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Time Course of Regeneration of Minced Frog Muscles Estimated by the Level of Energetic Substrates

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Summary. 1. Gastrocnemius muscles of frogs have been excised, chopped and put back into the leg.

2. The weight of the implanted muscles increases until the 8th day after the operation and then decreases to 1/3 of that of the control by the 30th day.

3. The inulin space is twice as high in regenerating muscle as in the unoperated controls.

4. Adenosine nucleotides are in an enzymatic equilibrium set by the ratio creatine/phosphorylcreatine during regeneration of the muscle.

5. Glycolytic intermediates (hexose monophosphate, fructose-1,6-diphosphate, α-glycerophosphate and lactate) have been measured during the course of muscle regeneration up to the 30th day. The results suggest that the glycolysis of regenerating muscle is very active.

6. The total creatine (sum of creatine and phosphorylcreatine) is very low after the operation: 5% of that of the controls; it rises sharply after the 8th day, and reaches 30% of that of the control on the 30th day.

7. The amount of total creatine seems proportional to the progress of the muscle regeneration. It is suggested to use this substance as a biochemical reference for biochemical works on muscle regeneration.

Key words: Regeneration of muscle – Creatine – Creatine phosphate – Adenosine nucleotides – Glycolytic intermediates.

INTRODUCTION

A remarkable case of muscle regeneration was reported by Studitsky in 1959. He had dissected out a rat gastrocnemius muscle, and cut it into small pieces of about 1 mm^3; the muscle thus minced was put back into its original place in the rat leg. After a few weeks, a new muscle had grown, which was innervated and contractile. This interesting phenomenon was confirmed by Carlson, who also reviewed and discussed the works recently published in the field of regeneration of muscle (Carlson, 1968, 1972). Most of these studies were aimed at the morphology of muscle regeneration, and little is known of the biochemical control of this process or of the physiological properties of regenerated muscle (for a review see Carlson, 1972, 1973).

A basic problem is to measure the progress of muscular regeneration. Morphological studies have shown that it follows a characteristic sequence. First, the minced muscle degenerates while the myofibrils and the other sarcoplasmic structure fragment and eventually disappear; secondly, spindle-shaped cells appear and proliferate; thirdly, the myogenic cells of this population fuse to form multinucleated myotubes in which typical cross-striations appear, and fourthly, the centrally located nuclei migrate toward the periphery of the fibre, and the sarcoplasm and myofibrils hypertrophy. In frogs these four phases last about 1 week each, but they are not simultaneous throughout the minced muscle, since there exists a craniocaudal and a centripetal gradient of development.

The morphological evidence, specific and descriptive as it is, does not however provide us with an easy method for a quantitative evaluation of the net amount of muscular tissue which originated from the mince, or that is truly regenerated. It is known that free creatine (C_f) and phosphorylcreatine (PC) are substrates which occur in high concentrations in striated muscles. Their sum, which will be called total creatine (C_T), is quite constant in muscular tissue, amounting to 20–30 μmol/g wet weight (Maréchal, 1964). The total creatine concentration correlates well with the maximal isometric force which is probably the best estimate of a muscle's "size" (Wilkie, 1968). In fact the total creatine content of a muscle is a better index of the amount of muscular tissue than is its dry or wet

weight, and has often been used as such in many studies on the energetic metabolism of muscle (for a full discussion of this problem, see Wilkie, 1968). Therefore, it is obviously of interest to know the time course of the total creatine concentration during the degenerative and regenerative stages of minced muscles. At the same time, this study might shed some light on the energy metabolism of the regenerating muscles, and so various other key metabolites were measured: the adenine nucleotides and some intermediates of the glycolytic pathway, in order to enlarge the overall experimental base.

METHODS

A. The Grafting Procedure

Orthotopic autografts were performed in December and January on adult *Rana temporaria* obtained from Ireland. The frogs were kept at room temperature (20° C) in small individual chambers with continuously running tap water.

On day zero, the frog was anesthetized by immersion in MS 222 (Sandoz) diluted 1:1000 in tap water. A skin incision was made from the ankle to the popliteal area. The Achilles tendon was severed and the gastrocnemius was dissected up to about 0.5 cm from its femoral insertion. At that level the gastrocnemius nerve was dissected as far as possible and the muscle was transversely cut. The piece of muscle was placed in a few drops of sterile 0.6% saline and chopped with scissors into small pieces of about 1 mm^3. The mince was put back under the skin flaps except for some gross fragments of connective tissue which were discarded. The skin incision was sutured with 6-0 silk (Ethicon) at 2 mm intervals. Apart from sterilizing the instruments and glassware coming into contact with the muscle, no other special precautions were taken. Infection, rupturing of sutures or other possible postoperative complications were not observed in the present experiments.

B. Design of the Experiment

The experiments were performed on 24 frogs. The right gastrocnemius was minced and autografted as described in the previous section, and the left gastrocnemius was used as control. In order to survey the most important stages of the regeneration processes, the two gastrocnemius muscles were removed each time in 3 frogs, at 1, 2, 4, 8 days (degenerative stage) and 15, 30 days (early regenerative stage) after implantation. They were analysed for the amounts of eleven different substrates.

For the 8th and 30th day however 6 animals were used rather than 3, because we wished to obtain more accurate information for these two periods: the beginning of second week sees the first appearance of myotubes and the end of the first month is important because no more muscle fibers are formed thereafter and the later evolution consist of maturation and growth (Carlson, 1968). In view of the number of chemical manipulations, it was not possible to analyse more than 6 pairs of muscles a week. The 24 operations were timed in such a way as to provide 3 frogs a week for analysis each having a randomly chosen postoperative delay.

In 10 animals of this series, 2/3 of the mince was replaced rather than the whole of it, in order to obtain some information on the influence of the amount of grafted tissue on the magnitude of the regeneration.

C. Chemical Procedures

a) Extraction Procedure. The regenerated and contralateral gastrocnemius muscles were dissected out, and transfered to an oxygenated Ringer's solution containing 1 μC/ml of inulin-H^3 in which they incubated for 5, 15 or 60 min at 22° C. The muscles were then lightly blotted between dry filter papers and frozen by rapid immersion in isopentane cooled to − 160° C with liquid N_2. The frozen operated muscle presented two parts: the 0.5 cm proximal intact muscle and the large distal portion which had been minced; they were separated and the proximal portion was discarded. The distal portion and the control muscles were weighted, still frozen, pulverized and extracted with 0.5 N perchloric acid (Maréchal, 1964).

b) Chemical Analysis. Creatine was measured by its reaction with diacetyl-α-naphthol (Ennor and Stocken, 1948); after acid hydrolysis (10 min at 65° C in 0.4 N HCl then neutralization with NaOH) the total creatine (creatine + phosphorylcreatine) was measured by the same reaction. An allowance of 11% was made for creatinine formation during the acid hydrolysis of phosphorylcreatine. Phosphorylcreatine itself was computed as the difference between total creatine and creatine. The analysis of adenosine nucleotides and intermediates of the glycolytic pathway are described in Bergmeyer (1970).

c) Inulin Space. The radioactivity of the extracts and of suitably diluted aliquots of the medium were measured with a liquid scintillation spectrometer (Packard Tricarb 3375).

d) Controls and Expression of the Results. The contralateral gastrocnemius muscles were used as control. The means and standard errors of the substrate concentrations are computed in micromoles per gram wet weight. The metabolites in minced muscles are expressed as percentages of the control values.

RESULTS

A. Gross Changes

After the grafting procedure the operated leg remains quite normal for a few days. Around the 3th day, its diameter increases as a result of an inflammatory oedema, reaching a maximum at the end of the first week; then the oedema subsides and the diameter of the leg thins to half that of the contralateral within 3 weeks. If only 2/3 of the minced muscle is implanted, the swelling is less and it resolves sooner. The sutures fall out during the second week.

Internally the form of the mince is roughly the same as that of the original muscle. Within 24 hrs the fragments of the implanted tissue are glued together by fibrinous materials, so that the whole mass is strong enough to retain its shape when it is removed from the limb. By the end of the first week, the connections between the implanted fragments and the cut ends of the Achilles tendon and the proximal muscle stump have become fibrous and stronger. At that time the muscle, even the proximal stump, is very oedematous, and the individual muscle fragments are less distinguishable. From then on, the volume of the muscle decreases until at the end of the first month the diameter is between 1/3 and 1/2 of the contralateral gastrocnemius.

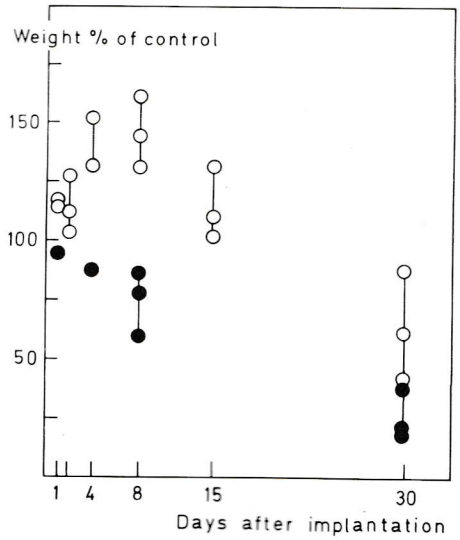

Fig. 1. The wet weight of grafted tissue, expressed as percent of controls, varies with time after implantation. The open circles represent muscles where the whole minced tissue was replaced and the filled circles those where about two-thirds of minced was replaced

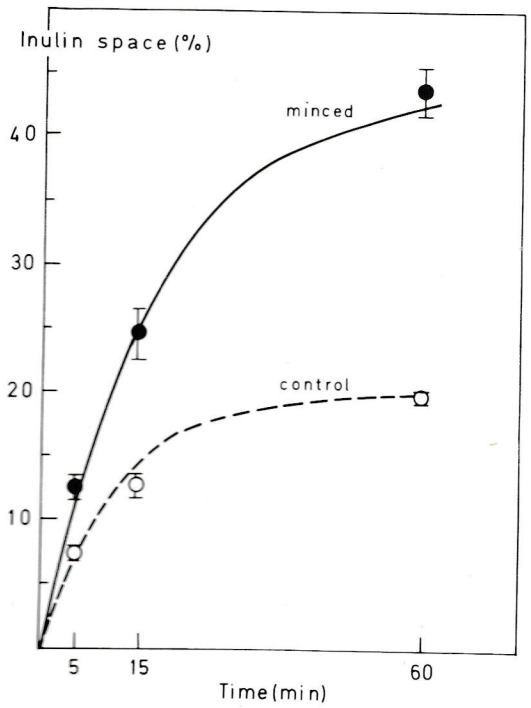

Fig. 2. Inulin space (percent of whole muscle volume) of minced (upper curve) and control (lower curve) muscles incubated *in vitro* for 5, 15 and 60 min. As the inulin space does not depend on the age of the regenerate, the results were pooled: each point represents the mean of eight experiments. The vertical bars represent standard error

Thirty days after implantation, some frogs could actively extend their foot, and some contractile activity of the gastrocnemius was observed. It may be, however, that this contractile activity originates only in the proximal muscle stump which was not removed during the operation; the regenerated portion would then only act as a passive transmitter of the tension. It has not yet been possible to evaluate the contribution of the regenerated muscle to the force which extends the foot.

B. Weight of Minced Muscles

Fig. 1 shows how the wet weights of the minced gastrocnemius, expressed as a percentage of the contralateral muscle, change with the time after the implantation. Two groups are represented. In the first, the removed muscles are entirely implanted at the time of operation (open circles). Their weights increase rapidly from the 1th day to a maximum at the 8th day. This phase corresponds to the swelling and oedematous reaction of minced tissue described in the previous section. Thirty days after the implantation, the regenerating muscles weight is about half the control. In the second group, 2/3 of the minced tissue is replaced into the leg (filled circles). These muscles show a much less intense inflammatory reaction, and there is no maximum. However at 30 days, they are significantly smaller than those of the first group despite the absence of oedema and the disappearance of all original muscle fragments at that postoperative delay (Carlson, 1968). The difference in weight observed between the two groups suggests that the amount of tissue regenerated depends on the amount of implanted muscle fragments, in agreement with the concept that the regenerating muscles develop from structures present in the grafted tissue (Kaspar *et al.*, 1971).

C. The Inulin Diffusion-Space

Any interpretation of the substrate concentrations must take into account any change in the extracellular space of the analyzed tissue. We estimated this space by observing the rate at which labelled inulin penetrated into regenerated and contralateral gastrocnemius.

The results, expressed as percentages of the muscular volume, are described in Fig. 2. The lower curve shows the diffusion of inulin into controls. Each point is the mean with its standard error (vertical bars) of eight control muscles. This can be fitted by an inverse exponential curve, time constant 12 min, corresponding to an inulin space at 60 min of 20 % of the muscular volume. The upper curve shows the inulin space of minced muscles; the postoperative delay has no significant influence on the inulin space. From the first to the 30th day after implantation, the inulin space re-

mains twice that of control muscles. Further, the time constant of inulin diffusion is increased (18 min) in minced muscles. These facts suggest that within 24 hrs after the operation 60% of the minced tissue has become separate from the extracellular fluid even though it was composed almost entirely of subcellular fragments. The persistence of the same inulin space 30 days after implantation requires a further explanation; perhaps in regenerating muscles of frogs there is a large number of small muscle fibres and a greater quantity of connective tissue (Carlson, 1968) or an increase of the permeability of the cells to inulin. This latter phenomenon might be a distinctive possibility, as Goldspink (1966), reviewing the litterature, points out that embryonic muscle fibers seems to be very permeable to inulin.

In the present work, the concentration of cellular substrates (C) has been expressed in micromoles (Q) per gram of muscular wet weight (W). If we assume that the density of the tissue is one, its weight is equal to the sum of the cell volume (V_c) and the extracellular volume (V_e). If all the substrates measured are intracellular, which is practically true here, the intracellular concentration (C_i) is:

$$C_i = \frac{Q}{V_c} = \frac{C(V_e + V_c)}{V_c}$$

In the following, we shall give the concentration uncorrected for the extracellular space. This is justified in view of the fact that extracellular space does not change during the post-operative period. However, if one wishes, there is a simple correction: the intracellular concentration may be estimated by dividing the concentration in control muscles by 0.8, and in minced muscles by 0.6. The ratios of the intracellular concentrations are obtained from the ratios of the whole muscle concentrations by dividing them by $0.6/0.8 = 0.75$.

D. Creatine, Phosphorylcreatine and Creatinine

The total creatine (C_T) content of minced muscles varies much with the time after implantation. Fig. 3 shows that the C_T is less than 5% of controls from the first to the eighth postoperative day, twice this value at the 15th day and it reaches about 30% of controls at the 30th day. Since C_T concentration is as far as is known proportional to the amount of muscle tissue, such a time course seems to indicate that a) during the first week the muscle is degenerating and its fragments are no longer functional and b) from the second week on, the muscle tissues is regenerating up to at least the 30th day. One may observe a clear correlation between the increase of creatine content and the morphological and histochemical features of minced gastrocnemius.

The great variation of C_T concentration found at the 30th postoperative day may partially be explained by the amount of tissue grafted, since the analysis of variance indicates a somewhat ($P \simeq 0.1$) higher value of C_T when the whole of the minced muscle was re-

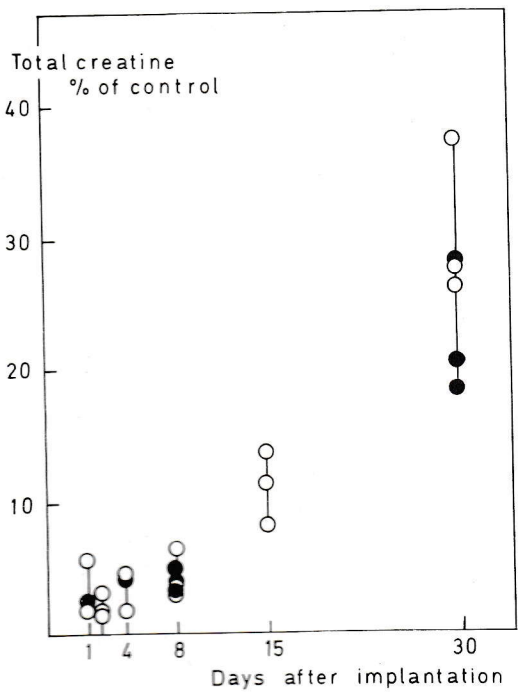

Fig. 3. The total creatine content of minced muscles, expressed as percent of controls, as a function of time after implantation. The open circles refer to muscles where the whole minced was reimplanted into the original space; filled circles to those where about two-thirds was reimplanted

implanted. This suggests as a first approximation that the amount of implanted fragments not only increases the weight of the regenerates but also the amount of muscular tissue which they will rebuild.

Phosphorylcreatine presents us with special problems. As shown in Table 1, its concentration in the minced muscle changes in a way quite similar to that of total creatine. However, the concentration of this substrate is highly dependent on the previous activity of the muscle because phosphorylcreatine is rapidly split to creatine and phosphate during muscular contraction (for a review see Maréchal, 1972), and it may be asked how far the values obtained are representative of resting muscles.

A rapid survey of the literature shows that in the frog gastrocnemius the resting values of PC reported by several authors are quite similar to those shown in Table 1: 10.19 µmol/g (Lundsgaard, 1930); 13.7 µmol/g (Feuer, 1955); 14.80 ± 2.86 (Cerretelli *et al.*, 1972); 14.2 ± 0.55 (Ambrosoli and Cerretelli, 1973). The more recent values reported are however slightly higher than the one reported here, by about 1.5 to 2.0 µmol/g.

It is difficult to conclude whether this discrepancy reflects genuine differences due to biological variations, or whether it is caused by differences in the preparation procedures. Cerretelli *et al.* (1972) and Ambrosoli

Table 1. Total creatine, phosphorylcreatine, creatinine concentrations and PC/C_T ratios of controls and minced muscle regenerates in the frog

Substrates	Controls	Minced muscles regenerates, % of controls, days after implantation					
		1	2	4	8	15	30
1. Total creatine µmol/g	22.28 ± 0.58	3	2	3	4 ± 0.5	11	26 ± 2.7
2. Phosphorylcreatine µmol/g	11.88 ± 0.62	2	2	3	4 ± 0.7	12	29 ± 2.9
3. PC/C_T mol/mol	0.53 ± 0.02	65	70	87	87 ± 8.6	106	110 ± 2.1
4. Creatinine µmol/g	3.50 ± 0.24	12	3	14	13 ± 0.5	27	33 ± 4.7
5. *n*	24	3	3	3	6	3	6

The means are reported with their standard error when the number of measurements is equal or higher than 6.

and Cerretelli (1973) froze their specimen between cooled pliers; the rate of freezing of the muscle should then be higher than in our case, where the muscles were immersed in the freezing mixture.

The rate of PC splitting is about 3 µmol/g for a sartorius muscle at 20° C (Canfield *et al.*, 1973) during the first second of a tetanus. Thus, we may account for the discrepancy referred to above if the muscles had contracted tetanically for about 0.5 sec when they were immersed in the cooled isopentane. This seems reasonable, and, moreover, explains two other observations. The first is the low PC/C_T ratio: here it is 0.53 which is much lower than those found by other authors: 0.66 (Canfield *et al.*, 1973), 0.72 (Maréchal, 1964), 0.81 (Curtin and Woledge, 1974), 0.80 (Homsher *et al.*, 1975). If we assume that 2 µmol/g of PC have been split during the contraction induced by the freezing procedure, this ratio becomes 0.62.

Another observation is that muscles regenerating for 30 days have more (110%) PC than the controls. This may be explained if we assume a) that the regenerating muscle cells have the same resting PC concentration than the control muscles, and b) that the tetanus induced by the freezing is shorter in regenerating than in control muscles, because the regenerating muscles are so much thinner.

The creatinine content of the muscles was measured in order to shed some light on the rapid disappearance of the total creatine from minced muscles after implantation. The results shown on Table 1 exclude the possibility that PC is retained in the muscles under its cyclic form, creatinine. After the operation, there occurs a large net loss of creatinine, nearly as great as that of PC. The recovery of creatinine during the postoperative days parallels that of PC. Therefore we may conclude that PC escapes very rapidly from the minced muscles (either as creatine or as creatinine).

E. The Adenosine Nucleotides

The findings are shown in Fig. 4 and Table 2.

The ATP content of the control muscle, 4.06 µmol/g, is quite similar to that reported by the other authors:

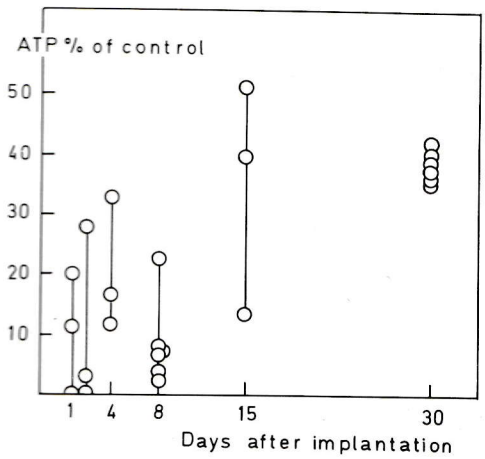

Fig. 4. The ATP content of minced muscles, expressed as percent of controls, as a function of time after implantation

Table 2. Adenosine nucleotides in controls and minced gastrocnemius muscle regenerates 30 days after implantation in the frog

Adenosine nucleotides	Controls µmol/g	Minced muscles µmol/g	% of controls
ATP	4.06 ± 0.19	1.57 ± 0.05	39
ADP	1.01 ± 0.07	0.38 ± 0.06	38
AMP	0.14 ± 0.09	0.06 ± 0.01	43

Each value is the mean with its standard error ($n = 6$ muscles). Controls are contralateral gastrocnemius.

3.79 (Cerretelli *et al.*, 1972), 4.2 (Ambrosoli and Cerretelli, 1973).

Canfield and Maréchal (1973) demonstrated that ATP, ADP, AMP, PC and creatine are in an equilibrium catalysed by only two enzymes, the PC-ATP phosphoryltransferase and the myokinase. An important assumption, necessary for this hypothesis, is that 80% of the acid-soluble ADP is bound and therefore not available for this equilibrium. In agreement with these authors, we found that the ratios ADP/ATP = 0.25 and AMP/ATP = 0.035 correspond to the ratio creatine/phosphorylcreatine predicted by

Table 3. Substrates of the glycolytic pathway of controls and minced muscle regenerates in the frog

Substrates	Controls µmol/g	Minced muscles regenerates, % of controls, days after implantation					
		1	2	4	8	15	30
Glucose-1-P + glucose-6-P	0.66 ± 0.06	3	3	2	2 ± 0.3	16	31 ± 8.0
Fructose-6-P	0.23 ± 0.03	5	8	4	4 ± 0.2	0	42 ± 7.6
Fructose-1,6-P_2	0.09 ± 0.02	18	8	32	55 ± 17.5	0	93 ± 15.8
α-Glycero-P	0.20 ± 0.05	21	4	5	46 ± 2.6	28	55 ± 14.3
Lactic acid	2.13 ± 0.22	69	45	59	74 ± 12.2	111	110 ± 20.0
n	24	3	3	3	6	3	6

The means are reported with their standard errors, when the number of measurements is equal or higher than 6.

their hypothesis, provided that 85% of the acid-soluble ADP is assumed to be bound.

The regenerating muscle at 30 days (Table 2) shows the adenosine compound concentrations which are predicted from the corresponding creatine/phosphocreatine ratios. This suggests strongly that the equilibrium of the energetic substrates is similar to that in the control muscle. It remains of course to be seen whether this equilibrium persists during the tetanization, as is the case for normal muscle (Canfield and Maréchal, 1973).

After the operation, the time course of the nucleotide concentrations parallels roughly that of PC (see Fig. 4). There is, however, one important difference which is mainly seen in the first 2 weeks after the operation. The scatter of the data is large, much larger, comparatively, than that of PC. One possible reason is that the numerous mononuclear cells which are present in the degenerating muscle (Carlson, 1968) contain variable amounts of ATP, or, conversely, that the degenerating muscle contains a variable number of mononuclear cells, each having similar amount of ATP.

F. Metabolites of the Glycolytic Pathway

Table 3 gives the general pattern for some metabolites of the glycolytic pathway during the degenerative and early regenerating stages of minced muscles, in comparison with their contralateral controls.

Lactic acid is quite abundant in the control muscles: 2.13 µmol/g. This value is higher than that reported for gastrocnemius muscles by Cerretelli *et al.* (1972) and Ambrosoli and Cerretelli (1973) who both found 1.6 µmol/g. However, the difference is not large, about 0.6 µmol/g, and is perhaps ascribable to the difference in temperature: they kept the isolated gastrocnemius at 4° C, while we kept the frogs and the muscles at 20° C before freezing them. Another possibility is that glycolysis was triggered during the freezing. There is some disagreement among authors as to the rate at which glycolysis might start during a tetanus. It seems that it might start quite rapidly at 20° C (Canfield *et al.*, 1973). However, even after a tetanus lasting 12 sec at 20° C, the lactic acid production of frog sartorius muscle is still moderate: about 1.54 µmol/g (Maréchal *et al.*, 1975) to 2.02 (Maréchal and Lebacq, 1972). A production of 0.6 µmol/g of lactate would require a tetanus lasting 4 sec. It seems to us highly improbable that the isolated gastrocnemius would have been stimulated for such a long time, either by our manipulations, or during the freezing. It might well be that the rather high amount of lactate found in the muscle reflects the normal state of affair of a resting gastrocnemius.

However, the operated muscles do contain large amounts of lactate, especially if compared to the amount of PC or ATP found there. This is noteworthy during the degenerative stage (first 8 days after operation): the amount of lactate is about half that of the control muscles, in spite of the fact that the PC and creatine content of the degenerating muscles is very low. This might be a consequence of the anaerobic activity of the numerous mononuclear cells characteristic of this stage, which probably obtain their energy mainly from the glycolytic pathway (Rifenberick *et al.*, 1974). As the histological and the histochemical observations show complete loss of glycogen 12 hrs after operation, and also an absence of blood supply (Schmidt, 1968; Snow, 1973), it has to be assumed that enough glucose can still diffuse from the surrounding tissue, in order for the anaerobic metabolism of the population of mononuclear cells to continue. During the regenerating stage (15–30th days after implantation), the amount of lactate is still high, and eventually gets higher than in the control muscles. This last fact probably needs a different explanation from the apparently similar finding for PC. While PC is very much dependent on the rate of freezing, this is not true for lactate. As we have discussed above, it is not likely that our manipulative processes could have increased the lactate content of the dissected muscles. Therefore,

it is probable that the lactate content of the muscles operated on 30 days previously, being higher than their contralateral controls, is not due to an unintended stimulation of the muscle, but is rather to be ascribed to the anaerobic metabolism of the regenerated tissue.

Fructose-1,6-diphosphate and α-glycerophosphate show variations similar to those of lactate, although due attention must be paid to the large standard errors obtained for these substrates. The discrepancies in time-course between the amount of hexose monophosphates, fructose-1,6-diphosphate and α-glycerophosphate indicate changes in reaction fluxes, which may perhaps depend on changes in the enzymes concerned (fructose-6-phosphate kinase, aldolase, etc.).

The hexose monophosphates (glucose-1-P, glucose-6-P and fructose-6-P) do not follow the pattern set by the other glycolytic substrates. They rather show a post-operative time-course similar to that of phosphorylcreatine. We have no explanation for this intriguing fact.

DISCUSSION

The time-course of adenosine components and glycolytic intermediates in minced muscles regenerating for 30 days after implantation has been described in the section on results. We refer to this section for a short discussion of these aspects, whilst we shall now focus to a practical problem of much importance in muscle regeneration studies: the evaluation of the amount of contractile material. Such a problem might seem trivial, but it is in fact complicated by a number of factors.

The most direct way to solve this problem would be to measure the amount of one contractile protein, for exemple actin. Such measurements are difficult to perform, because it is necessary to isolate quantitatively the protein. There is, in fact, no contractile protein which is easy to measure quantitatively. A somewhat less direct approach would be to measure the acid-soluble adenosine-diphosphate. According to Seraydarian *et al.* (1962), about 62% of it is bound to F-actin. However, ADP is bound to many other proteins and enzymes as well, and there is no guarantee that the ratio of F-actin to the other ADP-binding proteins remains constant during the course of the regenerative process. Quite to the contrary our results show a large scatter of the amounts of ADP found in the regenerating muscles; this fact indicates that the fraction of ADP bound to F-actin changes unpredictably during regeneration, and, accordingly, the ADP content of the muscle is not an acceptable estimate of the amount of F-actin.

A third method, which is very popular, is to use the weight as an estimate of the amount of muscular tissue. Such a procedure has been critically discussed by Carlson (1963) and Wilkie (1968), who pointed out how difficult it is to measure accurately the weight of a muscle which has been put into a Ringer solution. They discuss the differences between "drained wet weight" and "blotted wet weight". Such subtleties are important only if changes in weight of a few percent are of some concern, or if the amount of tissue is less than 10–20 mg, which is not the case in our studies. Other objections to the use of the wet weights are more important in the frame of our experiments. First, there is no necessary link between the wet weight and the amount of contractile material which has been resynthesized. Morphological observations are common, which show that the regenerating muscle contains various and numerous mononuclear cells, dense connective tissue and fewer muscles fibres (Carlson, 1972). Secondly, the wet weights vary quite much, as shown in our Fig. 1, depending on the time after operation and on the amount of implanted tissue, because there are variable proportions of oedema and connective tissue. It is interesting to recall here that the extracellular volume, as evaluated by the inulin diffusion space is twice that of control muscle throughout the postoperative time.

Another version of the weight method is the "dry" weight. This method is obviously impractical when the chemical measurements have to be made on a muscle extract, perchloric in our case.

A fourth method is to measure the maximum tetanic tension. This method is surely one of the best from a physiological point of view, as it evaluates the amount of muscle which is functional for the animal. It has been used by Salafsky (1971) who found that regenerated muscle of mice could develop a tetanic tension of approximately 45% of control tension, 75 days after the operation by which the muscles had been minced, and Carlson and Gutmann (1972) who found that 100 days after implantation, the minced rat gastrocnemius could develop a low tetanic tension, about 10% of controls, despite the fact that their weights were about half of the controls. We have not found in the literature any information on the tension developed by frog muscle regenerates.

A fifth method is to use the total creatine content (C_T = sum of creatine and phosphorylcreatine) as an estimate of the "size" of the muscle. Total creatine content is quite well correlated to the tetanic tension and heat production; this correlation is better than that shown by the weight (Wilkie, 1968). It is thus a reasonable asumption to hypothesize that the amount of C_T in a muscular tissue bears a constant relationship to the amount of contractile proteins in the intact

muscle cells. Although there is still no conclusive proof of such an hypothesis, it is interesting to discuss it in the frame of our experiments.

Fig. 3 shows that the time course of C_T after the operation is very different from that of the weight (Fig. 1) and of nucleotide concentration (Fig. 4). One can divide it into two periods, which corresponds quite well with the two morphological and histochemical stages which have been described (Carlson, 1968; Snow, 1974). During the first stage, which lasts about 2 weeks, the minced muscles have a very low amount of C_T, less than 5% of that of the controls. This fact indicates that there is then very little functional muscle. What little is left does not depend on the size of the implant, because as little C_T is found in the cases where the whole muscle is reimplanted (Fig. 3, open circles) as in the cases where about 2/3 of the minced is reimplanted (filled circles). It is interesting to point out here that the wet weight is very different in these two situations (see Fig. 1) and, therefore it is useless as an index of the amount of muscle tissue. The low amount of C_T corresponds to the "degenerative stage" characteristic of this period, which consists in a destruction of the implanted tissue and a proliferation of undifferentiated monoblastic cells.

During the second stage, which begins about 2 weeks after operation, the amount of C_T in the implanted tissue increases sharply; it is about 30% of that of the control muscles at the 30th day. Despite the increase in C_T, the wet weight of the regenerating tissue decreases. These facts suggest again that the wet weight is useless as an estimate of the magnitude of muscle regeneration, but that C_T is a better estimate of this processes. Indeed, during this period, there occurs the "early regenerative stage", characterized by fusion of the myoblastic cells into myotubes, appearance of cross striated fibres and overall tissular reorganisation.

All these facts suggest strongly that the amount of C_T is well correlated with the amount of functional muscle tissue present in the regenerating muscles.

If such an hypothesis is accepted, one can understand why the C_T content of muscle which regenerate since 30 days is significantly higher when the whole minced muscle has been reimplanted then when only 2/3 of it has been reimplanted (compare open circles with filled circles of Fig. 3): this difference might mean that there is more muscle regenerated in the former case than in the latter case. This interpretation is in agreement with the concept that the regenerated muscle is reconstructed from materials present in the minced implants (Kaspar *et al.*, 1971; Carlson, 1972).

This discussion indicates that the amount of C_T is a worthwhile estimate of the amount of functional muscular tissue. We suggest that it should be used as a reference in the works where biochemical parameters are studied during the course of muscle regeneration.

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