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THE EXCHANGE OF ^{18}O BETWEEN WATER AND PHOSPHATE COMPOUNDS IN ISOLATED FROG SARTORIUS MUSCLE UNDER CONDITIONS OF NEGATIVE WORK†

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Frog sartorius muscles were soaked at 0°C for 2 minutes in a Ringer solution enriched with $H_2{}^{18}O$. Five minutes after the first contact with the isotope, they were stimulated tetanically for 7.7 sec. One muscle of the pair remained isometric, the other one was slowly stretched. Some muscles were poisoned with fluorodinitrobenzene or dinitrophenol. The muscles were frozen rapidly after the last stimulus and PC, ATP and P_i were chemically analysed. Phosphate isolated from these compounds was analysed for ^{18}O content by mass spectrometry. The water of the muscles is near the isotopic equilibrium with the water of the surrounding Ringer solution. P_i has a specific activity which is about one-quarter to one-fifth of that of water. This indicates that P_i split from PC or ATP is not recycled during the tetanus. PC has a specific activity which is about 90% of that of P_i. ATP has a specific activity much lower than that of P_i or PC. This is explained by the fact that both the terminal phosphates of ATP were used for isotopic analysis. If one assumes that ATP-P_γ is in isotopic equilibrium with PC, then ATP-P_β has a specific activity which is about one-third of that of P_i. Pre-treatment of the muscles with FDNB does not influence the specific activities of P_i or ATP; that of PC is however very low. This indicates that the Lohmann reaction is the only path which involves PC. Stretch does not change ^{18}O specific activity of P_i, ATP or PC. However, no conclusions can be drawn, because these compounds are so close to isotopic equilibrium. Stretched muscles use less ATP or PC than their isometric control. The ratio of the negative work received by the muscles to the amount of spared ~P was 8.7 kcal/mole for unpoisoned muscles and 25 kcal/mole for fluorodinitrobenzene-treated muscles. Estimation of this ratio was not possible for the muscles poisoned with dinitrophenol. Thus the experiment yielded no decision as to whether work imposed upon contracting muscle causes a synthesis of ATP or an inhibition of its breakdown. They did, however, yield detailed information on other aspects of the sparing of metabolism by negative work.

When work is done upon active muscle by an imposed stretch, its equivalent does not appear wholly as heat. The heat evolved by such a muscle may or may not be more than the isometric heat under the same conditions, but if it is more, the difference is less than the imposed work itself. Phenomena of this sort were noted by Fenn (1924). His paper, otherwise, dealt mainly with the opposite situation which has become known as the Fenn-effect. In its original form (*cf.* Mommaerts, 1969, 1970), this means that a muscle performing work accumulates about the same amount of heat as one contracting isometrically, implying that an extra-amount of energy equivalent to the work is generated. In the reverse case of the imposed negative work, the implication is that energy has been spared.

The subject has been investigated at several occasions with the myothermal method (Abbott, Aubert and Hill, 1951; Hill and Howarth, 1959). Using very slow stretches, the limiting case was demonstrated that the stretched muscle develops quite accurately the same amount of heat as the one isometrically contracting (Abbott and Aubert, 1951), though Hill and Howarth (1959) also obtained the same result with rapid stretches. In these limiting cases, the imposed work was said to have been absorbed entirely by the muscle, or to have disappeared as far as its thermal detectability is concerned. Most probably, this occurred by an economizing of the concurrent metabolism. For the positive Fenn-effect, a thermo-chemical balance was given by Mommaerts, Seraydarian and Maréchal (1962) and by Carlson, Hardy and Wilkie (1963), and, quite precisely, by Maréchal (1964). Efforts to demonstrate the biochemical equivalent of the reverse Fenn-effect were made by Maréchal (1964) who achieved a partial balance in accord with the intermediate speeds of stretch employed, and by Infante, Klaupiks and Davies (1964) who likewise reported a partial balance. The

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present investigation was first of all aimed at clarifying this point, and has indeed achieved a balance for the case of very slow stretches, in that the chemical energy spared during the stretch is equal to the work done on the muscle.

Secondly, there is the question as to the mechanisms of this economizing effect. It could be that the imposition of mechanical work forces a synthesis of ATP from ADP and P_i, on the analogy of what has recently been shown for the reversal of a Na^+-transport mechanism (Glynn and Lew, 1970). On the other hand, it could be that the effect of stretch is to prevent the occurrence of a certain amount of breakdown. These alternate possibilities will be designated as the hypothesis of resynthesis versus that of inhibition.

One would be inclined to attempt a choice between these mechanisms by the application of ^{32}P-phosphate, which would be incorporated into ATP in the case of a forced synthesis. However, general experience with the use of labelled phosphate in living muscle, reviewed critically by Carlson (1963), has shown that externally provided P_i penetrates but slowly into the muscle cell, and seems to reach isotopic equilibrium with all relevant phosphorylated compounds as it enters. We refer to the accompanying paper by Gillis and Maréchal (1974) in which the same problem is taken up for a glycerol-extracted muscle fiber into which labelled phosphate distributes without permeation barriers. In order to study the problem in living muscle, we have resorted to the use of $H_2{}^{18}O$ which penetrates rapidly and which, once there, enters into phosphorylated compounds by a variety of unidentified exchange reactions which, according to Fleckenstein, Gerlach, Janke and Marmier (1960), leads to a preferential labelling of P_i and of ATP and PC, in that order. Thus barring complications due to morphological or kinetic compartmentalization, the occurrence or non-occurrence of mechanochemical synthesis of ATP could be decided.

METHODS

1. *The Muscles*

Sartorius muscles (*Rana pipiens*) were kept for 2 to 4 hours after dissection in phosphate-free Ringer solution (95 mM NaCl; 2.5 mM KCl; 1.0 mM $CaCl_2$; 1.0 mM $MgSO_4$; 20 mM $NaHCO_3$; 95% O_2 and 5% CO_2; pH: 7.3; 0°C). Some muscle pairs were poisoned with 0.4 mM 2,4-dinitrophenol (DNP) or 1-fluoro-2,4-dinitrobenzene (FDNB) for 1 hour at 0°C. Four to six muscle pairs were treated together with exactly the same treatment, and were later pooled, in order to get enough material for the isotopic analysis.

2. *Loading with ^{18}O*

The muscle pairs were transferred to 2 ml phosphate-free Ringer solution enriched with $H_2{}^{18}O$ (4 to 10% Atom ^{18}O). The muscles were blotted gently just before they were soaked into the $H_2{}^{18}O$-Ringer, in order to minimize dilution of the heavy water. They were left 2 min in the Ringer solution. Then the two muscles of the same pair were placed on the stimulation assembly, tetanized, and frozen exactly 5 min after their first contact with $H_2{}^{18}O$. This timing is important and is to be discussed later on. (See results.)

3. *Mechanical Arrangements*

The muscles while on the rapid freezing apparatus of Mommaerts and Schilling (1955) were kept at 0°C in 95% O_2–5% CO_2 saturated with water. The two muscles of the same pair were stimulated together with square pulses (0.5 msec, 20/sec). The tension was recorded separately for each muscle by capacitance gauges (Schilling, 1960). One muscle remained isometric (at l_0+2 mm); the other one was stretched about 5 mm (within lengths l_0-2 mm and l_0+4 mm) by a Levin-Wyman ergometer (1927) at a slow rate (0.7 mm/sec) with a delay of 400 msec (l_0 varied from 31 to 35 mm). The last stimulus triggered the freezing device, which was timed in such a way as to operate just at the end of the stretch; the muscles had no time to relax. In a few experiments the rate of the stretch was higher (4 mm/sec or 26 mm/sec) and accordingly the tetanus duration was shorter (1.8 sec or 0.65 sec).

4. *Chemical Analysis*

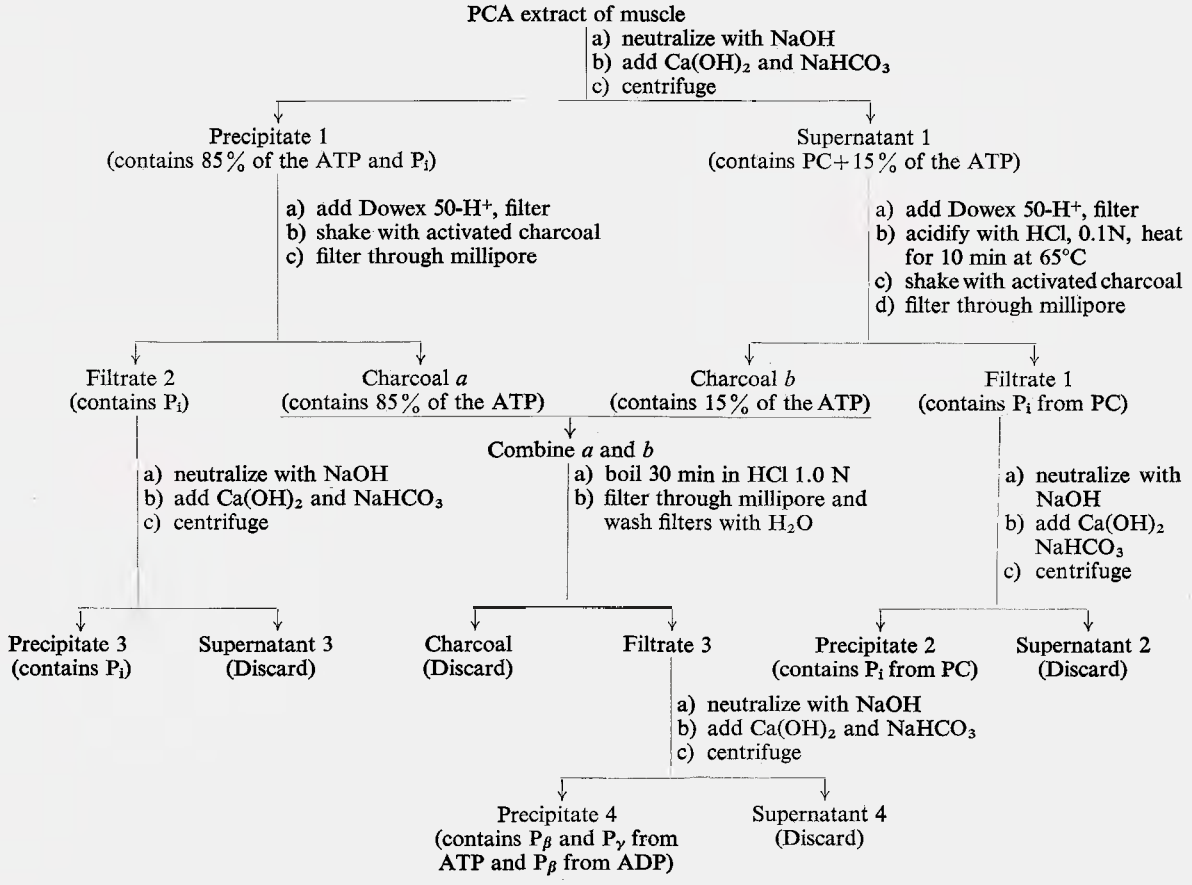
a) *Muscle extracts.* Four to six muscles were ground in a stainless steel cartridge (−196°C), and extracted twice with 3 ml of 0.25 N $HClO_4$, according to Seraydarian, Mommaerts, Wallner and Guillory (1961). After centrifugation (Lourdes 14.000 rpm for 10 min) the extracts were neutralized with 10 N NaOH (indicator: phenolphtaleine) and they were made up to 8 ml with water. Aliquots were taken from the supernatant for ATP (20 μl) and for free (C_F) and total (C_T) creatine (0.1 ml).

b) *Isolation of the phosphate fraction P_i, ATP and PC* (Scheme I). Inorganic phosphate: 6 ml of the neutralized muscle extract was precipitated with 2 ml of a $Ca(OH)_2$ mixture and 1 ml of 0.05 M sodium bicarbonate, according to the procedure described by Seraydarian, Mommaerts and Wallner (1961). This precipitate contains, in addition to P_i, 85% of the ATP. The precipitate was centrifuged and washed twice with 2 ml of 10% alcohol saturated with $Ca(OH)_2$. The washings were added to the supernatant (supernatant 1). The precipitate was dissolved in 1 g Dowex-50 H^+ slurry (Dowex-50 H^+ was prepared in the following way: 200 g cation exchange resin AG 50 W-X8, 100–200 mesh, H^+ form analytical grade, from Bio-Rad, is mixed with 400 ml of 1N HCl, and then with 500 ml of glass-distilled water; this cycle is repeated twice. The final washing fluid should give a faint blue color when tested with bromcresol green 0.1%; the pH is then higher than 4.5). The Dowex was separated by filtration, and was washed with CO_2-free water, until the combined volumes were 5 ml. ATP was removed by adding 25 mg activated charcoal and shaking for 5 min, (100 g Norit A washed with 2 liters of 1 N HCl, 2 liters of 1 M NH_4OH, then washed with distilled water until neutral, and dried 18 hours at 110°C), then filtered on a 0.45 μ millipore filter. The inorganic phosphate of the filtrate was precipitated with 4 ml of the $Ca(OH)_2$ mixture and 2 ml of 0.050 M Na-bicarbonate (P_i precipitate).

The calcium of supernatant 1 was removed with 1 g Dowex-50 H^+, followed by filtration and washings as described earlier. Phosphocreatine is hydrolyzed by this treatment, but to make sure that all the PC is split the solution was further acidified with 0.2 ml of 5 N HCl (final concentration 0.1 N) and heated for 10 min at 65°C. The ATP, which is not hydrolyzed by this treatment,

SCHEME I

Separation of phosphate fractions



was removed by activated charcoal, filtered on 0.45 μ millipore filter and combined with the ATP separated from precipitate 1. Inorganic phosphate from PC was precipitated by the $Ca(OH)_2$ mixture (PC-precipitate).

The combined charcoal powders which had absorbed the ATP were boiled for 30 min in 3.0 ml 1 N HCl. This hydrolyzed the γ and β phosphate from ATP and ADP. There is practically no other nucleotide to consider in such a muscle extract. The charcoal hydrolyzate is filtered on a microfilter and the charcoal is washed three times with 1.0 ml H_2O; the washings were combined with the filtrate. After neutralization with 0.3 ml of 10 N BaOH, the phosphate was then precipitated with the $Ca(OH)_2$ mixture (ATP-precipitate).

c) *Purification of the phosphate fractions.* So far three phosphate precipitates have been obtained. Precipitate 2 contains the phosphate coming from PC. Precipitate 3 contains the inorganic phosphate from the muscle extract and precipitate 4 contains the β and γ phosphates from ADP and ATP. These phosphate samples are not pure enough for the isotopic analyses, and were further purified according to the procedure of Boyer, Graves, Suelter and Dempsey (1961). Each precipitate was dissolved in about 1 ml of a Dowex-50 H^+ slurry; and the solution with the slurry was poured over a small column (3 mm $\times$ 2 cm) filled with the same slurry. The eluate was evaporated to dryness in a rotary evaporator and redissolved in 0.6 ml H_2O. The solution was adjusted to pH 4.0 with NaOH by using 20 μl Bromcresol green 0.1%. An aliquot of 50 μl was removed for phosphate analysis (Berenblum and Chain, 1938). If the amount of phosphate was lower than 1 μmole, isotopically normal phosphate was added. An aliquot was placed in a sealing tube. The solution was slowly evaporated in an oven at 55°C, and 25 mm Hg pressure, avoiding any boiling. The residue was redissolved in a few drops of water by rinsing the sides of the tube and reevaporated to dryness. The samples were then ready for the isotopic analysis.

d) *Isotopic analysis.* The ratio $^{18}O/^{16}O$ was measured with a mass spectrometer, using the guanidine hydrochloride method described by Boyer *et al.* (1961). The results are expressed as atom % ^{18}O excess, meaning that the value of ^{18}O which occurs naturally (0.206%) has been subtracted from the isotopic content measured with the mass spectrometer.

e) *Chemical analysis.* ATP and ADP were measured with an enzymatic method (Boehringer kit). Free and total creatine were measured with the diacetyl method (Ennor and Rosenberg, 1952); inorganic phosphate was measured by the method of Berenblum and Chain (1938).

f) *Recovery.* A statistical analysis was performed in order to estimate the loss between the first step of the extraction procedure (where aliquots of ATP and PC were taken), and the final stage of purification of the phosphate sample. All the data obtained were pooled, disregarding the treatments the muscles had received (type of poisoning, or mechanical conditions). For PC, the mean content was 13.22 μmole/g, as evaluated before any purification procedure; it was 11.32 μmole/g, as estimated from the inorganic phosphate content readv for ^{18}O analysis. A linear regression analysis yielded a regression coefficient between the two sets of data of 0.903 ± 0.057 ($n = 34$). The recovery is therefore about 90%. Similar results were obtained for ATP.

RESULTS

A. *Mechanograms and ATP-PC Hydrolysis*

Figure 1 reproduces a typical record for one experiment. One muscle was stimulated for 7.7 sec isometrically (l_0+2 mm). The other muscle of the pair was stimulated at the same time; its tetanus developed at first isometrically; 400 msec after the first stimulus, it was stretched at a very slow rate (0.7 mm/sec). The two muscles were frozen together, before any relaxation could set in. The time of freezing was matched to the lengthening in such a way that the freezing occurred just when the stretch movement had stopped.

The stretch begins only when the tension has reached the tetanic level. This has been deliberately designed because if it were not so, the stretch would begin during the rising phase of the tension. The elastic work on the muscle series elastic component and on the recording system would be, at least in part, done by the ergometer. The muscle itself would have *less* work to perform, and therefore would presumably use less ATP (or PC). The result would be an economy of ATP hydrolysis, and an apparent effect of the stretch on the ATP hydrolysis. The artefact is obviated if the stretch begins when the tetanic tension is constant.

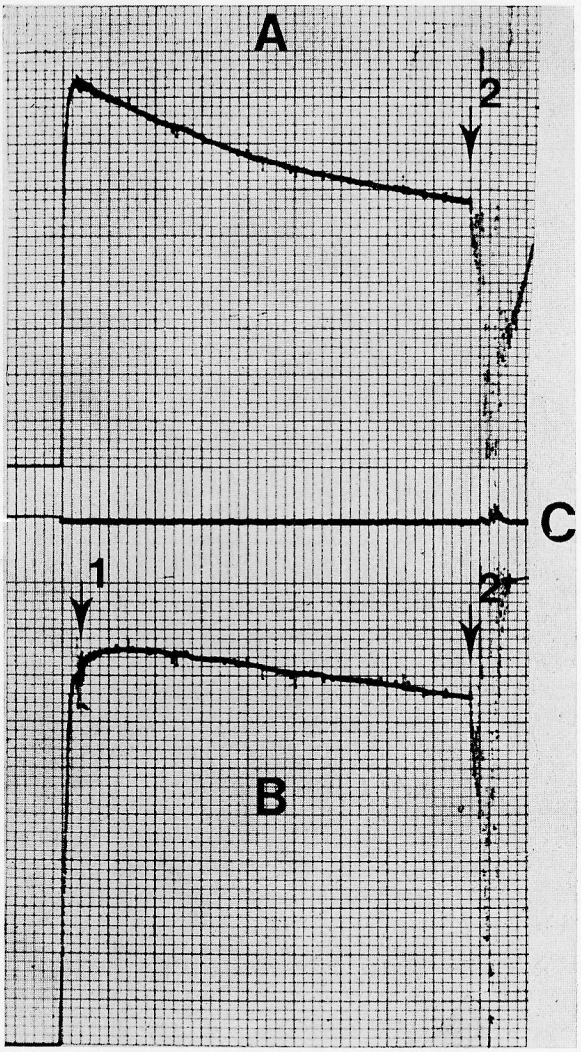


FIGURE 1 Mechanograms of frog sartorius muscles tetanized for 7.7 sec at 0°C. One muscle of the pair is held isometric (A). The other one is stretched with an ergometer (B). The stretch begins at the time shown by the arrow labelled 1. Both muscles are frozen together at the end of the movement (arrows 2). Line C shows the stimulation. Scales: 20 g or 1.75 sec for one large square.

The very slow rate of stretch was also chosen on purpose. In the myothermal investigations of Abbott, Aubert and Hill (1951), it was shown that at a very slow rate of stretch the heat production could actually be made *lower* than that of the isometric control, in spite of the fact that work was done on the muscle. If the work done on the muscle could be used to synthesize ATP, this condition seemed therefore the best one to use in experiments designed to detect the reality of such a synthesis.

Three series of experiments were performed. In the first one, the muscles were in O_2, in the second one, they were poisoned with fluorodinitrobenzene (FDNB), and in the third one, they were poisoned with dinitrophenol (DNP). Table I reports the main findings.

The weights of the muscles (4 to 6 pooled sartorius) are the same for the stretched series and for the isometric controls. The maximum isometric tension (P_0) is slightly higher for the FDNB muscles than for the normal muscles. This difference, not statistically significant, has been observed before (see Dumoulin and Maréchal, 1970). The maximum tension is somewhat lower for the DNP treated muscles, for reasons unknown. The negative work received by the muscles is nearly the same for the three treatments. It can be said that as far as mechanical conditions are concerned, there are therefore no meaningful differences between them.

In the unpoisoned muscles, stretch does not influence the content in ATP, but the stretched muscles have *more* PC than the isometric ones. The difference is 1.14 ± 0.36 micromole/g ($n = 12$). The content of creatine is correspondingly *lower* in the stretched muscles than in the isometric control: this difference is -0.85 ± 0.40. The economy in PC should exactly balance the economy of free creatine; they are not significantly different, and we may assume that they are two evaluations of the same phenomenon; thus, the best estimate of the economy of PC during the stretch is the mean of these two evaluations: *i.e.*, 0.99 micromole/g.

The negative work performed by the ergometer on the muscles equals 369 gcm/g or roughly 8.6 mcal/g.

Obviously the economy of PC is balanced somehow by the mechanical work received by the stretched muscle. We may define a "mechano-chemical coefficient" for negative work, which is the ratio negative work/economy in PC: it is equal to 8.7 kcal/mole. Its significance will be discussed later.

In FDNB-treated muscles, the PC is much higher than that found in the normal muscles, by 9.1 μmoles PC/g. This occurs because FDNB blocks the Lohmann reaction. One may therefore assume that PC concentration of FDNB muscles is the same as the PC concentration of normal unstimulated muscles. The difference represents then the energetic cost of a 7.7 sec isometric tetanus. It can be compared to the energetic cost of isometric tetani previously published. According to

TABLE I

Effect of stretch on the chemical changes during a tetanus

	ATP μmoles/g	Phosphocreatine μmoles/g	Free creatine μmoles/g	Weight mg	P_0 Kg/cm²	Negative work gcm/g
1. Normal muscles ($n = 12$)						
Stretched muscles	3.21	13.80	15.37	602.0		369±19
Isometric muscles	3.20	12.66	16.22	610.0	2.25±0.11	
Differences	0.01	1.14	−0.85	−8.3		
S.E. of differences	±0.06	±0.36	±0.40	±6.6		
2. FDNB poisoned muscles ($n = 10$)						
Stretched muscles	0.81	20.97	8.76	561.0		366±28
Isometric muscles	0.64	21.79	8.18	561.0	2.33±0.05	
Differences	0.17	−0.82	0.58	0.0		
S.E. of differences	±0.04	±0.37	±0.55	±3.0		
3. DNP poisoned muscles ($n = 4$)						
Stretched muscles	2.73	5.80	28.05	655.0		350±18
Isometric muscles	2.81	7.83	29.18	644.0	1.92±0.07	
Differences	−0.08	−2.03	−1.13	11.0		
S.E. of differences	±0.08	±0.97	±0.55	±8.0		

Maréchal and Mommaerts (1963), muscles (*Rana pipiens*) would hydrolyze about 2.5 μmoles PC/g for a 7.7 sec isometric tetanus. According to Maréchal (1964) muscles of *Rana temporaria* use somewhat more: 4.20 μmoles PC/g. The energetic cost of the experiments described here (9.1 μmoles PC/g) is at least twice that expected from the conclusions of earlier works. A possible explanation of this discrepancy is that the muscles were not at 0°C, but at a somewhat higher temperature. The manipulations had to be performed rapidly once the muscles had been loaded with ^{18}O; they were stimulated about 1 min after they had been hung on the electrode assembly. This time is perhaps too short to cool the muscle to 0°C once they had been exposed to the room air. As the Q_{10} of maintenance metabolism is about 5 (see Aubert 1956, p. 159), a muscle temperature of 4–5°C would account for the discrepancy.

There is an effect of stretch on the ATP content of FDNB poisoned muscles, the stretched muscles have 0.17 μmole/g *more* ATP than the isometric controls. As the myokinase reaction is certainly active (Cain and Davies, 1962) the difference in ~P used might equal $0.17 \times 2 = 0.34$; however, this is a maximum figure. The mechanochemical coefficient for negative work is 366 gcm/0.34 μmole ~P, or at least 25 kcal/mole. This is about three times the value obtained in unpoisoned muscles. It seems therefore that FDNB interferes with the absorption of negative work by the muscle.

In DNP poisoned muscles, the PC is much lower than in normal muscles. This is of course a well-known effect of DNP (see, for instance, Fleckenstein, Janke, Davies and Krebs, 1954). The ATP level is however much higher than in the FDNB poisoned muscles. It is interesting to notice that the time course of tetanic tension was not much different in the three series of experiments. This indicates that tetanic tension does not depend much on the PC or the ATP concentrations (Murphy, 1966), at least not over this range. The effect of stretch seems to be opposite to that found in unpoisoned muscles. However, the standard deviations of the data are large, and more experiments are required before such a conclusion may be accepted.

B. ^{18}O *Exchanges in Tetanized Muscles*

1. *The exchange of water*

Muscles were soaked for two minutes in a Ringer solution enriched with ^{18}O; they were then blotted, hung on the stimulation assembly, tetanized and frozen exactly 5 min after the first contact with the heavy water. A number of muscles were successively soaked in the same Ringer solution. It was observed that the volume "*m*" and the specific activity "*a*" of the Ringer solution decreased, as the experiments progressed. The analysis of the losses provides some information on the exchange of water between the muscles and the surrounding Ringer solution.

a) *The loss of volume* arises because the muscles which were soaked in the Ringer were previously *blotted* (in order to prevent as much as possible the dilution of the Ringer solution) whereas the muscles

which were taken out from the solution were only *drained*. The difference in weight between blotted and drained muscles was not measured in our experiments, to reduce the time of manipulations. According to Carlson, Hardy and Wilkie (1967), it is equal to 12% of the blotted muscle weight. The loss of fluid from the Ringer solution equals therefore 0.11 drained weight W. We observed that the water loss computed from this formula agrees well with the weight loss of the Ringer solution. Therefore, if m_0 is the initial weight of the Ringer solution, and W_1, W_2, ... W_n are the weights of the muscles successively soaked in it, the weight (m_n) of Ringer which remains after n muscles have been soaked is:

$$m_n = m_0 - 0.11(W_1 + W_2 \ldots W_n) \qquad (1)$$

The individual weights $W_1 \ldots W_n$ of the muscles were not measured. We measured the total weight W_T of all the muscles pooled together in an experimental series. If we assume that all the muscle pairs have nearly the same weight, *i.e.*, $W_1 = W_2 = W_3 = W_n = W_T/n = \overline{W}$, equation (1) becomes:

$$m_n = m_0 - 0.11(n)\overline{W} \qquad (2)$$

An estimate of the error introduced by this assumption may be obtained from the distribution of $\overline{W}$. For example, $\overline{W}$ was computed in seven experimental series of normal muscles; its mean was 249 mg, and its S.D. was 22 mg. This S.D. is an estimate of the S.D. of the individual muscle weights, if it is multiplied by $\sqrt{n}$ ($n \simeq 4$ or 5). This is true only if there are no batch effects which would affect the W_T's; otherwise, this estimate would be too high. The assumption is better justified in the case of muscles treated with FDNB ($\overline{W}$ = 221 mg; S.D. = 3 mg; 6 series) but it is not as good for muscles treated with DNP ($\overline{W}$ = 205 mg; S.D. = 56 mg; 4 series).

b) *The specific activity* of the Ringer solution is initially a_0. It decreases as the experiments progress, because water exchange occurs with the muscles. If this equilibrium is complete, it is easy to compute how a should change when muscles are soaked in turn. The amount of muscle water equals 77% of its weight (Wilkie, 1968). The specific activity of the Ringer solution after the first muscle pair has been soaked in it is:

$$a_1 = \frac{a_0 m_0}{m_0 + 0.77 W_1} \qquad (3)$$

The specific activity of muscle natural water does not enter the computation if a_0 is expressed in Atom % excess ^{18}O. It is easy to show that after the nth muscle pair, the specific activity is:

$$a_n = a_0 \frac{m_0}{m_0 + 0.77\overline{W}} \cdot \frac{m_1}{m_1 + 0.77\overline{W}} \cdot \frac{m_2}{m_2 + 0.77\overline{W}} \cdots \frac{m_{n-1}}{m_{n-1} + 0.77\overline{W}} \qquad (4)$$

Or introducing equation (2):

$$a_n = a_0 \prod_{n=1}^{n=n} \frac{m_0 - 0.11(n-1)\overline{W}}{m_0 - 0.11(n-1)\overline{W} + 0.77\overline{W}} \qquad (5)$$

The ratio a_n/a_0 is the observed ratio of the Ringer specific activities before and after an experimental series. The π product is easily computed from the observed weight of the Ringer solutions and of the muscles.

For unpoisoned muscles, the ratio a_n/a_0 for 7 experiments equals 0.847 ± 0.022, and the π product equals 0.816 ± 0.038; for FDNB treated muscles (5 experiments) the data are respectively 0.874 ± 0.036 and 0.866 ± 0.054. It is clear that the differences between the a_0/a_n ratio and the π product are negligible. The data are perfectly compatible with the result expected if muscle water is in complete isotopic equilibrium with Ringer water.

This finding is in good agreement with the former studies on the rate of water diffusion into muscle; for example Elford (1970) finds that 90% of HTO exchange in 2 min from the sartorius muscle water into the surrounding.

2. *The ^{18}O Incorporation into Inorganic Phosphate*

a) *Comparison with the ^{18}O specific activity of the Ringer solution.* The specific activities of ^{18}O which were found in the inorganic phosphate fraction (P_i) of the muscle extracts (abbreviated ^{18}O-P_i) vary within wide limits (Figure 2).

The main source of this variation comes from the variation of the ^{18}O specific activity of the water into which the muscles were soaked. This varied from 0.6 to 8.4 Atom % excess ^{18}O. The variation of the P_i specific activity is strongly correlated with that of the Ringer solution. There is some uncertainty as to which specific activity of the Ringer solution should be used for the comparison. Each muscle of the pool has been exposed to a Ringer solution whose activity is a function of the number of muscles which were soaked previously. ^{18}O-P_i of the pooled muscles is therefore some mean of the activities of the muscles of the set. One should compare it to some means of the activities of the

Ringer solution in which the muscles bathed. Such calculations cannot be done with accuracy, because the activities of the Ringer solution were measured *before* and *after* the experimental run, but not after each muscle had been in it. One could theoretically use Eq. 4 to compute such a mean, but it seemed simpler to compare the results to the activities of Ringer solution measured *after* the experiments. (This activity is smaller than the true mean activity.) It is plotted in Figure 2 with the corresponding P_i specific activities (in Atom % excess ^{18}O). The various lines indicate the functions expected for a ratio of specific activities (^{18}O-P_i)/(^{18}O-Ringer) indicated by the associated fraction. This ratio is always lower than 1/2; *i.e.*, that isotopic equilibrium is never obtained. It is usually close to 1/5 to 1/4, but may be as low as 1/20. In fact, the mean line (assuming that it must go through zero) indicates a ratio of 1/5.

There is no difference between stretched muscles (filled symbols) and isometric muscles (open symbols) in this respect, even if the differences between paired data (muscles of the same pairs) are analysed. Three results on resting muscles (crosses) also fit in the general frame. The type of treatment on the muscles, normal, FDNB or DNP poisoned, has also no influence on this ratio.

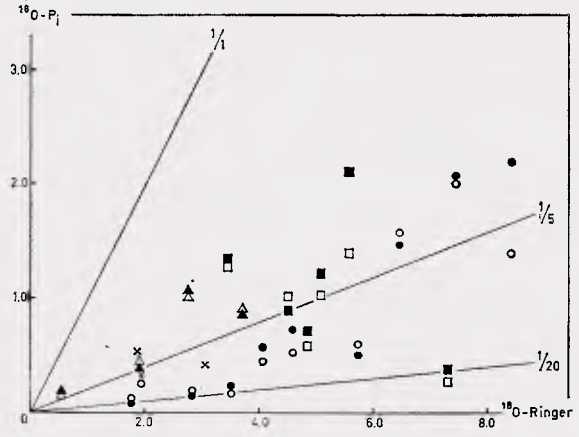


FIGURE 2 The dependence of the specific activity (in atom % excess) in ^{18}O of muscle inorganic phosphate as a function of the specific activity in ^{18}O of the Ringer solution. One muscle of the pair was stimulated isometrically (open symbols), the other one was stretched slowly during the tetanus (filled symbols). Three sets of muscles were not stimulated (crosses). Nine sets of muscle pairs were in O_2 (round symbols), six sets of muscle pairs were poisoned with fluorodinitrobenzene-N_2 (square symbols) and four were poisoned with dinitrophenol (triangular symbols). Two data of FDNB experiments are not plotted, because the corresponding specific activity was not measured. The experimental points should lie on the line 1/1, if the isotopic equilibrium is obtained; they should be on the lines 1/5 and 1/20 if the specific activity of P_i is 1/5 or 1/20 of that of the Ringer.

The interpretation of this ratio is made difficult because knowledge on the cellular distribution of the perchloric acid soluble P_i is lacking. If all of it is freely exchangeable in sarcoplasm, then a ratio of 1/5 indicates that each phosphate atom has been hydrolysed from an organic compound only *once* on average (see Janke, Marmier and Fleckenstein, 1965). This is equivalent to the concept that P_i released during contraction is not reused immediately by synthetic processes, or, if it is reused, the new compound is not hydrolysed during the contraction. Absence of any glycolytic or oxydative recovery processes (*i.e.*, reactions that use P_i, in contrast to the Lohmann or the myokinase reaction) seems well possible in a tetanus of 7.7 sec at 0°C (see Gilbert, Kretzschmar, Wilkie & Woledge, 1971).

A 1/5 ratio is also compatible with another concept: that there are two P_i pools. The first one is in isotopic equilibrium with water, the second one is not. The relative size of these two pools must be 1/4. The size of the exchangeable phosphate pool would be about equal to the "true" inorganic phosphate of resting muscle (Seraydarian *et al.*, 1961).

b) *The effect of the stretch.* In Table II, ^{18}O-P_i of stretched and isometric muscles are compared for various treatments.

In normal muscles, the mean specific activity of stretched muscles is 1.232 and that of the isometric control is 1.109; the difference ($+0.123 \pm 0.092$) is not significant. In these experiments the rate of the stretch was very low (0.7 mm/sec). If this rate is increased to 4 mm/sec or 26 mm/sec, there is also no difference between stretched and isometric muscles.

In muscles poisoned with FDNB, the mean activity is 0.911 for stretched muscles, and 0.780 for isometric muscles. Again here, the difference (0.131 ± 0.093) is not significant.

The muscles poisoned with DNP have ^{18}O specific activity (excess atom %) for P_i of 0.630 (stretched) and 0.647 (isometric). The difference (-0.017 ± 0.036) is insignificant. The specific activities are lower than those found for the normal and the FDNB series. This is explained by the lower activity of ^{18}O of the Ringer used in these experiments.

c) *The effect of stimulation and temperature.* In one experiment, one muscle of a pair was left

unstimulated and the other one was stimulated isometrically for 6 sec. They were frozen after the last stimulus, before the relaxation. The other details of the experiment were the same as those of the main series. The ratio of P_i to Ringer specific activity was 1/6 for the resting muscles and 1/8 for the stimulated muscles, thus practically the same.

In a second experiment, one muscle of the pair was left at 0°C, and the other was left at 18°C, both remained unstimulated. The ratio of the specific activities P_i/Ringer was 1/3.5 for the warm muscles, and 1/7 for the cold muscles. These experiments support the conclusions of Janke *et al.* (1965) that the turn-over of P_i is increased by temperature but not by stimulation.

3. ^{18}O *Specific Activity in Phosphorylcreatine*

Table III reports the ^{18}O specific activity of the phosphate isolated from PC (in Atom % excess, abbreviated ^{18}O-PC).

In normal muscles stretched at a slow rate (0.7 mm/sec), the mean specific activity (0.799) is not different from that of the isometric control (0.793).

TABLE II

^{18}O specific activity of P_i (Atom % excess)

Treatment	Normal			FDNB	DNP
Rate of stretch (mm/sec)	0.7	4	26	0.7	0.7
(*n*)	(9)	(3)	(2)[a]	(8)	(4)
Stretched muscles means	1.232	0.920	(0.159)	0.911	0.630
S.E.M.	±0.249	±0.181	(0.088)	±0.218	±0.205
Isometric muscles means	1.109	0.940	(0.191)	0.780	0.647
S.E.M.	±0.230	±0.191	(0.115)	±0.157	±0.198
Differences means	0.123	−0.020	(−0.032)	0.131	−0.017
S.E.D.	±0.092	±0.042	(−0.027)	±0.093	±0.036

TABLE III

^{18}O specific activity in phosphate isolated from PC (Atom % excess)

Treatment	Normal			FDNB	DNP
Rate of stretch (mm/sec)	0.7	4	26	0.7	0.7
(*n*)	(8)	(3)	(2)[a]	(8)	(4)
Stretched muscle means	0.799	0.563	(0.100)	0.075	0.258
S.E.M.	±0.202	±0.090	(0.163)	±0.015	±0.092
Isometric muscles means	0.798	0.607	(0.212)	0.067	0.251
S.E.M.	±0.217	±0.104	(0.168)	±0.010	±0.061
Differences means	0.001	−0.043	(−0.112)	0.008	0.007
S.E.D.	±0.027	±0.020	(−0.005)	±0.007	±0.031

[a]The results of the two experiments are given between brackets.
S.E.M.: Standard error of the means
S.E.D.: Standard error of the mean of the differences between paired measurements.

Similar results are obtained if the rate of stretch is increased to 4 mm/sec or 26 mm/sec. The results are also similar for muscles poisoned with dinitrophenol.

In muscles poisoned with FDNB, the ^{18}O-PC is again not different for stretched and isometric muscles. The magnitude of this activity is however very low, and in little excess over the natural ^{18}O activity. This finding is in agreement with the concept that FDNB blocks the only reaction (the Lohmann reaction) in which PC is involved.

The lack of any effect of stretch on the isotopic content of PC is by itself not a sufficient proof that such an action has not happened, because its detection rests on an assumption which should now be examined. The assumption is that the phosphoryl group of PC is not in isotopic equilibrium with the inorganic phosphate of the muscles (via ATP-building reactions and the Lohmann reaction).

In Figure 3 the specific activity of the phosphate isolated from PC is plotted as a function of the specific activity of the inorganic phosphate isolated from the same muscles. The data divide clearly into two groups: those of normal and DNP muscles, and those of FDNB-treated muscles. For this latter group, there is no dependence of ^{18}O-PC on ^{18}O-P_i but there is one for the former group.

The line which is drawn on the graph is the relation expected if the two compounds are in isotopic equilibrium. It takes into account the fact that the phosphate recovered from the muscle PC contains 3 O-atoms for the phosphoryl group, and one O-atom introduced into the phosphate when PC is hydrolysed in unlabelled water. Thus complete equilibrium is obtained if 3 O-atoms of the phosphate from PC are labelled, and 4 O-atoms from inorganic phosphate. The slope of a regression line of ^{18}O-PC on ^{18}O-P_i is 0.70 ± 0.05 ($n = 28$) for the normal muscles. This demonstrates that muscle PC is practically in isotopic equilibrium with P_i. The stretch does not influence these relative specific activities. The slope in DNP-muscles may be different, but too few experiments were made to decide the issue. Clearly, FDNB muscles are different.

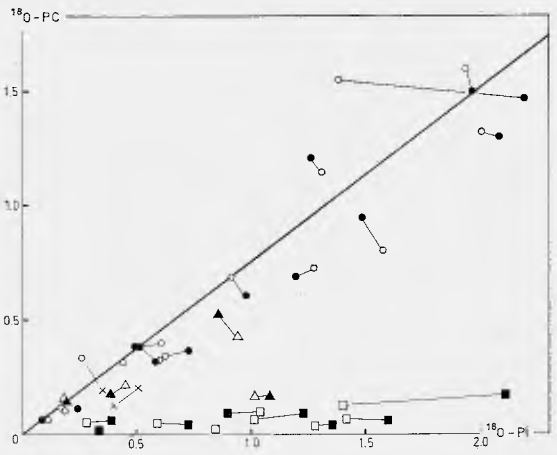


FIGURE 3 The correlation between the specific activity in ^{18}O of the phosphorylcreatine (^{18}O-PC), in Atom % excess, as a function of the ^{18}O specific activity in the muscle P_i.

Same symbols as for Figure 2. Data obtained on paired muscles are joined by short lines. They should lie on the thick line if isotopic equilibrium was obtained.

Three experiments were performed on resting muscles (Crosses on Figure 3). The one which is the farther right on the graph gives the results of muscles held at 18°C through the physiological part of the experiment. For the two other ones on the left, the muscles were held at 0°C. No influence of the change of temperature may be ascertained. The three points are however lower than the points for the stimulated muscles, be they paired (as is the case of one experiment) or not. This may indicate that a 7.7 sec stimulation increases the turn-over of PC although this conclusion is contrary to that of Janke *et al.* (1965). The points from resting muscles are *higher* than those obtained on stimulated muscles poisoned with FDNB. This again emphasized the fact that the isotopic exchange of ^{18}O between PC and P_i requires the activity of the Lohmann enzyme.

4. *The ^{18}O Activity in ATP*

a) *The isotopic equilibrium between ATP-P_γ and ATP-P_β.* The ^{18}O specific activity observed in ATP (symbolized ^{18}O-ATP) is reported in Table IV.

The interpretation of these results is somewhat complicated by the fact that the technique used did not allow us to isolate ATP-P_β from ATP-P_γ: the isotopic analysis had to be performed on a mixture of the two terminal phosphate groups of ATP.

Comparison of Table IV with Tables II and III shows that for any muscle, ^{18}O-ATP is lower than ^{18}O-P_i and ^{18}O-PC. This must mean that ATP-P_β and P_γ are not in general isotopic equilibrium with PC or $_iP$. The first hypothesis to be considered is that ATP-P_β does not exchange at all. In this case the ^{18}O-ATP-P_γ is twice ^{18}O-ATP (if specific activities are in % Atom excess), so that, from our

results, the true specific activity of ATP-P_γ equals 1.2 ^{18}O-P_i (see Table V).

In this hypothesis, ^{18}O should be introduced into ATP-P_γ by another mechanism than incorporation of labelled P_i by a synthesis reaction with ADP. ATP-P_γ does not exchange O atoms directly with water, and myosin or actomyosin do not catalyze such an exchange reaction (Levy *et al.*, 1962). No other reaction is known, which might account for a ^{18}O specific activity higher in ATP-P_γ than in P_i.

On the other hand, Fleckenstein *et al.* (1960) have found that the ratio of the ^{18}O specific activities in ATP-P_γ and ATP-P_β is 100/22. An exchange of labelled P between ATP-P_γ and ATP-P_β could well occur through the interplays of the Lohmann and the myokinase reaction, both of

TABLE IV

^{18}O specific activity in phosphate isolated from ATP (Atom % excess)

Treatment	Normal			FDNB	DNP
Rate of stretch (mm/sec)	0.7	4	26	0.7	0.7
(*n*)	(9)	(3)	(2)[a]	(9)	(4)
Stretched muscles					
means	0.451	0.340	(0.046)	0.505	0.175
S.E.M.	±0.095	±0.119	(0.025)	±0.107	±0.045
Isometric muscles					
means	0.437	0.353	(0.045)	0.493	0.151
S.E.M.	±0.100	±0.117	(0.030)	±0.108	±0.040
Differences					
means	0.014	−0.013	(0.001)	0.012	0.024
S.E.D.	±0.042	±0.003	(−0.005)	±0.015	±0.019

TABLE V

Ratio ^{18}O-ATP to ^{18}O-P_i

Treatment	Normal			FDNB	DNP
Rate of stretch (mm/sec)	0.7	4	26	0.7	0.7
(*n*)	(9)	(3)	(2)[a]	(7)	(4)
Stretched muscles					
means	0.620	0.578	(0.668)	0.735	0.543
S.E.M.	±0.032	±0.041	(0.765)	±0.102	±0.106
Isometric muscles					
means	0.582	0.588	(0.731)	0.680	0.541
S.E.M.	±0.034	±0.058	(0.813)	±0.094	±0.090
Differences					
means	0.036	−0.010	(0.063)	0.055	0.002
S.E.D.	±0.044	±0.024	(0.048)	±0.047	±0.045

The ^{18}O-ATP data of Table IV have been corrected for the two natural O atoms introduced into ATP by the *in vitro* hydrolysis performed for the preparation of inorganic phosphate. The results of Table V indicate the mean ^{18}O-activity of the P_β and P_γ of ATP, as a percentage of the ^{18}O-activity of the inorganic phosphate of the same muscle.

[a]The data for the two experiments are given between brackets.

S.E.M.: Standard error of the means.

S.E.D.: Standard error of the mean of the differences between paired measurements.

which are active during a tetanus (Canfield and Maréchal, 1973). Isotopic equilibrium between ATP-P_β and ATP-P_γ is however not obtained, as ^{18}O-ATP is lower than ^{18}O-PC. It seems reasonable to assume that ^{18}O-ATP-P_γ is equal to ^{18}O-PC, as Janke *et al.* (1965) have found. Then, ^{18}O-ATP-P_β can be computed as $1.23-0.93 = 0.30$ for normal muscles. This is in good agreement with the results of Fleckenstein *et al.* (1960).

For FDNB muscles, however, it is not possible to compute ^{18}O-ATP-P_β in this way, because PC is not labelled due to the inhibition of the creatine-ATP kinase. The ^{18}O-ATP is however not much different from that found in normal muscle (Table IV); if referred to ^{18}O-P_i it is somewhat higher (Table V). Myokinase is still active in FDNB treated muscle, but the Lohmann enzyme is not (Cain and Davies, 1962). That inhibition of creatine phosphate kinase does not bring about a decrease of ^{18}O-ATP-P_β presumably indicates that other reactions (mainly ATP synthesis from ADP) are the main pathways for an ^{18}O exchange between ATP-P_γ and ATP-P_β.

b) *The effect of the stretch and of stimulation.* As for the study of ^{18}O incorporation into PC, it is better to consider the specific activity of ATP relative to the P_i activity, *i.e.*, the ratio ^{18}O-ATP/^{18}O-P_i (Table V). For normal muscles stimulated isometrically, this ratio is 0.582 ± 0.034 and 0.620 ± 0.032 for the stretched muscles. The difference -0.036 ± 0.044 is not significant. Similar results are obtained for FDNB poisoned muscles, and for those treated with DNP.

Three resting muscles had ratios of 0.48, 0.53 and 0.55; this is somewhat lower than the mean ratio found in stimulated muscles; it may indicate that stimulation increases it. This inference is supported by the fact that in one experiment, stimulated muscles were compared with paired resting ones; the ratio was 0.75 in the former case, and 0.50 in the latter case.

DISCUSSION

Two main conclusions emerge from the experimental data:

1) After a 7.7 sec tetanus at 0°C, the muscles which have been slowly stretched (0.7 mm/sec) have more PC and ATP than paired muscles tetanized isometrically for exactly the same duration.

2) After labelling the phosphate compounds by an exchange with Ringer enriched in ^{18}O, no difference could be detected in specific activity of P_i, PC or ATP between muscles which have been stretched and muscles which have been stimulated isometrically.

The meaning of these findings should now be assessed.

A. *The Mechano-Chemistry of Muscles during a Slow Stretch*

The original version of the problem has been stated in the Introduction as it presented itself on the basis of myothermal findings. It is a curious historical point that one has found it self-evident that work done upon an active muscle should appear there as heat, just as one took it for granted that work returned to the muscle in relaxation should do so. As noted briefly with reference to the latter case (Mommaerts, 1970), this applies only under one of these conditions: that the work be imposed in an irreversible manner; or that, if it is applied reversibly, the receiving structure should have rubber-like thermo-elastic properties. The same conditions apply here, but it is known that active muscle (Hill, 1952) and also muscle in rigor (Aubert, 1956) is not rubber-like, but norm-elastic. During the course of a stretch-experiment, various thermo-elastic effects occur, which cancel and are discounted in the final result (Hill and Howarth, 1959). As to the final outcome, the imposed work then can have two fates: heat or some other form of internal energy. In the latter case, the internal energy could be that of a chemical compound synthesized or spared, *i.e.*, ATP or PC, and the major significance of our results is to have specified it in that sense by finding that in slow stretches where no extra heat appears (Abbott, Aubert and Hill, 1951) the spared PC is the chemical equivalent of the work imposed to the muscle, with satisfactory approximation.

At this point, it is convenient to introduce a quantity which has been called the mechano-chemical coefficient for work transduction (Maréchal, 1964). It is defined by the ratio of the work produced or received by the muscle to the extra amount of PC split (in the case of positive work) or spared (in the case of negative work). This coefficient is easily computed for our experimental results. In the experiments reported here, it is equal to 8.7 kcal/mole for muscles stretched in normal conditions. It is numerically close to the enthalpy changes of PC hydrolysis. This suggests a com-

parison with the metabolism of a shortening muscle. Mommaerts *et al.* (1962) concluded that for the positive Fenn-effect, "the terms (describing chemical changes) specifically associated with work do not lead to additional heat." This means in other words:

$$Q_w = m_w T\bar{\Delta}S + m_w \bar{\Delta} F - W \simeq 0 \qquad (1)$$

if Q_w is the additional heat displayed by a working muscle, and m_w the molar amount of chemical changes associated with the work.

This conclusion was based on the experimentally verified fact (Mommaerts *et al.*, 1962; Maréchal, 1964; Wilkie, 1968) that the mechano-chemical coefficient for positive work transduction is numerically equal to the enthalpy change for PC hydrolysis†:

$$\frac{W}{m_w} = \bar{\Delta}H \qquad (2)$$

In the case of negative work, W should be replaced by $-W$ and m_w by $(-m_w)$ (the amount of PC spared). As it is found experimentally that Eq. (2) does still hold in this case, one must conclude that the Q_w produced by the muscle as the result of the stretch should have been near zero, its total heat during the stretch equal to the maintenance heat. This is exactly what Abbott *et al.* (1951) found for muscles stretched at very slow rates similar to those used for the present experiments. This places the sparing effect in stretched muscle in exact symmetry to the Fenn-effect in the same muscle.

This conclusion is not true anymore for muscles poisoned with FDNB: the mechano-chemical coefficient equals then 25 kcal/mole or more. This is more than twice the enthalpy changes for the only energy-yielding reaction which then occurs, the ATP hydrolysis. This situation is similar to that of unpoisoned muscles stretched at a rate about six times (0.42 cm/sec) higher than that used here (0.7 mm/sec): the mechano-chemical coefficient equals then 37 kcal/mole (Maréchal, 1964, p. 134). It allows us to estimate in these experiments which proportion of negative work has been stored as internal energy, and which proportion has been dissipated as heat. The stored internal energy which corresponds to the spared PC (or ATP) equals $m_w\bar{\Delta}H$, according to Eq. (2). The heat Q_w dissipated from the negative work is simply the difference:

$$Q_w = W - \bar{\Delta}H/m_w \qquad (3)$$

Or the fraction of negative work which is dissipated as heat is a function of the mechano-chemical coefficient (W/m_W):

$$\frac{Q_w}{W} = 1 - \frac{\bar{\Delta}H}{W/m_w} \qquad (4)$$

We predict thus that in FDNB poisoned muscles stretched at 0.7 mm/sec (0°C), 56% of the negative work done on it should be dissipated as heat, while for normal muscles stretched at the same rate, no increase in heat production should occur during the stretch. Another expression would be that conditions allowing the reversible absorption of imposed work lead to a largely irreversible absorption after FDNB-poisoning. Thus far, there had been no indications that the chemo-mechanical transduction process is altered in its characteristics by FDNB (see Bárány, Bárány and Bailin, 1968).

When thus seeing the Fenn-effect and the sparing effect as exactly symmetrical counterparts, it would seem tempting to conclude that as work performance implies a chemo-mechanical transduction of ATP splitting energy into work, negative work must then mean a mechano-chemical synthesis of ATP. This does not follow. In the light of the theory of Huxley (1957), modified by Mommaerts (1969), the transduction process occurs at the cross-bridges; we represent this as a change of interactive attachment of myosin to actin which leads to a translation of the I-filaments over a distance of a few hundred Angströms, while splitting an ATP molecule. It is entirely plausible that, when movement is forced in the opposite direction the coincident splitting of ATP is inhibited. Since the accompanying paper of Gillis and Maréchal (1974) comes out in support of the inhibition hypothesis, this will indeed be our view.

However, we must explain one seeming paradox at this level of formal analysis. The general trend has been to associate higher tension with higher metabolism, both in twitches and in the maintenance of a tetanus (see Mommaerts, 1969 for an overview). Experimentally this is amply documented for the isometric situation, *e.g.*, when the

† We must point out, however, that this specific form of the Fenn-effect is a rather coincidental one; the extra metabolism generated with work can also be more, or often less, than its equivalent to the work (Mommaerts, 1970). It seems to equal it for frog muscles at 0°C, and it is to this special case that the investigation of the Fenn-effect has been mainly restricted.

active tension is varied as a function of length (Aubert, 1956; Maréchal, 1964; Gibbs, Ricchiuti and Mommaerts, 1966; Smith, 1972; Homsher, Mommaerts, Ricchiuti and Wallner, 1972). It has also been accepted by Gibbs, Mommaerts and Ricchiuti (1967) and by Homsher *et al.* (1972) for the tension associated energy in isotonic shortening, as the basis for their independent method for the evaluation of the shortening heat. Now, however, we are proposing that, when muscle is stretched and shows an even higher tension, its metabolism is inhibited as a result of this. Is this a contradiction? It is not. At the mechanistic level, the difference is that now the cross-bridges are forced to move in the opposite direction; at the formal level, we recall that for the case of lengthening, the force-velocity relations show a discontinuity (Katz, 1939; Aubert, 1956), and we recall that the two lines of thought are united in A. F. Huxley's theory (1957). Thus we believe the view to be plausible and consistent.

B. *The Mechanism of the Absorption of Negative Work by the Active Muscle*

We have sought to bring a decision between the two hypotheses as stated in the Introduction. Having eliminated the use of ^{32}P which attains complete isotope equilibration as it penetrates, we placed our hope upon the application of ^{18}O-water. The first condition of its application was met when we found that external water exchanges in a few minutes with internal water (in agreement with Elford, 1970).

The second condition, the agreement with the results of Janke *et al.* (1965) on the rectus abdominus muscle, required the bulk of our experiments to be tested.

As to its first part, agreement was obtained: inorganic phosphate becomes labelled only to a specific activity 0.2 to 0.25 that of the water. If not due to compartmentalization, this means that P_i is cycled only once during the 5 minutes of contact with labelled water. This means of course that inorganic phosphate split from ATP (or PC) incorporates one ^{18}O-atom from the water according to the equation (Koshland, Budenstein and Kowalsky, 1954):

$$ATP + H_2{}^{18}O \longrightarrow ADP + H_3PO_3{-}{}^{18}O \quad (5)$$

The inorganic phosphate which is liberated does not react a second time with $H_2{}^{18}O$, *i.e.*, it seems metabolically inert. This finding throws doubt on the physiological relevance of the phosphate oxygen exchange reaction observed during the "intermediate" reaction (Levy and Koshland, 1959; Yount and Koshland, 1963) or during the "medium" exchange reaction (Dempsey and Boyer, 1961). In experiments with glycerinated fibres (Dempsey, Boyer and Benson, 1963) ^{18}O exchange (medium type) increased with the load on the fibres. If such exchanges occurred *in vivo* as well, one should expect a maximum rate of exchange as the contractions are isometric, at maximal force. The ratio $^{18}O\text{-}P_i/H_2{}^{18}O$ should then equal 3/8, if it is 1/4 for incorporation by hydrolysis + 1/8 for medium exchange incorporation. As the ratio found rarely exceeds 1/4, and is nearer 1/5, we conclude that there is no indication that "intermediate" or "medium" exchange plays a physiological role during the muscle contraction. It may be that a muscle contraction of 7.7 sec at 0°C is much too short for these reactions to have any significant effect, as they are barely detected after an incubation of 2 min at 25°C (Benson, Dempsey and Benson, 1967).

From here on, however, our results differed from those of Janke *et al.* (1965) on the rectus abdominus muscle, in which P_i becomes labelled precociously. In our case, the specific activity of ^{18}O in PC, and presumably that of the P_γ of ATP, is 90% that of P_i. This difference is too small to allow any conclusions from our finding that the specific activity of PC is not increased by stretch, in view of the limits of error as they emerged. In fact, it appears that even with the results of Janke *et al.* (1965) the experiment would not be promising. We know that negative work can induce a sparing of 1 micromole PC/g; this is about 1/13 of the amount of PC left at the end of the tetanus (see Table I). If this spared PC is synthesized from ATP formed from ADP and P_i under the action of the stretch, the increase in ^{18}O specific activity of PC, is easily computed. If results similar to those of Janke *et al.* had been obtained, the isometric muscles would have had 12 μmoles PC/g at an ^{18}O activity equal to 5% of that of P_i; the PC synthesized by the stretch should have an ^{18}O activity equal to that of P_i, and therefore, the total PC found in the stretched muscles should equal 13 μmoles/g at an ^{18}O activity equal to 12% of that of P_i $\left(0.12 = \frac{(12 \times 0.05) + (1 \times 1.0)}{13}\right)$. The difference in ^{18}O activity between isometric and stretched muscles should equal 7% of the specific activity of

P_i. The standard error for diffference between paired muscles is about equal to 4% (n:4 to 9). This reasoning leads to the conclusion that there is not much chance to obtain positive results in favor of the reversal hypothesis by examining how stretch can influence ^{18}O exchange between P_i and PC, even if the best conditions are obtained.

Previously, Infante *et al.* (1964) had concluded that suppression of metabolism, not synthesis, occurs. Their differences (4 mm stretches) are of the order of 0.15–0.20 μmole ATP in the FDNB muscle, similar to ours for a 5 mm stretch, but much less than ours for the PC sparing in unpoisoned muscle. Since they only determined the amounts of ATP used but not its net synthesis, if any, there was nothing in their experiments to allow a choice between the two explanations. The decision cannot be decided by balance studies alone. Since our effort, too, turned out unsuccessful, the matter is undecided, but for the accompanying investigation of Gillis and Maréchal (1974).

C. *PC and ATP Equilibrium in Muscle*

One other result should be mentioned. It is that FDNB inhibits completely the incorporation of ^{18}O into PC, although it does not change this incorporation into P_i or ATP. FDNB is known to inhibit the phosphoryl transfer between ATP and PC. This poison should also block the incorporation of ^{18}O into PC, if the Lohmann reaction is the only reaction in which the phosphoryl group of PC is involved. That it does so, effectively, is a strong argument for the hypothesis that PC is involved in no other reactions than the Lohmann reaction. Especially the concept that PC could phosphorylate directly the contractile proteins, for example actin, hypothesis of Carlson and Siger (1960), is difficult to hold.

The clearance of ATP by adenylate kinase does not involve phosphate oxygen exchange with water (Levy *et al.*, 1962), but this enzyme can still catalyse incorporation of ^{18}O into ADP-P_β. The labelled ADP can then be reutilised to form ATP labelled both at the P_β and P_γ, either through the Lohmann reaction, or through the myokinase reaction. The fact that FDNB does not influence the specific activity of $P_\beta + P_\gamma$ may indicate that the dissociation of ATP by the myokinase reaction is the limiting process. This is in agreement with the very low level of AMP in resting muscle, as found by Canfield and Maréchal (1973).

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