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Title Phosphorus Distribution in the Muscle During a Deficiency of the Anti-stiffness Factor

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In studies of the metabolism of the anti-stiffness factor van Wagtendonk observed that a deficiency of the anti-stiffness factor resulted in an abnormal distribution of the acid soluble phosphorus in the livers and kidneys of the experimental animals. The most striking changes observed were the increase of the inorganic P and the decrease of the easily hydrolizable P during the deficiency. The alkaline serum phosphatase was lower than in non-deficient animals of the same age group.

Since the muscle is a tissue with high rate of metabolism and one in which the carbohydrates are a prominent substrate, it was considered important to trace the distribution of the acid soluble phosphates in that tissue.

This thesis represents an attempt to determine the distribution of adenosinephosphates, creatine-phosphate and inorganic phosphate in the Rectus femoris muscle of guinea pigs during the deficiency of the anti-stiffness factor as compared to controls and groups of animals treated with the extracted anti-stiffness factor.

In progressively older animals on stock diet the creatin-phosphate and adenylphosphate fractions in the muscle increase slightly while the inorganic phosphate content falls. During the deficiency diet the muscle adenylphosphate

and creatine-phosphate decrease, and the inorganic phosphate increases. These changes become more pronounced the longer the animals are on the deficiency diet. In animals on the deficiency diet with supplemented extract of the anti-stiffness factor the distribution of P compounds commences to return to normal. The total P content of the muscle remains approximately the same for all groups.

The deficiency disease is also accompanied by a rise of the Ca level. Ca accelerates the breakdown of adenosinetriphosphate, which is followed by a decomposition of creatine-phosphate. The increased concentration of inorganic phosphate may therefore be attributed indirectly to the preceding derangement of the Ca distribution. Ca also prevents the resynthesis of creatine and inorganic phosphate into creatine-phosphate.

Since the increased concentration of inorganic phosphate or the transfer of Ca ions may cause a higher permeability of the muscle membrane, the observed increase in plasma P may be a result of the deranged muscle metabolism.

PHOSPHORUS DISTRIBUTION IN THE MUSCLE DURING A DEFICIENCY OF THE ANTI-STIFFNESS FACTOR

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PHOSPHORUS DISTRIBUTION IN THE MUSCLE DURING A DEFICIENCY OF THE ANTI-STIFFNESS FACTOR

Introduction

For a long time phosphate transfer was believed to be important only in the alcoholic fermentation; but after intensive studies of intermediate reactions involved in this breakdown and investigations of the relations between muscular action and metabolism, it became evident that the phosphate radical is introduced into compounds not only to facilitate their breakdown but also as carriers of energy (50). These findings threw a new light on the old conception of muscle action which associated contraction with glycogen breakdown to lactic acid. Lundsgaard's (56) experiments, linking creatine phosphate* breakdown to muscular contraction, when interpreted in that light were important facts in that revision.

As early as 1907 Urano (31) suggested the existance of a creatine containing complex on the basis of dialysis experiments with isolated muscle. In 1914 Folin and Denis (35) demonstrated in vivo that muscle creatine exists in bound form. Cr-ph, besides adenylic acid, is one of the two particularly important phosphate esters in the metabolism of

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* The following abbreviations will be used:
cr-ph -- creatine phosphate
AA -- Adenylic acid
ATP -- adenosinetriphosphate
ADP -- Adenosinediphosphate

muscle tissue (44). It is present exclusively as an unstable ester of phosphoric acid in the muscle and nervous tissue of the vertebrates (17, 37, 50). Eggleton and Eggleton (14, 15, 16, 17) discovered it in frog muscle and called it "phosphagen", while Fiske and Subbarow (30, 31) isolated cr-ph from cat muscle.

Cr-ph liberates proportional amounts of creatine and inorganic phosphate in acid solution and when first estimated quantitively was therefore confused with inorganic phosphate (87).

In the muscle over one-half of the total creatine content was found to exist in combination with phosphorus and about 70% of the available P tied to creatine, so that there is only a slight surplus of creatine over P (50).

Already Eggleton and Eggleton (15, 17) and Fiske and Subbarow (31) observed that cr-ph, when decomposed during a long series of contractions, was reconstructed quite rapidly during recovery in oxygen. Likewise, anaerobically, cr-ph was resynthesized very effectively at the expense of glycogen (68).

Meyerhof and Suranyi (62) observed that large amounts of heat were released by enzymatic decomposition of cr-ph; in measuring the ultimate heat energy of cr-ph and glycogen breakdown, Meyerhof, Lundsgaard, and Blaschke (60) found them to be the same and capable of doing the same amount of mechanical work. The combination of these experiments

clearly linked energy transfer through phosphate breakdown to muscular contraction.

Lundsgaard (56) noticed the remarkable efficiency of glycolysis. During the decomposition of one half mole glucose to lactic acid approximately two moles cr-ph are formed and the active heat energy of glycolysis is utilized for the conversion into phosphate bound energy. Even in the absence of glycolytic energy, energy-rich phosphate bonds are easily available from stored cr-ph (77) and the conversion of glycolytic or combustion energy into energy-rich phosphate linkages is relatively easy; it is therefore safe to assume that the energy utilized in muscular activity is always derived from the energy-rich phosphate bond, supplied by glycolytic or oxidative foodstuff breakdown (50).

The entire chain of glycolytic reactions from hexosediphosphates to lactic acid is made up of reversible reactions with the exception of phosphopyruvate breakdown. The energy yielding reaction is conditioned by the energy-rich phosphate turnover, i.e. by the eventual liberation of energy-rich phosphate bonds; the aerobic reaction is one of the factors to reverse the glycolytic reaction. Lipmann (50), Needham and Pillai (73), Meyerhof, Ohlmeyer and Mohle (61) have shown that one half of the phosphate taken up in the oxidation can be transferred to creatine. The transfer of energy-rich phosphate linkages can best be understood by studying their role in the intermediate reactions of glycolysis.

In the mechanism of anaerobic metabolism glucose, hexosemonophophate, AA and ADP act as phosphate acceptors during alcoholic fermentation, while creatine and glycogen play that role during muscle glycolysis (49). Adenosinetriphosphate and cr-ph act exclusively as ph-donors (45). The ph-cr and phosphopyruvic acid compete as phosphate donors to the adenylic acid system (57). In alkaline solution adenylic acid reacts more rapidly with phosphopyruvic acid than with cr-ph; in acid and neutral solutions the reverse is true.

In the working muscle inorganic phosphate is constantly liberated at a higher rate than glycogen is esterified; it originates not from cr-ph but from ATP (57). ATP is a universally present cell constituent whose first phosphate is joined in an energy poor linkage to ribose; the PO-P linkages are readily hydrolized enzymatically, liberating energy (50). ATP is in enzymatic equilibrium with creatine according to the equation:

$$ATP + cr \rightleftharpoons ADP + cr\text{-}ph$$

In the resting or recovering muscle ADP accepts ph from cr-ph, phospho-pyruvate or glycerophosphate to form ATP. Exactly where glycogenolysis begins and where phosphorylation of adenylic acid is taken over by pyruvic acid, rather than cr-ph, is not yet known. Even in the smallest muscle tension some lactic acid formation can be pointed out by chemi-

cal investigation (69).

Dephosphorylation of ATP is accompanied by the liberation of free energy:

$$ATP \rightleftharpoons ADP + H_3PO_4 + 11{,}000 \text{ cal.}$$

Lundsgaard (56) showed in his experiments with the iodoacetate poisoned muscle that the contractile system was charged with energy supplied by the liberation of inorganic phosphates from cr-ph and ATP. Since the muscle contracted at the expense of cr-ph and ATP alone, it was exhausted in the state of rigor as soon as these dephosphorylating sources were depleted. The energy furnished thereby was most likely used for the relaxation of the contracting muscle and therefore the relaxed state may be considered the charged state of the contractile system. There is reason to believe that ATP is not dephosphorylated spontaneously, but rather by an enzymatic reaction (myosin acting as the phosphate transfer system).

With his experiments Lundsgaard opened up a new trend in the study of phosphate esterification, by associating ATP directly with the restoration of the contractile substance. Up to then the hydrolysis of phosphocreatine was regarded as most likely the major cause of muscular contraction (31). Since the attachment of the phosphate to the organic molecule is accompanied by increased acidity, Fiske and Subbarow (30) believed that the hydrolysis of cr-ph would liberate

enough base, under optimum conditions, to neutralize a considerable part of the lactic acid formed during muscular contraction. This theory has met with opposition by Meyerhof and Lohmann(59) who observed that the hydrolysis of cr-ph, in brief periods of stimulation, proceeds much more rapidly than the production of lactic acid. They therefore regarded the contraction as a response to the increased acidity. Embden (20) even believed that lactic acid formation does not take place until after contraction has occurred.

Sacks (80, 81) still regards the role of cr-ph in the muscle as serving to neutralize the lactic acid formed, therefore opposing the Embden-Meyerhof theory of shuttling the energy-rich phosphates back and forth between creatine and the adenylic acid system. But Lundsgaard's experiments have brought the adenylic acid system into the foreground. Lohmann (54) proposed the change from cr-ph to ATP after he found that the hydrolytic breakdown of cr-ph occurred only by way of intermediate formation of ATP and subsequent hydrolysis of the latter through adenylpyrophosphatase. Lipmann (50) does not accept this argument as justifiable because hydrolytice destruction and utilization need not take the same routes, and he believes no decision can yet be taken on the origin of energy rich phosphate bonds, due to the lack of experimental proof.

The present conception of muscle contraction regards cr-ph as by-product, not a direct intermediate product of energy-rich phosphate transfer. Creatine takes up phosphate from an oxidative or glycolytic system through ATP, and on contraction gives it up exclusively to the adenylic acid system in the presence of Mg ions; this represents the transmission of phosphorylating energy to the contractile system, since it can take part as phosphate-acceptor and phosphate-donor (55).

The ATP is originally formed during the glycolytic breakdown (or oxydative synthesis). One of the energy-rich phosphate linkages generated in this oxidation-reduction reaction is removed through the adenylic acid system and eventually can appear in cr-ph.

1,3 di-ph-glyceric acid + ADP $\rightleftharpoons$ 3 ph-glyceric acid + ATP

ATP + ph $\longrightarrow$ [inorganic ph / cr-ph / glucose / hexose di-phosphate] + ADP

ADP can again be phosphorylated by cr-ph with the aid of transphosphorylase. Cr-ph may therefore be regarded as a storage for the energy-rich phosphate and it will act as a reserve donor of the phosphate radical for the resynthesis of ATP from adenylic acid or ADP (77). Lipmann (50) sees in this the operation of pyrophosphatase as an outlet for the adenylic acid system to adjust the flow of the energy-rich phosphate, in case of over-production, much in the manner

of a valve.

The breakdown of ATP occurs almost simultaneously with the contraction of the fibrils. ATP is therefore the phosphate donor to the contractile protein, myosin, and the breakdown of cr-ph, glycolysis and oxidation only serve to restore the adenylic acid system to a constant level of phosphorylation. Both the cr-ph and ATP breakdown precede the glycolytic breakdown of carbohydrates (56). This interpretation ties together two, until then isolated facts: 1) the energy transfer from the carbohydrate breakdown to the muscle fiber through the phosphate cycles (32). 2)The discovery of the elongated or anisometric character of the particles of muscle myosin, which through X-ray studies lead to the establishment of muscular contraction as molecular contractibility of protein chains (66, 67, 2).

Engelhardt and Ljubimova (21) found that myosin, the chief muscle protein, possesses adenosinetriphosphatase properties. This was confirmed by Szent-Györgyi and Banga (84). According to Barron (7) the adenylphophatase activity of the muscle is associated with the myosin for after repeated purifications of myosin by precipitation, the protein remained just as active in splitting off one PO_4 group from ATP (51) but had lost the power peculiar to the mash, or once precipitated myosin, of converting ADP to AA. Barron therefore seems justified in suspecting in myosin not one

particular compound, but rather several protein-complexes, each catalyzing a specific reaction. In the sequence of reactions he confirmed Lohmann's findings that the splitting ATP precedes that of cr-ph.

Myosin I facilitates the reaction:

$$ATP \rightleftharpoons ADP + H_3PO_4 + 11{,}000 \text{ cal.}$$

It seems to be a protein-Ca compound for Ca is the most effective activator. (Engelhardt and Ljubimova 22, Needham 72).

Myosin II, which is precipitated myosin, catalyzes:

$$ADP \rightleftharpoons AA + H_3PO_4 + 11{,}000 \text{ cal.}$$

(Bailey 4)

Myokinase facilitates the reaction:

$$2\ ADP \rightleftharpoons ATP + AA$$

(Kalckar 44)

Transphosphorylase is important in two reactions:

$$ATP + cr \rightleftharpoons ADP + cr\text{-}ph$$

$$ATP + 2\ cr \rightleftharpoons AA + 2\ cr\text{-}ph$$

(Lehmann 47, Lohmann 51, 52)

Szent-Györgyi (85) found in muscle another fiber protein which he termed "actin". If a thread of actomyosin was placed into a solution of $MgCl_2$ plus the Mg salt of ATP, a complex was formed without which the form of the thread is changed. After addition of KCl the thread contracts to 33% of its original length and in this state the adenosintri-

phosphatase is split off more easily than when actomyosin is in the relaxed state.

Some relation may exist between the enzymic properties and the mechanical response of the myosin thread to the action of ATP (21), thereby controlling the relative optical anisometry and shape of the myosin particles and possibly their intermicellar forces (74, 45) so that myosin might be phosphorylated and dephosphorylated, occupying thereby the last link in the chain of simultaneous transfers of ph ions and energy.

Needham et al (74) therefore believe that ATP donates its ph to the contractile protein, myosin, which has adenyl-pyrophosphatase activity and phosphate would be transferred from ATP to some part of the protein molecule which would simultaneously extend, thereby changing chemical to mechanical energy. Extended myosin would then be in phosphorylated form and charged with energy. During nerve stimulation the physical changes would be accompanied by the liberation of inorganic phosphate and energy made available for the use in contraction.

Triose-ph + ph $\rightleftharpoons$ phosphoglyceryl-ph

Phosphoglyceryl-ph + ADP $\rightleftharpoons$ ph-glycerate + ATP + cr $\rightleftharpoons$ ADP + cr-ph

ATP + contracted Myosin $\rightleftharpoons$ ADP + relaxed ph-myosin

relaxed ph-myosin $\rightleftharpoons$ contracted myosin + ph

(Modification of Kalckar 45)

The use of isotopes in experimentation has been of great value, especially in the coordination of "vivo" and "vitro" results and by this method it is possible to demonstrate reactions that cannot otherwise be detected in vivo. The use of radioactive inorganic P in studies of muscle contraction was introduced by Hevesy (41) in 1924 and has been continued by many other groups with more or less complete agreement of results. The main cause of differences seems to be traceable to the fact that the muscle tissue is contaminated by the presence of plasma, which also contains the injected radioactive P, and thereby the radioactive, so-called inorganic phosphate fraction, of the muscle as a whole is increased. Another problem to consider is that of the location of the radioactive P, either in the connective tissue or within the muscle cell itself (45).

Flock and Bollman (34) observed that the uptake of P^{32} in the acid labile phosphate groups in ATP is much greater than in the stable phosphate group. Two ph radicals of ATP are exchangeable with the inorganic phosphate of the tissue; of these the first group which is hydrolizable with myosin, takes up the greatest amount of P^{32}. The third phosphate group which is attached to the ribose, takes up least of the P^{32}.

The behavior of phosphocreatine and inorganic phosphate in the working and resting muscle has been most successfully

studied by Bollmann and Flock (8). They found that the hydrolysis and resynthesis of 80% of the cr-ph can be accomplished without appreciable change of its radioactivity. The inorganic ph fraction of the muscle contains much greater radioactivity than the cr-ph and since the latter retained its content of P^{32} this must be interpreted that the phosphate liberated by the hydrolysis of cr-ph of exercised muscle did not mix to any extent with the inorganic ph originally there; therefore the resynthesis must have taken place with the lower radioactive ph of the previously hydrolized cr-ph. Apparently then, the presence of labeled phosphates in certain fractions of ph-containing compounds is independent of the working process.

In this connection it is interesting to note that the cr-ph is present in greatest amounts in white muscle, least in red muscle (27) (76). This might mean that muscles capable of rapid energy output are in resting condition richer in phosphagen than those intended for lower rate of energy expenditures (17).

Bollman and Flock (8) worked on a very plausible explanation of their experiments; they consider the possibility that the ph liberated during muscular activity may be bound chemically or separated physically in the muscle from the inorganic ph originally there, prior to the extraction with CCl_3COOH. The phosphate might have a special function in

connection with the contractile system, hydrolysis of the organic phosphate being accompanied by phosphorylation of myosin, resynthesis by dephosphorylation of the protein. Whenever the radioactivity increased in the inorganic phosphate it might be ascribed to the influx of plasma which is higher in radioactivity, though the labeled ph increase is very small in comparison to the large volume of blood flowing through the active muscle.

The participation of anaerobic processes in the aerobically working muscle was investigated by Flock, Ingle and Bollman (33). They found that during the first minute of work the ph-cr and glycogen decreased rapidly, followed by a delayed decrease of ATP and accompanied by a slow increase of inorganic phosphates, but rapid accumulation of lactic acid and hexose monophosphate. During the next fifteen minutes of activity there was a gradual return to the normal level in all but the cr-ph and glycogen whose concentration remained unaltered. The experimenters concluded therefore, that since the concentration of the labile substances was little altered by increased aerobic activity, they must be involved in the early phases of work, but little in steady state activity.

The far-reaching importance of the phosphorus metabolism can be best appreciated in studies of its distribution during nutritional muscular dystrophy. A dystrophy producing

diet may be one deficient in vitamin E, as the one suggested by Goetsch and Pappenheimer (65). The dystrophy could be cured or prevented by a supplement to the deficiency diet of fresh alfalfa, or lettuce and vitamin E (wheat germ oil), or dry alfalfa and vitamin E, or whole wheat germ. Therefore there must be two factors involved in cure and prevention; both of them present in fresh green alfalfa or whole wheat germ oil, one present in lettuce and dry alfalfa. The other factor was destroyed by drying or extraction with water or alcohol or by ethereal Fe Cl_3 (65).

Histological changes in the degenerating muscle are accompanied by alterations in the chemical composition and in functional behavior. The most striking of these is the diminishing cr content (38), which is roughly proportional to the degree of degeneration (43). There is also a corresponding decrease in total solids, total N, and glycogen, an increase in cholesterol, a gain in Na Cl with corresponding loss of K and Mg and finally an increase in Ca. (64, 25) the latter was also evidenced histologically (25). Vitamin E deficient diet does not affect the total P content of the tissue (92) but the ph-cr and ATP portions decrease while the soluble ester P and inorganic phosphates make up a larger part of the total acid soluble P compared to normal (78).

Friedman and Mattill (36) observed that the O_2 con-

sumption was elevated during muscular dystrophy. Since oxidation and phosphorylation are coupled in muscle, an increase in rate of the oxidative processes would be paralleled by an increase in P turnover. Kaunitz and Pappenheimer (46) found that the high O_2 consumption in muscle during vitamin E deficiency precedes the morphological changes. An increase in P turnover in tocopherol deficiency was observed by Houchin (42) and Weissberger and Harris (92) and explained by the former as not due to a stimulation of the P metabolism directly, but as a result of the lack of the oxidation inhibitor, vitamin E, on the succinoxidase system.

The low creatine content of dystrophic muscle (43) and the accompanying creatinuria (38) also suggest that an elevated P metabolism accompanies muscular dystrophy. An excessive and continuous loss of cr from the muscle would impair the resynthesis of cr-ph and therefore the transfer of ph in the carbohydrate cycle. The deficient utilization of ph would have in consequence the higher rate of ph turnover (92) as shown by the increase in the P*:P ratio (92,8).

Ni (15) also reported a decreased cr-tolerance of dystrophied animals who were able to store only 15-37% of the ingested cr, while controls retained 49-53% of the cr administered.

Muscular dystrophy can also be effected on a diet lacking in the "anti-stiffness factor" described by Wulzen and

Bahrs (93, 3) and isolated by van Wagtendonk and Wulzen (91) from raw cream. In a dosage of 0.1γ the anti-stiffness factor was able to cure stiffness induced by the skim milk diet within four days (89).

Animals on the deficiency diet exhibited as overt symptoms stiffness of the joints, while autopsy revealed dystrophied muscle and Ca deposits in the tissues.

In studies of the metabolism of the anti-stiffness factor, van Wagtendonk observed that a deficiency of the anti-stiffness factor resulted in an abnormal distribution of the acid soluble phosphorus in the liver and kidneys of the experimental animals. The most striking changes observed were the increase of the inorganic P and the decrease of the easily hydrolyzable P during the deficiency (88). The alkaline serum phosphatase is lower than in non-deficient animals of the same age groups (89). Since the muscle is a tissue with a high rate of cr/metabolism, and as shown in the preceding discussion, one in which the carbohydrates are a prominent substrate, it was considered important to trace the distribution of the acid soluble phosphates in that tissue. The following description of experiments represents an attempt to determine the distribution of ATP, cr-ph and inorganic phosphates in the Rectus femoris muscle of guinea pigs during the deficiency of the anti-stiffness factor, as compared to controls and groups of animals treated with the extracted anti-stiffness factor.

Experimental

a) Method

The colorimetric determination of cr-ph is based on the fact that generally only the inorganic phosphoric acid forms a reducible molybdenum complex with molybdic acid, while the phosphoric acid esters do not react. The esters can therefore be analyzed either by specific reactions of the organic component or by determination of the inorganic phosphate, liberated by the hydrolysis of the ester after specific reactions.

The method employed in this investigation is a modification of those described by Fiske and Subbarow (28, 29) and Stone (82, 83). Table I reproduces the steps followed in this experiment.

Since traumatic injury of the muscle brings about extraordinarily rapid cleavage of cr-ph (26) the muscle was placed at once after removal into a mixture of dry ice and ether. The tissue was kept in the frozen state overnight. The solution was made alkaline immediately after maceration, thereby preventing further decomposition of cr-ph (13). The changes in the phosphate content of the frozen muscle are very slight (58, 1).

The acid components of the filtrate were extracted with ice cold CCl_3COOH and the inorganic phosphates in solution

were precipitated from the protein free extract as Ca salts (13, 28).

The adenylphyrophosphate fraction was separated from the inorganic phosphate by boiling with 5 N HCl; in the seven minute hydrolysis two of the three phosphate groups were split off (82) which then could be determined according to the method by Fiske and Subbarow (28). The sum of the inorganic and pyrophosphate was calculated from the final reading and the amount of pyrophosphate found by the difference.

The P content of cr-ph was determined by the method of Fiske and Subbarow (28) as the "acid unstable P".

In the colorimetric analysis of P, Molybdate II was used, as indicated in Table I. The solution was prepared according to Fiske and Subbarow (28) as 2.5% NH_4 molybdate dissolved in 3 N H_2SO_4. The dye, aminonaphtholsulfonic acid, also suggested by Fiske and Subbarow (28) acts as oxidizing agent; in a mixture of molybdate and phosphomolybdate only the latter is reduced to "molybdate blue" (86). The reduced solution was read on a Klett colorimeter against a known standard. The number of mg phosphorus per 100 gm muscle were calculated from the following equation:

$$\text{mg P/100 gm muscle} = \frac{\text{conc. of standard} \times \text{own reading} \times \text{dilution}}{\text{reading of standard}}$$

Three groups of guinea pigs, a total of 116 animals, were used in this investigation. The first group of 29 animals served as control group, and was raised on a stock

TABLE I

Frozen Muscle Macerated with ten times its Volume CCl_3COOH and filtered

↓

Alkalized to phenolphthalein

↓

4 cc Filtrate 1 cc 10% $CaCl_2$
After 10 minutes Standing
Centrifuged 10 minutes at 3000 RPM

CREATINE-PHOSPHATE

Supernatant fluid ← Decanted — Ppt. washed with 4 cc H_2O 1 cc $CaCl_2$ saturated with $Ca(OH)_2$; centrifuged

↓

Diluted to 10 cc

↓

5 cc Filtrate
1 cc Molybdate II
0.4 cc Dye

↓

Read on Klett after ten minutes

(Total dilution: 500 times)

Ppt. dissolved in 4 drops 5 N HCl

↓

Diluted to 8 cc

ADENYLPYROPHOSPHATE (FINAL READING - INORGANIC PHOSPHATE)

↓

4 cc aliquot
1 cc 5 N HCl
boiled in H_2O bath 7 min.

↓

Cooled

↓

Total Filtrate
1 cc Molybdate II
0.4 cc Dye

↓

Diluted to 50 cc

↓

Read on Klett after ten minutes

(Total dilution: 2500 times)

INORGANIC PHOSPHATE

↓

4 cc aliquot
1 cc Molybdate II
0.4 cc Dye

↓

Diluted to 50 cc

↓

Read on Klett after ten minutes

(total dilution: 2500 times)

diet of the following composition:

Rolled barley	ad lib.
Greens	ad lib.
Straw	ad lib.
iodized salt	

The second group of 51 animals received a diet deficient in the anti-stiffness factor, described by v. Wagtendonk (88) as follows:

Skim milk powder	16 g.
Water	84 g.
Ferric chloride	0.25 mg.
Copper sulfate	0.25 mg.
Autoclaved straw	ad lib.
Iodized salt	ad lib.

The diet was given twice a day. To the morning feeding a solution of the water soluble vitamins was added in such a concentration that the average daily intake per animal of the individual vitamin was:

Thiamin hydrochloride	0.2 mg.
Pyridoxine hydrochloride	0.1 mg.
Riboflavin	0.5 mg.
Nicotinic acid	1.0 mg.
Pantothenic acid	0.1 mg.
Inositol	10 mg.
P-aminobenzoic acid	2 mg.
Choline	50 mg.
Biotin(S.M.A. conc S 200)	0.01 mg.

To the evening diet was added a solution of the fat soluble vitamins in cottonseed oil. The average daily intake per animal was:

Beta carotene	150 I. U.
Viosterol	40 I. U.
Alpha-tocopherol	0.1 mg.
2-methyl-1,4-naphthoquinone	0.1 mg.

Ascorbic acid (50 mg.) was administered orally once per week.

The third group of 36 animals was raised on the same diet as group two, but received supplements of the anti-stiffness factor extract in different amounts and after various periods on the deficiency diet. Each group was divided into subgroups of progressively older age groups, with an attempt to have parallel age brackets for comparison in all three main groups. In the second group the time period on the deficiency diet increased with progressing age of the animals.

All animals were introduced into the experiment at the age of eight weeks, the age being roughly determined by weight.

b) Results

From Table II it is evident that in all animals on stock diet the cr-ph and pyrophosphate fractions increase slightly with progressive age. This substantiates the

TABLE II

	NORMAL			DEFICIENT				DEFICIENT ASF**			
Age in weeks	Inorg. P mg/100 g.	Cr-P mg/100 g.	ATP-ADP mg/100 g.	weeks on diet	Inorg. P mg/100 g.	Cr-P mg/100 g.	ATP-ADP mg/100 g.	Dosage of ASF**	Inorg. P mg/100g.	Cr-P mg/100g.	ATP-ADP mg/100g.
13	54.1	7.0	12.4								
14				1	48.7	7.8	18.7				
15				2	69.1	7.1	12.4				
16				3	51.6	10.0	14.8				
20	37.2	9.2	20.6	7	71.0		17.7	1000 U¥a	70.8	2.3	15.5
23				10	54.0	7.9	7.6	1000 U¥b	[illegible]	[illegible]	[illegible]
29				19	76.1	4.5	4.4	1/100 ɣ c	72.1	4.6	5.7
								10 ɣ d	66.4	5.6	7.8
41				28	65.2	4.2	3.8				
72	42.7	12.0	19.8								

*One unit was arbitrarily defined as follows: a solution of the compound in Wesson oil contains one unit per cc if, when 1 cc is administered daily for 5 consecutive days to a deficient animal, it alleviates the induced stiffness in this time. (91)

**ASF Anti-stiffness Factor

a 1000 Units of the anti-stiffness factor every day for the last week of the experiment.

b 1000 Units of the anti-stiffness factor every second day for the last 10 weeks of the experiment.

c 1/100 ɣ of the anti-stiffness factor every day for the last 5 days of the experiment.

d 10 ɣ of the anti-stiffness factor every day for the last 5 days of the experiment.

findings of Fainshmidt, Osinskaya and Ushakova (23). The cr-ph content of the adult animals' M. Rectus femoris of this experiment coincides with that found by Palladin and Apelbaum (76) for the M. biceps femoris.

During the deficiency diet there is a marked decrease of/pyrophosphate and cr-ph, paralleled by an increase in the inorganic phosphate fraction of the muscle, compared to non-deficient animals of the same age group. These variations become more pronounced the longer the animals are on the deficiency diet. There is an increase in the ph-cr and ad-pyrophosphate of some of the early deficient muscles, compared with the general trend, but this is not progressive with the period of deficiency.

In the groups receiving supplements of the anti-stiffness factor on the deficiency diet it can be noticed that the P distribution is beginning to return to normal. It is interesting to note that the administration of 1/100 γ of the anti-stiffness factor, each day for the last five days of the experiment does not change the P distribution of the deficient muscle. A supplement of 10 γ of the factor given the same time, however, does approximately return the P level to normal.

The total P content remains just about the same in the muscles of all groups. Riesser (79) observed large daily variations in the total P content of the guinea pig muscle;

the cause is unknown, but a certain relationship between the P content and meteorological conditions was suggested by Riesser.

Discussion

It is evident from the results presented in this thesis that the deficiency of the anti-stiffness factor in the diet of guinea pigs entails a deranged distribution of the acid soluble phosphorus compounds in the muscle.

At the same time it is interesting to note the apparent similarity between the symptoms produced by this deficiency diet and the characteristic symptoms associated with vitamin E deficiency. In both cases cr-ph and adenylphosphates decrease, while the inorganic phosphate content rises in the muscle. Both diets result in dystrophy of the muscle and Ca deposits in muscle tissue (25). Since the deficiency diet of this investigation includes an amount of alpha-tocopherol (0.1 mg. per animal every day) adequate as preventive dosage (92) the derangement of the P metabolism may therefore be attributed to the lack of the anti-stiffness factor in the diet.

One of the main differences between the effects of the diets lies in the further fate of these acid-soluble P compounds. Vitamin E deficiency is accompanied by severe creatinuria, which means that while the phosphate of the split cr-ph remains in the muscle, the creatine leaves and is excreted. In diets lacking the anti-stiffness factor we know that the split cr is not excreted (90),

but studies have not been conducted to determine whether the free cr remains within the muscle cell or travels to other parts of the body. For this reason alone it would be interesting to estimate quantitively the amount of free cr in the muscle. It is also important to affirm whether all the P determined in this study actually originates from metabolic processes in the muscle fiber itself or whether some of the P migrated to muscle cells from the plasma; this investigation could be best conducted with the aid of labelled P. Such an experiment seems especially urgent in the face of a study conducted by Hahn, Hevesy & Rebbe (39) who found that labelled P, originally located in the plasma, migrated to cells of the muscle, while an equal amount of non-labelled P migrated in the opposite direction. After four hours 1/80 of the total acid soluble P present in the cell migrated from the plasma to the muscle cell.

There is therefore a twofold possible origin of the deranged P distribution in the muscle:

1) It is a direct product of the muscle metabolism
2) It reflects an unbalanced condition of the plasma in which case the P in the muscle can either parallel the distribution of P in the plasma or vary from it due to the different rates of diffusion of the various compounds of P.

In either case the problem is one of the rate of

penetration of P ions, either into or out of the cell. This in turn is determined by:

a) the properties of the cell wall

b) the rate at which the inorganic phosphate is incorporated into organic compounds, either within or outside the cell, in order to prevent it from diffusing back from where it came.

Eggleton (11) believed that the membranes binding the muscle cell are relatively impermeable to phosphate. Though Fenn (24) doubts this statement because it fails to explain the removal of high inorganic phosphates in the exercising muscle, it seems to hold generally true, especially when considering that the inorganic phosphate remains within the muscle in heightened concentration for some time after the exercise stops; the relative impermeability of the muscle membrane might prevent a rapid penetration of the phosphate to the outside of the cell.

Furthermore, Embden (19) explained that fatigue is accompanied by increase in permeability of the membranes around the muscle fiber and on the basis of this he devised a theory of muscle contraction in which a sudden increase in permeability is an integral part of the response to stimulus. This idea was further developed by Needham, Shen, Needham and Lawrence (74) and Needham (69) suggested that the contraction of the protein particles in the fibril (myosin) would be

caused by a sudden contact with the resynthesized ATP; she proposed that the contact was occasioned by a changed permeability. The actual cause for the change in permeability is not understood, but maybe it is a result of the transfer of ions, rather than due to a physical alteration in the membrane itself. The change may be occasioned by a breakdown of the compound originally present, and in particular the transfer of Ca ions (40) which always accompanies the deficiency.

Coming back to the first question of the origin of the deranged P distribution in the muscle, one might venture to suggest that if Embden is correct in associating increased permeability of the membrane with exercise, i.e. increased presence of inorganic phosphate, the same might hold true here; the deficiency of the anti-stiffness factor may cause an increased rate of P turnover with consequent increase of inorganic phosphate production and increased permeability of the membrane, so that the increased plasma concentration of P is a result of the speeded up muscle metabolism.

Yet we also know from experiments by Bollman and Flock (8) that the resynthesis of cr-ph in the muscle is accomplished directly from the products of hydrolysis. This way the rate of penetration of P into or out of the muscle is independent of the working process. If this is the case, the increased concentration of inorganic phosphate is probably due to the

stored P originally formed by the breakdown of cr-ph and adenylphosphates, i.e. phosphates are broken down faster than they can leave. The failure of inorganic phosphate and creatine to be resynthesized into cr-ph might be due to the inhibiting effect of Ca (4, 9, 10, 75) which is found in the tissues in increased concentration in this deficiency disease.

Needham (72) showed that the breakdown of ATP to ADP and H_3PO_4 is greatly activated by Ca, so that the increase of inorganic phosphates might be a response to the raised Ca level, which in itself is a symptom of the deficiency disease. The breakdown of cr-ph following the breakdown of ATP, as expected (52, 53) is brought out in the experimental data of this investigation by a faster and earlier drop of ATP phosphorus.

Since v. Wagtendonk (88) observed a deranged P distribution in kidney and liver, the concentration of these compounds in the plasma might also find their origin there. If they remain in ionized form there is a good possibility that some of them might penetrate into the tissues, e.g. the muscle (though care must be taken not to confuse P compounds in the connective tissue surrounding the muscle with P compounds within the muscle fiber itself). On the other hand the inorganic phosphate of the plasma might be traced to its origin in the muscle and then there would be little reason to assume that it would diffuse back--if the process is one

of diffusion and not osmosis.

The discussion of this investigation might well be compared to a lock and key. The key represented by experimental data may pass into the keyhole but fail to turn if not synchronized with the characteristics of the lock. The body of evidence must be complete, in harmonious working order and filed to fit into the experimental picture as a whole. If the key is to play its part as a functioning unit all its composing fragments must be obtained and pieced together secondarily. The subject of this thesis presents but one more small fragment in the primary construction of the key and all discussion as to its outline and function can at its best be considered only speculation.

Summary

In progressively older guinea pigs on stock diet the muscle cr-ph and adenylphosphate fractions increase slightly, while the muscle inorganic phosphate content falls slightly. During the deficiency diet there is a marked decrease of adenylphosphate and cr-ph, paralleled by an increase in the inorganic phosphate fraction of the muscle. In groups receiving supplements of the anti-stiffness factor on the deficiency diet the P distribution is beginning to return to normal.

The rise of inorganic phosphates in the muscle may be due to the raised Ca level which accompanies the deficiency disease. Ca accelerated the ATP breakdown which is followed by an increased rate of decomposition of cr-ph. The derangement of the P metabolism in the muscle is intimately connected to the properties of the cell wall of muscle fibers and the rate of penetration of ions through it. The raised P level of the plasma may be a result of the speeded up P metabolism in the muscle, increased permeability of the muscle membrane favoring transfer of the inorganic phosphates. The increased Ca level of the deficiency or the greater concentration of inorganic phosphates may be responsible for the change in permeability of the cell membrane.

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