown in the model. If both the actin polymerization and depolymerization reactions, with their coupled dephosphorylation and transphosphorylation, are fast compared to the CPT reaction in solution, and that reaction in turn is fast compared to the mitochondrial phosphorylation of ADP, then the results of Chance and Connelly would obtain. Contraction and relaxation could correspond to polymeri-

zation and depolymerization, or *vice versa*; in either case the sarcoplasmic PC would drop and the C and P_i would increase. The increased C and decreased PC would drive the sarcoplasmic CPT reaction in the direction of ADP formation but only very slightly because the equilibrium actually favors ATP production. The slight increase in ADP would stimulate mitochondrial activity, which would gradually reduce the ADP level in accord with Chance and Connelly's observations.

Other significant facts with which this model is in accord are: (*a*) the finding of no net ATP breakdown, but that PC hydrolysis is the net energy yielding reaction; (*b*) the stoichiometry of 1 PC per G-actin per twitch clearly suggests that the G-F transformation of actin is implicated in a major way in the contraction cycle; and (*c*) the identification of contraction with actin agrees with the findings of Huxley and Niedergerke (1954) and Huxley and Hanson (1954) that only the I-band, which contains actin, Hanson and Huxley (1957), shortens during contraction. Certainly at the moment, one cannot say that this model corresponds to the true state of affairs. It does, however summarize many, if not all, of the observations made on the chemical events which occur in intact muscle, without doing great violence to the results obtained on model systems and purified contractile proteins. No role is assigned to myosin, not because we think it has none, but because we have been unable to assign it one, except possibly the dephosphorylation of actin bound ATP during polymerization; indicated by a question mark in the scheme.

Further Remarks The IAA-nitrogen–poisoned muscle emerges from these studies as the most suitable system presently available for the study of muscle mechanochemistry in the intact cell, with a minimum of interference from non-essential metabolic reactions. With care and caution one can hope to obtain from a study of this system a reasonably accurate picture of the mechanochemical events of contraction. Such a picture when viewed in the light of studies on model systems and contractile proteins should form a basis for formulating a cogent theory of the molecular mechanism of muscular contraction.

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