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Microenvironment and Enzyme Function: Control of Energy Metabolism During Muscle Work

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SYNOPSIS. The mobilization and utilization of glycogen and lipids as fuel for muscular activity has been reviewed with emphasis on regulatory mechanisms. Data are reviewed and presented which show that epinephrine-induced glycogenolysis is mediated through cyclic 3′,5′-AMP formed from neurohormonal activation of adenyl cyclase. The cyclic nucleotide, acting as a cofactor for protein kinase, facilitates the conversion of phosphorylase *b* kinase to the activated form, which in turn catalyzes the conversion of phosphorylase *b* to phosphorylase *a*, thus facilitating glycogenolysis. Data are described which indicate that when skeletal muscle is induced to contract by electrical stimulation, phosphorylase *b* to *a* conversion takes place without increased intracellular levels of cyclic 3′,5′-AMP and without conversion of phosphorylase kinase to the activated form. The suggestion is made that phosphorylase activation under these conditions is facilitated by ionized calcium. Evidence is reviewed which shows that phosphorylase kinase is greatly stimulated by extremely low concentrations (of the order of 10^{-7} M) of this cation. Thus, Ca^{++} may serve not only to couple the electrical and mechanical events of muscle contraction (excitation-contraction coupling), but the metabolic events as well. The hormonal regulation of lipolysis in adipose tissue is reviewed and the role of lipids, especially FFA, as fuel for muscular activity in various organisms is discussed. Data are reviewed which suggest that the ability of an organism to derive energy from FFA for muscular activity is dependent upon its aerobic work capacity.

Our understanding of how nature regulates metabolic activity has expanded rapidly in recent years. At the molecular level, metabolic control is effected in two basic ways: by regulation of enzyme activity and by regulation of enzyme synthesis. Regulation of enzyme activity (pre-formed enzyme) may involve kinetic effects of substrates, cofactors, or products; conversion of inactive enzyme to an active form; and such chemical and physical parameters as ionic environment, intracellular architecture, pH, and temperature. In addition, the catalytic activity of certain *allosteric* proteins may be affected either positively or negatively by the presence of specific effector molecules, usually metabolites which bear no structural relationship to either the substrate or product and which bind to the enzyme at a site other than the catalytic site (Monod, Changeux, and Jacob, 1963). Modulation of enzyme activity by these mechanisms should be rapidly effective, within seconds or minutes, and enable the organism to adapt to sudden or "acute" physiological circumstances. Regulation of metabolism by changes in enzyme synthesis, the second mechanism mentioned above, since it involves genetically directed formation of new protein, might necessarily require more time, hours or longer.

During muscular activity an organism or tissue may require sudden and profound increases in metabolic activity. Fuel reserves must be mobilized and energy-yielding processes marshalled and precisely regulated to provide increasing energy demands. When activity ceases, all processes must be readily reversed to preserve homeostasis. One might expect that in such "acute" circumstances those regulatory mechanisms which affect enzyme *activity* would be of crucial importance. Mechanisms which affect the *amount* of enzyme available, involving protein synthesis, would likely play a lesser role. These latter mechanisms might provide for more

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"chronic" or adaptive regulation and could affect the ultimate *capacity* of an organism for muscular work. To review the broad implications of regulation of energy metabolism is beyond the scope of this presentation. I wish only to examine factors which act to regulate the mobilization and utilization of the primary energy reserves, carbohydrate, particularly glycogen, and fat. We are all aware that organisms and tissues within organisms differ markedly in their ability to mobilize and to extract energy from these two "fuel" sources. It should become clear from what follows that nature makes liberal use of those mechanisms just cited for regulating the supply and utilization of these materials for muscular work.

GLYCOGEN AND GLYCOGENOLYSIS

The mechanism by which glycogen is utilized as an energy source has been vigorously investigated. (For a review, see Drummond, 1967). During physical activity this fuel is mobilized not only from skeletal muscle; the liver serves as a major reserve from which glucose, the product of glycogenolysis in this tissue, is transported by the blood to working muscles and other tissues. In this presentation we have chosen not to discuss the contribution made by the liver, nor factors which facilitate liver glycogenolysis. Instead, our remarks will be confined exclusively to those mechanisms which are operative in contractile tissues, particularly skeletal muscle. Much interest in this area has been prompted by the realization that glycogenolysis and glycolysis are closely geared to the process of muscle contraction (Cori, 1956; Danforth, Helmreich, and Cori, 1962; Karpatkin, Helmreich, and Cori, 1964). The enzymology of glycogenolysis and factors which regulate this process are summarized in Figure 1. Phosphorylase, the enzyme which degrades glycogen to glucose-1-phosphate exists in mammalian skeletal muscle in two forms, phosphorylase *a* and phosphorylase *b*. Phosphorylase *b* differs from the *a* form in that it has half the molecular weight and is

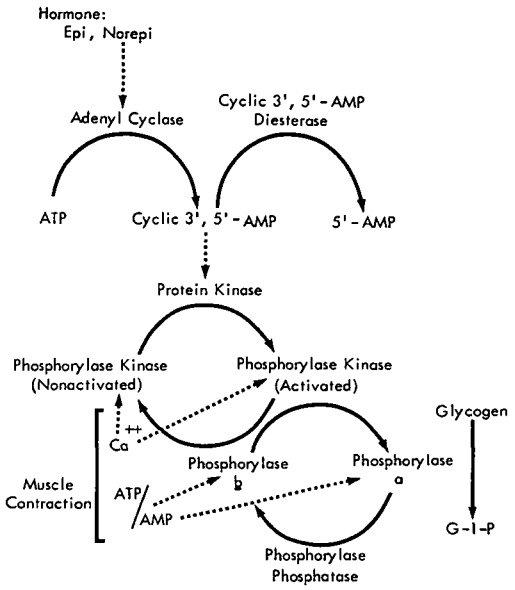

FIG. 1. Metabolic pathway of glycogenolysis and some regulatory mechanisms.

catalytically inactive except in the presence of relatively high concentrations of 5′-AMP. In most instances it can be envisaged as a physiological reservoir of inactive enzyme. Phosphorylase *a* is active in the absence of 5′-AMP and can be considered to be the physiologically active form. In resting mammalian skeletal muscle the enzyme exists almost exclusively in the *b* form. The two proteins are interconvertible and enzymes catalyzing their interconversion have been extensively studied. The conversion of phosphorylase *b* to phosphorylase *a* (phosphorylase activation) is catalyzed by the enzyme phosphorylase *b* kinase; the reverse reaction (phosphorylase inactivation) is catalyzed by phosphatase.

Phosphorylase activation as a regulatory mechanism. Cori (1956) was the first to demonstrate that the increased rate of glycogen breakdown which occurred when muscle was induced to contract by electrical stimulation was accompanied by conversion of phosphorylase *b* to phosphorylase *a*. Similar results were obtained when isolated muscle was exposed to epinephrine. Danforth, Helmreich, and Cori (1962) found that when frog sartorius was

stimulated tetanically, phosphorylase *a* levels rose from near 0 to 100% of total enzyme activity within 3 seconds; activation by epinephrine occurred much more slowly. From their studies they concluded that changes in phosphorylase *b* kinase rather than in phosphorylase phosphatase were responsible for phosphorylase activation. These, and other studies, established that increased glycogenolysis as mediated by the sympathetic nervous system (*via* epinephrine and norepinephrine) (See Himms-Hagen, 1967) and by muscular activity occurred primarily as a result of phosphorylase *b* to *a* conversion.

Much information regarding the mechanism of this conversion has come from *in vitro* studies on phosphorylase *b* kinase. Krebs, Graves, and Fischer (1959) found that this enzyme could be extracted from resting rabbit muscle in a form that was essentially inactive at physiological pH (pH 7 and below). Above pH 7 activity rose sharply to a maximum at pH 8.5. The enzyme present in partially purified preparations could be activated *in vitro* by incubation with ATP and Mg^{++}. This activation was markedly facilitated by minute concentrations (10^{-8} to 10^{-7} M) of adenosine 3′,5′-cyclic phosphate (cyclic 3′,5′-AMP) (Krebs, Graves, and Fischer, 1959; Krebs *et al.*, 1964). Activation was especially marked when the enzyme was subsequently assayed at pH 6.8 where the nonactivated enzyme had very low activity. Activation was of lesser magnitude when the subsequent assays were performed at pH 8.2 where the nonactivated enzyme had appreciable activity. Activation by ATP and cyclic 3′,5′-AMP was thus accompanied by an increased ratio of activity at pH 6.8 to 8.2. This activity ratio has been used as a quantitative index of activation of the enzyme under various experimental circumstances. Such studies were highly relevant to an understanding of how adrenergic amines (epinephrine and norepinephrine) facilitate phosphorylase *b* to *a* conversion. The work of Sutherland and his associates (Sutherland, Oye, and Butcher, 1965; Sutherland and Robison, 1966; Robison, Butcher, and Sutherland, 1968) established that adrenergic amines as well as other hormones cause increased intracellular levels of cyclic 3′,5′-AMP in a variety of tissues, including skeletal muscle. This was shown to take place by neurohormonal or hormonal activation of adenyl cyclase, an enzyme located primarily in the plasma membrane of cells, which catalyzes the formation of the cyclic nucleotide from ATP (Murad *et al.*, 1962; Klainer *et al.*, 1962). Thus, in the presence of neurohormone (epinephrine or norepinephrine), stimulation of adenyl cyclase could result in increased formation of cyclic 3′,5′-AMP which would facilitate the conversion of nonactivated phosphorylase kinase to the activated form; this enzyme would then catalyze phosphorylase *b* to *a* conversion resulting in stimulation of glycogenolysis (Fig. 1). One question remains. How does the cyclic nucleotide mediate the activation of phosphorylase kinase? It does not seem to interact with phosphorylase kinase directly because DeLange *et al.* (1968) could demonstrate no significant binding to the purified nonactivated enzyme. The answer to this question has been provided by Walsh, Perkins, and Krebs (1968) who have discovered an enzyme which catalyzes an ATP-dependent phosphorylation of nonactivated kinase with concommitant conversion to the activated form. This enzyme has been called protein kinase (Fig. 1) (rather than phosphorylase *b* kinase kinase). Protein kinase in skeletal muscle has a specific requirement for extremely low concentrations of cyclic 3′,5′-AMP (K_a 7×10^{-8} M). It is not specific for phosphorylase kinase but phosphorylates other proteins, particularly histones. Protein kinase has been highly purified and extensively studied in a variety of tissues (Kuo and Greengard, 1969, 1970). It should be mentioned that activation of phosphorylase kinase by this system is enzymatically reversible. Thus, Riley *et al.* (1968) have discovered still another enzyme, a phosphatase, which catalyzes the reverse reaction, namely the conversion of activated phosphorylase kinase to the nonactivated form. The homeostatic function of this enzyme is obvious; whether it

has regulatory properties is not known.

Much evidence now exists that all these metabolic events occur in contractile tissue *in vivo* in response to adrenergic amines. Posner, Stern, and Krebs (1962) first showed that when epinephrine was injected into rats and frogs, cyclic 3′,5′-AMP levels rose in skeletal muscle, and phosphorylase *b* kinase was converted to the activated form concommitant with conversion of phosphorylase *b* to phosphorylase *a*. These events also take place and with extreme rapidity (within seconds or less) when epinephrine is injected into isolated perfused beating hearts (Robison *et al.*, 1965; Drummond, Duncan, and Hertzman, 1966; Namm and Mayer, 1968). Stimulation of the sympathetic nervous system with release of circulating epinephrine can thus initiate a complicated sequence of changes in the microenvironment of the muscle cell in which the "messenger," cyclic 3′,5′-AMP, formed by neurohormonal activation of adenyl cyclase on the cell membrane, facilitates the activation of key metabolic reactions leading to increased catabolism of glycogen. In order to reverse the process, several mechanisms are available to the cell, such as phosphorylase phosphatase and deactivation of activated kinase. Perhaps the most immediate mechanism for "turning off" the system would be destruction of the "messenger." This is readily accomplished by cyclic 3′,5′-AMP phosphodiesterase, an enzyme present in most tissues, which converts the cyclic nucleotide to 5′-AMP. The physiological importance of this enzyme has been repeatedly demonstrated (Robison, Butcher, and Sutherland, 1968).

It should be made clear at this point that the role of the sympathetic nervous system in the regulation of glycogenolysis in skeletal muscle during exercise is uncertain. It seems that very little information is available on blood levels of adrenergic amines during exercise (Himms-Hagen, 1967), and it is not known whether they rise to levels sufficiently high to stimulate these metabolic events in working muscles. Obviously this is a problem that needs serious attention.

The role of calcium ion in regulation of glycogenolysis. In their studies on phosphorylase *b* kinase, Krebs and his associates (Krebs, Graves, and Fischer, 1959) found that this enzyme was converted to an activated form when incubated not only with ATP and cyclic 3′,5′-AMP, but also with Ca^{++}. Calcium activation required the presence of a specific protein, kinase activating factor (Meyer, Fischer, and Krebs, 1964). Studies with highly purified kinase activating factor (Huston and Krebs, 1968; Drummond and Duncan, 1968) revealed that it was a calcium requiring proteolytic enzyme and that Ca^{++} activation of kinase proceeded by cleavage of a peptide or peptides from the enzyme. Because the reaction involved proteolysis and because it was irreversible, it is now considered of unlikely physiological significance. There is evidence, however, that Ca^{++} may be involved in a completely different manner in phosphorylase kinase activity. In their early experiments, Meyer, Fischer, and Krebs (1964) found that chelating agents such as EDTA[1] or EGTA caused marked inhibition of the enzyme and this was reversed by Ca^{++}. This indicated that phosphorylase kinase might have a requirement for extremely low concentrations of this cation. These observations have been extended by Ozawa, Hosoi and Ebashi (1967) who have shown that when the partially purified enzyme was assayed in the presence of EGTA, Ca^{++} at concentrations as low as 10^{-7} M (in excess of EGTA) caused significant stimulation. The ability of low concentrations of EGTA to inhibit phosphorylase kinase (in this case the enzyme highly purified from chicken breast muscle) is shown in Figure 2. When Ca^{++} was added in excess of EGTA, activity was completely restored. The inference from this type of experiment is that the normal assay components contain sufficient Ca^{++} as contaminants to allow enzyme activity. The effect of Ca^{++} can be demonstrated only by first complexing endogenous calcium with a

[1] Abbreviations used are: EDTA, ethylene diamine tetraacetic acid; EGTA, ethyleneglycol *bis* (β-aminoethyl ether)-N, N′-tetraacetic acid.

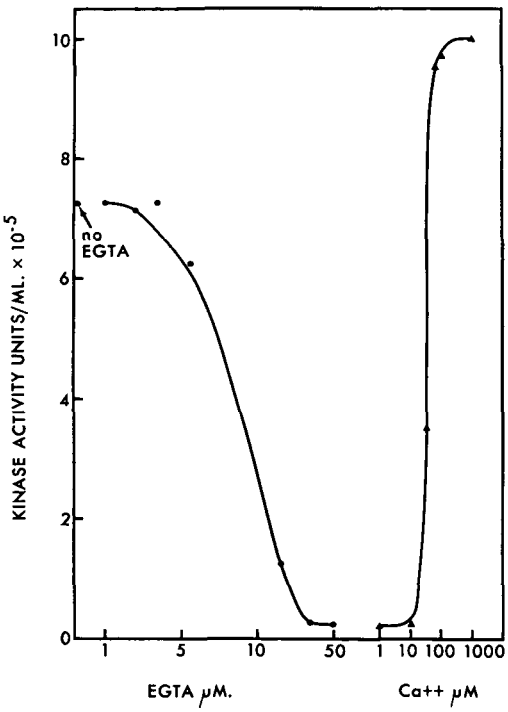

FIG. 2. Inhibition of phosphorylase kinase by EGTA and reactivation by Ca^{++}. The enzyme was purified from chicken breast muscle and assayed as described by Drummond, Harwood, and Powell (1969). EGTA was present at 50 μM in those tubes to which Ca^{++} was added.

highly specific chelating agent. Recently, Brostrom and Krebs (1970) have provided more direct evidence for the involvement of Ca++ in kinase activity. They have assayed the enzyme in Ca++-free reagents and, using this approach, have actually been able to determine the K_a for Ca++ for both the activated and nonactivated enzyme. The K_a in each case was found to be of the order of 10^{-7} M. It thus seems reasonable to suggest that Ca+ may have a profoundly important role in regulation of glycogenolysis. This function for the cation is indicated in Figure 1. It is now widely recognized that ionized calcium released from storage sites, or transported across the plasma membrane of the cell, serves to couple the electrical and mechanical events of muscle contraction. It would be entirely logical if nature used it at the same time to couple the metabolic events which provide the energy for the mechanical response.

It should be realized that the studies just described were performed using *in vitro* systems with the partially purified enzyme. Is there such a function for Ca+ in contracting muscle? Could this cation be involved in phosphorylase activation which occurs when muscle contracts by electrical stimulation, or is cyclic 3′,5′-AMP the only stimulus? Posner, Stern, and Krebs (1965) examined phosphorylase activation in frog sartorius and rat gastrocnemius made to contract by electrical stimulation. In contrast to epinephrine which produced both increased cyclic 3′,5′-AMP levels, and conversion of phosphorylase kinase to the activated form, electrical stimulation produced no increase in cyclic nucleotide in the tissues and seemed to cause only marginal activation of phosphorylase kinase. We have investigated phosphorylase activation in isolated frog sartorius and rat diaphragm and in rat gastrocnemius *in situ* when these tissues were made to contract by electrical stimulation and when treated with epinephrine (Drummond, Harwood, and Powell, 1969). The data in Figure 3 show that when isolated rat diaphragm was incubated with epinephrine (10 μg/ml for 5 min), phosphorylase *a* levels rose sharply as expected (compare *bar* B with *bar* A), and phosphorylase kinase was significantly converted to the activated form as evidenced by increased pH 6.8 to 8.2 activity ratios (compare *bar* G with *bar* F). When the muscle was made to contract by electrical stimulation at 50 pulses per second, either directly or through the phrenic nerve, phosphorylase was markedly activated (compare *bars* C, D, and E with *bar* A), but there was no significant activation of phosphorylase kinase (compare *bars* H, J, and K with *bar* F). Similar experiments using rat gastrocnemius are shown in Figure 4. Injection of epinephrine intracardially caused significant phosphorylase *b* to *a* conversion in the gastrocnemius (compare *bar* B with *bar* A) and this was accompanied by conversion of phosphorylase kinase to the activated form (compare *bar* F with *bar*

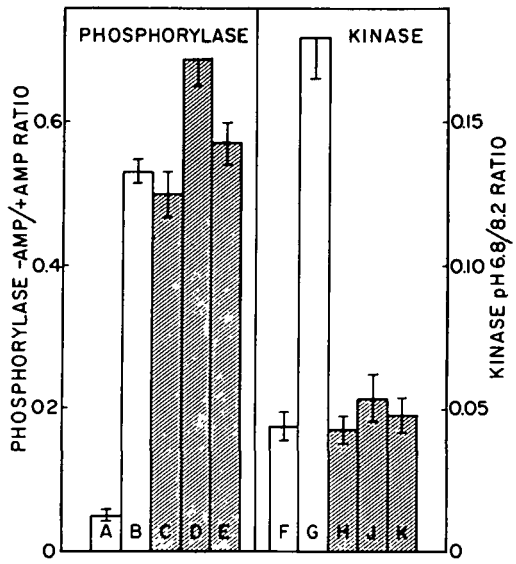

FIG. 3. Effect of electrical stimulation and epinephrine on phosphorylase and phosphorylase kinase in isolated rat diaphragm. Hatched bars represent electrical stimulation; open bars epinephrine treatment. *Bars* A and F, control; B and G, 10 μg of epinephrine per ml for 5 min; C and H, electrical stimulation at 50 pulses per sec for 2 seconds; D and J, stimulation at 50 pulses per sec for 10 seconds; E and K, stimulation for 10 seconds through the phrenic nerve. Tissues were frozen and assayed as described by Drummond, Harwood, and Powell (1969). (Reprinted with the permission of the Journal of Biological Chemistry).

E). When the muscle was made to contract by electrical stimulation at 8 and 20 pulses per second, phosphorylase *a* levels rose (compare *bars* C and D with *bar* A), but again there was no apparent conversion of phosphorylase kinase to the activated form (compare *bars* G and H with *bar* E). Identical results were obtained in experiments using isolated frog sartorii. Under experimental conditions identical to these, epinephrine, when injected intracardially, greatly elevated cyclic 3′,5′-AMP levels in rat gastrocnemius (Fig. 5, compare *bar* C with *bar* A), and when added to rat diaphragm *in vitro*, large increases in the cyclic nucleotide also occurred (compare *bars* G and H with *bar* D). Electrical stimulation, however, had no effect on cyclic 3′,5′-AMP levels in rat gastrocnemius (compare *bar* B with *bar* A) or on rat diaphragm *in vitro* (compare *bars* E and F with *bar* D). Thus, in these tissues, phosphorylase activation caused by muscle contraction differed from that produced by epinephrine in that it proceeded under conditions in which there was no detectable conversion of phosphorylase kinase to the activated form and no increase in tissue levels of cyclic 3′,5′-AMP. It seems that phosphorylase activation during muscle contraction may be basically different from that involving adrenergic amines. It is realized, however, that experiments using electrical stimuli in isolated systems do not precisely simulate muscular activity as it occurs in the whole organism. In the intact animal, adrenergic stimulation could provide sufficient circulating catecholamine to facilitate glycogenolysis in working muscles through 3′,5′-AMP. Our lack of information on this possibility has been mentioned previously.

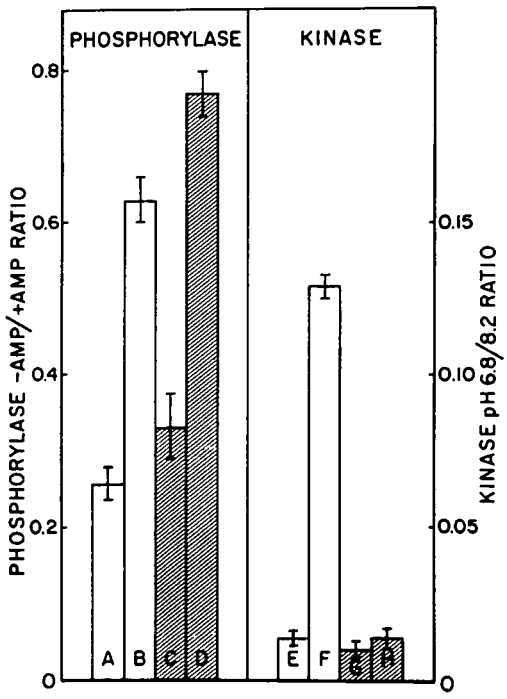

FIG. 4. Effect of electrical stimulation and epinephrine on rat gastrocnemius *in situ*. *Bars* A and E, control; B and F, 5 μg of epinephrine per Kg given intracardially for 40 sec; C and G, electrical stimulation at 8 pulses per sec for 5 seconds; D and H, stimulation at 20 pulses per sec for 5 seconds. Assays were performed as for Figure 3. (Reprinted with the permission of the Journal of Biological Chemistry).

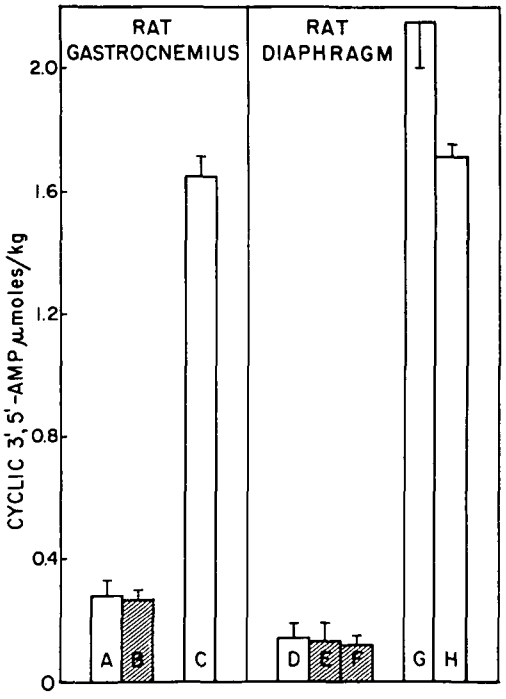

FIG. 5. Effect of epinephrine and electrical stimulation on cyclic 3',5'-AMP in skeletal muscle. *Bars* A and D, controls; B, stimulation at 20 pulses per sec for 5 seconds; C, 5 μg per Kg epinephrine intracardially; E and F, electrical stimulation at 50 per sec for 2 and 10 sec respectively; G and H, 10 μg of epinephrine per ml for 1 and 5 *min* respectively. Determinations of cyclic 3',5'-AMP were made as described by Drummond, Harwood, and Powell (1969). (Reprinted with the permission of the Journal of Biological Chemistry).

We have considered the possibility that Ca++ may be responsible for phosphorylase activation in these isolated tissues during electrical stimulation, through an action on phosphorylase kinase. This possibility was especially attractive since, as has already been mentioned, ionized calcium is released into the cell during the excitatory phase of muscle contraction where it plays a fundamental role in excitation-contraction coupling and in the development of tension. In experiments designed to test this, isolated hemidiaphragms were bathed in Tyrode's solution at 37° and stimulated at 50 pulses per second for 2 seconds to establish a basal level of tension. A chelating agent, either EDTA or EGTA, was then added in varying concentrations (usually 1 to 2 mM) for 15 minutes. A tetanic stimulus was then applied as before and the tension measured. Depending on the degree of removal of Ca++ from the tissue, tension generated varied between 0 and 100% of that produced initially. Tissues were instantly frozen while the stimulus was being applied, homogenized, and assayed for phosphorylase *b* and total phosphorylase (Drummond, Harwood, and Powell, 1969). The relationship between contractile tension and phosphorylase activation under these conditions is shown in Figure 6 (panel A for EDTA as the chelating agent and panel B for EGTA). The regression line was calculated by standard statistical methods. The data show that when tension development was reduced by restricting the availability of ionized calcium, a correlation existed between phosphorylase activation and tension development. We consider these data to support the possibility that ionized calcium may play a role in facilitating glycogenolysis during muscle contraction, presumably by virtue of its ability to stimulate phosphorylase kinase.

The regulatory role of 5'-AMP and ATP. One additional regulatory mechanism for glycogenolysis must be mentioned. It has already been stated that phosphorylase *b* is catalytically inactive *in vitro* except in the presence of 5'-AMP. The K_a of the nucleotide is 5×10^{-5} M. When reliable values for determination of adenine nucleotides became available, it was apparent that concentrations of 5'-AMP in tissues were considerably above the K_m for phosphorylase. For example, in aerobic heart the concentration of this nucleotide was found to be 4×10^{-4} M, sufficient to keep phosphorylase *b* active at all times (Morgan and Parmeggiani, 1964*a*). ATP is a powerful inhibitor of 5'-AMP on the enzyme (Parmeggiani and Morgan, 1962) and it is generally considered that phosphorylase *b* activity is normally kept in restraint by this inhibitory action. Considerable evidence exists that this action of 5'-AMP on phosphorylase *b* may be important physiologically *in vivo*. For example, Cornblath *et al.* (1963) and Morgan and Parmeggiani (1964*a*,*b*) have shown

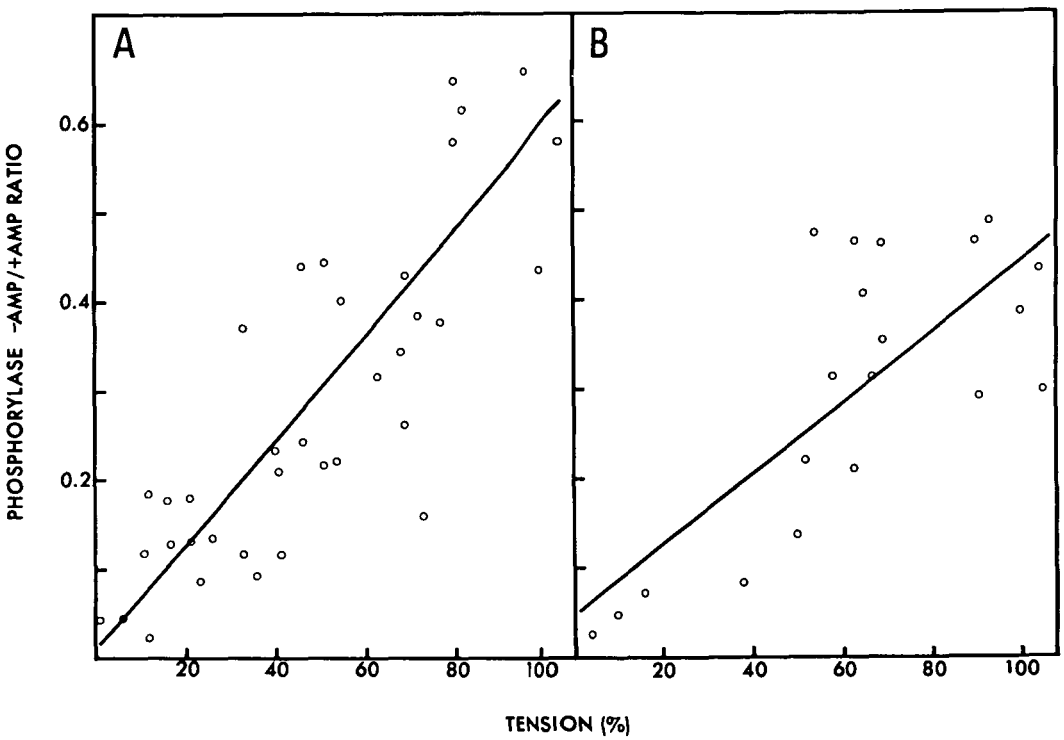

FIG. 6. Effect of EDTA (panel A) and EGTA (panel B) on phosphorylase activation and tension in isolated rat diaphragm following tetanic stimulation. See text for experimental details.

that the rapid rate of glycogenolysis that occurs in perfused hearts subjected to anoxia can best be explained by the interactions of 5′-AMP, ATP, and inorganic phosphate on phosphorylase *b*. During anoxia, ATP levels would decline, removing the inhibitory action and, at the same time, 5′-AMP levels would increase. Danforth and Lyon (1964) have examined the relative physiological importance of 5′-AMP on phosphorylase *b* in working skeletal muscle. For their studies they employed an inbred strain of mouse (I strain) which contains no phosphorylase *b* kinase in the skeletal muscle and has, as a consequence, phosphorylase only in the *b* form. During tetanic stimulation of the caudiofemoralis muscles in I strain mice, phosphorylase remained in the *b* form, while in normal controls, under identical conditions, 70% of the total activity appeared as phosphorylase *a* in six seconds. Glycogen, nevertheless, was degraded rapidly in muscles from both groups of mice; the maximal rate being about twice as great in the tissues from normal animals. Such data suggested that in normal muscle both phosphorylase *b* to *a* conversion and stimulation of phosphorylase *b* activity by 5′-AMP could occur during muscle contractility. The kinetics of phosphorylase has been much studied recently and it is clear that the enzyme belongs to the allosteric group of proteins capable of undergoing interaction with its positive effector, 5′-AMP, and being inhibited by the negative effector, ATP (See Atkinson, 1965, 1966). This action of 5′-AMP and ATP on phosphorylase is indicated in Figure 1.

Regulation of glycogenolysis in lower organisms. Most of our understanding of regulation of glycogenolysis has come from studies on mammalian tissues. Certain lower organisms also rely heavily on glycogen as fuel for muscular activity and an examination of these may yield valuable information. One might ask: are the mechanisms involved analogous to those of

mammalian contractile tissues? The data available suggest they are qualitatively similar. When fish are subjected to vigorous swimming, their muscle glycogen stores are depleted with extreme rapidity and both muscle and blood lactate rise precipitously (See Drummond and Black, 1960; Drummond, 1967). Nakano and Tomlinson (1967) have reported that when rainbow trout were subjected to vigorous muscular activity (induced by chasing) muscle phosphorylase *a* levels rose significantly and tissue cyclic 3′,5′-AMP levels were sharply increased. At the same time, plasma catecholamines rose to extremely high levels. It is not possible to determine whether such changes were a consequence of increased muscular contraction or whether they were triggered by sympathetic stimulation due to the stress of fright. The studies do indicate, however, that regulation of glycogenolysis in fish is qualitatively similar to that in higher organisms.

Certain insects also utilize glycogen as a primary fuel for flight energy (Sacktor, 1965; Drummond, 1967). Insect flight muscles can contract at rates of several hundred per second and are the most mechanically active tissues in nature. On initiation of flight the metabolic rate may increase 100-fold; regulation of metabolism in the flight muscles must be a particularly dynamic process. Sacktor and Wormser-Shavit (1966) have shown that an intense rate of glycogenolysis is triggered on initiation of flight in the blowfly *Formia regina*. In order to learn something of the regulatory mechanisms involved, Childress and Sactor (1970) have extensively purified phosphorylase *b* and phosphorylase *a* from blowfly flight muscle and have carried out a detailed kinetic analysis of both enzymes. From their kinetic data, and previously determined concentrations of metabolites in flight muscle, they were able to make certain predictions regarding glycogenolytic regulation during flight. Specifically, they found the K_m of phosphorylase *b* for inorganic phosphate (P_i) at 0.1 mM 5′-AMP to be about 100 mM, well above the concentration found in muscle. The K_m for 5′-AMP at 8 mM P_i was 1 mM, approximately 10-fold that in muscle at rest. ATP strongly inhibited phosphorylase *b* at low 5′-AMP concentrations. The concentration of 5′-AMP in the muscle was 100-fold greater than its K_m for phosphorylase *a*. The K_m for P_i, at simulated *in vivo* conditions, was 8 mM, approximately the concentration found in the muscle. ATP did not inhibit phosphorylase *a*. From these data they concluded that the activity of phosphorylase *b* was too low to account for the rate of glycogenolysis during flight. Thus, the allosteric effects of 5′-AMP and ATP on this enzyme (See Fig. 1) seem not to be of relevance in flight energetics. They calculated that the activity of phosphorylase *a* under simulated *in vivo* conditions was adequate to account for glycogenolysis during flight provided at least 50% of the total enzyme was in the *a* form. The obvious experiment was to determine phosphorylase *a* levels in flight muscle at rest and during flight. Their data indicate that phosphorylase *a* levels in resting unmounted flies was 18% of total (probably high because of technical problems). After 30 seconds of flight, phosphorylase *a* levels rose to 72% of total! Thus, during flight in this insect, glycogenolysis is facilitated by phosphorylase *b* to *a* conversion. One can now ask the question: what is the mechanism of this phosphorylase activation? To provide the answer Hansford and Sacktor (1970) have studied the properties of phosphorylase *b* kinase from blowfly flight muscle. They found that the enzyme was strongly inhibited *in vitro* by EGTA and this was reversed by Ca^{++} at about 10^{-6} M in excess of chelating agent. This enzyme, like the mammalian enzyme, therefore, seems to have a requirement for extremely low concentrations of ionic calcium. They also found that the enzyme was stimulated by inorganic phosphate when this anion was present in the assay at physiological concentrations. On initiation of flight the concentration of inorganic phosphate in flight muscle is elevated instantaneously. At the same time Ca^{++} should be released from the sarcoplasmic reticulum. Hansford and Sacktor (1970) have concluded that it is

the availability of both Ca++ and inorganic phosphate, enhancing kinase activity about 20-fold, that is responsible for the phosphorylase *b* to *a* transformation during flight. Such studies certainly lend additional support to the central role of Ca++ in coupling not only the electrical and mechanical events, but also the metabolic events of muscle contraction. If cyclic 3′, 5′-AMP has a role in facilitating glycogenolysis in the working muscle of this organism, it is yet to be revealed.

Thus, it seems clear that nature has developed several mechanisms to regulate glycogenolysis. To recapitulate, these include: (1) a conversion of phosphorylase *b* to phosphorylase *a* through the mediation of cyclic 3′,5′-AMP, (2) conversion of phosphorylase *b* to phosphorylase *a* through the stimulatory effect of ionized calcium on phosphorylase *b* kinase, and (3) the positive allosteric effect of 5′-AMP on phosphorylase *b* which permits this enzyme to be catalytically active. We should add a fourth. Since the *in vitro* response of nonactivated phosphorylase kinase to pH is so extremely sharp (pH 6.8 to 8.2, activity ratios as low as 0.02 are frequently encountered), it is possible that slight changes in intracellular pH could significantly affect its catalytic activity *in vivo* which could be reflected in alterations in phosphorylase *a* levels. We do not fully understand the precise quantitative contribution or interrelationships of each of these mechanisms under every physiological circumstance. Perhaps cyclic 3′,5′-AMP primarily mediates glycogenolysis produced by sympathetic nervous system activity. Ionized calcium may perform its regulatory role during muscular exercise under conditions in which sympathetic tone is not greatly altered. There are also likely important interrelationships between cyclic 3′,5′-AMP and Ca++ which we do not fully understand. Stimulation of phosphorylase *b* activity by 5′-AMP may be operative primarily in those situations in which a tissue, because of prolonged and vigorous muscular activity or for other reasons, has acquired a significant level of oxygen debt. Each mechanism, and there may be others, unquestionably plays an important role in allowing an organism to meet every contingency; to adjust to and recover from the numerous physiological experiences which nature demands.

LIPIDS AND LIPOLYSIS

It is now widely recognized that free fatty acids (FFA) transported through the plasma are a readily available oxidizable substrate in many tissues including skeletal muscle and heart, and that lipid is the major fuel from which energy is derived in the post absorptive state when oxygen supply is adequate (Fritz, 1961). Organisms and tissues differ widely in their ability to utilize lipids as a major fuel source especially during physical activity (Fritz, 1961; Drummond and Black, 1960; Drummond, 1967).

Regulation of free fatty acid release from adipose tissue. The primary storage form of this fuel in higher organisms is triglyceride, deposited in physiological depots, adipose tissue. FFA are mobilized from these stores and released into plasma by a variety of physiological stimuli, particularly sympathetic nerve activity, hormonal secretion and exercise. Release of FFA into plasma is a consequence of hydrolysis of triglycerides (lipolysis) and this is generally recognized as the rate-limiting step. Lipolysis then is a likely site for controlling the release of FFA into the plasma. This has been amply demonstrated. Vaughan and Steinberg (1963) found that a variety of hormones, epinephrine, norepinephrine, ACTH, glucagon, TSH, and growth hormone, all stimulated lipolysis in isolated rat epididymal fat pads. These, and other studies (See Himms-Hagen, 1967) led to the conclusion that adipose tissue contained a hormone-sensitive triglyceride lipase that might possibly exist in active and inactive forms, analogous to phosphorylase. Much evidence now exists that hormonal activation of this enzyme is mediated through cyclic 3′,5′-AMP (Rizack, 1964; Butcher *et al.*, 1965; Butcher, Baird, and Sutherland, 1968). In particular, these hormones stim-

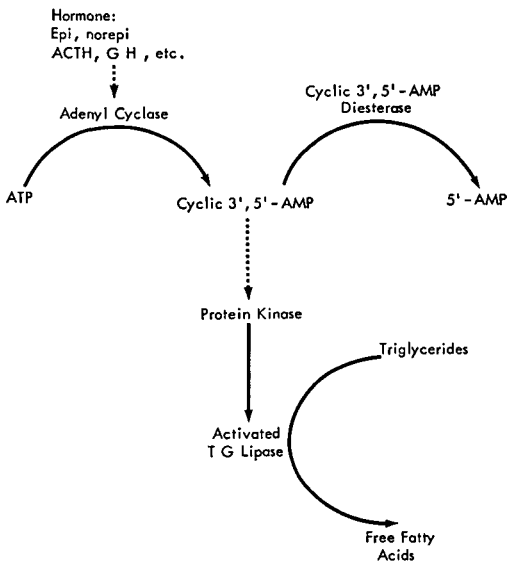

FIG. 7. Hormonal regulation of lipolysis in adipose tissue.

ulate increased levels of the cyclic nucleotide in adipose tissue and this event occurs immediately before lipolysis becomes evident (Butcher *et al.*, 1965). This would be expected to occur by a stimulatory action of these hormones on adenyl cyclase. This indeed is the case; the ability of hormones, particularly epinephrine, ACTH, and glucagon, to activate adenyl cyclase isolated from adipose tissue cells has been extensively studied by *in vitro* methods (Birnbaumer, Pohl, and Rodbell, 1969; Birnbaumer and Rodbell, 1969; Bär and Hechter, 1969; Vaughan and Murad, 1969). Thus, at least a partial understanding has emerged as to the regulation of this physiologically important process. A summary is shown in Figure 7. The analogy to glycogenolysis (Fig. 1) is readily apparent. The precise molecular nature of triglyceride lipase is, at the moment, not well understood. The enzyme has not been purified and active and inactive forms have not been isolated. It is known, however, (Corbin and Krebs, 1969) that protein kinase is present in adipose tissue, and it is reasonable to assume that it is involved in the activation of triglyceride lipase through the stimulus of cyclic 3′, 5′-AMP. It is becoming clear that at least under some physiological circumstances, nature may use the same "messenger" or signal, namely cyclic 3′,5′-AMP, to mediate the mobilization and utilization of both major fuels, glycogen and triglycerides. Again we should emphasize that the role of the sympathetic nervous system in mobilizing FFA from adipose tissue during physical exercise has not been completely clarified, although most evidence points to a prominent role (See Himms-Hagen, 1967).

The contribution of lipids to energy metabolism during work. It has been mentioned that organisms and tissues within organisms, differ in their ability to oxidize lipids during rest and during work. Heart and kidney, tissues which are adapted for aerobic metabolism, are known to oxidize lipids readily: skeletal muscle also uses this fuel extensively. Much evidence is available that the output of FFA from adipose tissue is considerably increased in mammals during exercise and more avidly taken up by working tissues. This is reflected in increased FFA turnover (increased output and uptake). Issekutz and coworkers (Miller, Issekutz, and Rodahl, 1963; Issekutz *et al.*, 1965; Issekutz, Miller, and Rodahl, 1966) have carried out a detailed examination of FFA utilization in working dogs. They found that during exercise (running on a treadmill) the turnover of FFA increased and this increase was always much greater in trained (fit) than in untrained (unfit) animals. The rate of increase in blood lactate was much greater in unfit animals than in trained specimens. Lactate infusions into normal resting dogs decreased the release of FFA from adipose tissue. They concluded that the rise of blood lactate during exercise is an important factor affecting the participation of adipose tissue in exercise metabolism. Since blood lactate reflects the capacity of aerobic mechanisms, it can be said that the ability of an organism to utilize FFA for energy during work depends on its aerobic work capacity. Capacity for aerobic work can be developed by physical training, and obviously involves adaptive mechanisms dependent upon alterations in enzyme syn-

thesis. The ability of lactate to decrease FFA output from adipose tissue seems to make considerable physiological sense, because it means that during periods of vigorous exercise when oxygen supplies become limiting, the product of anaerobiosis (lactate) suppresses the availability of a fuel (FFA) which can be utilized only aerobically.

Utilization of lipids for avian flight. Some organisms such as certain insects, particularly insects that migrate, and birds, especially those that indulge in prolonged flight, are much better adapted than most mammals to mobilize and to utilize lipids for vigorous and sustained muscular activity. Lipid metabolism in these creatures is attracting increasing attention. Birds, for example, suddenly store large deposits of fat in the premigratory period and this material serves as "high octane" fuel for the migratory journey which, in some species, may involve a flight of several thousand miles, nonstop! Such creatures must have an especially high aerobic work capacity and the architecture of the flight muscle must be exquisitely adapted for aerobic metabolism. George and his associates, (Chandra-Bose and George, 1964; Grinyer and George, 1969*a*; see particularly George and Berger, 1966) have carried out extensive studies on the architectural and functional features of various avian flight muscles, particularly those that are involved in flight, the pectoralis and the supracoracoideus. Briefly, flight muscles consist of two types of fibers—red and white, with a preponderance of the red variety. The red fibers are small in diameter, are copiously supplied with blood, contain large and numerous mitochondria, high concentrations of myoglobin, fat, and oxidative enzymes. White fibers are of larger diameter, contain fewer and smaller mitochondria, low oxidative capacity, high glycogen content, and low fat stores. Obviously, red fibers are especially adapted for aerobic work and for long and sustained activity. White fibers on the other hand, are adapted for anaerobic metabolism and for quick and sudden contractile activity. Such architectural and biochemical adaptation reaches its zenith in the hummingbird. This tiny creature is capable of prolonged flight and on its migratory journey moves nonstop across the Gulf of Mexico, a distance of over 500 miles! It possesses the incredible capacity for hovering flight and is able to fly both forwards and backwards. The flight muscles consist exclusively of red fibers (George, 1965). Grinyer and George (1969*b*) have recently examined the architecture of both the pectoralis and supracoracoideus muscles in the ruby throated hummingbird. The supracoracoideus, the chief elevator of the wing, is especially well developed in this organism. Some electronphotomicrographs of this issue are shown in Figure 8 and are taken from the data published by Grinyer and George (1969*b*). Mitochondria are so large (10 μ in length and 1.5 μ in width) and so dense that the number of myofibrils in an area may be almost unnoticed (Fig. 8A)! Large fat inclusions are also present (Fig. 8B). The mitochondria and fat droplets occur between myofibrils. Clearly this constitutes a most striking example of architectural and metabolic adaptation to permit exceedingly vigorous and prolonged aerobic work.

Utilization of intramuscular lipids. The studies described so far have dealt primarily with the delivery of FFA from adipose tissue stores to working muscles. Skeletal muscle, however, stores triglycerides intracellularly and we have just seen an example of this in the hummingbird (Fig. 8). The possibility must be considered that these "endogenous" stores can serve as fuel at rest or during activity. This would require a lipolytic release of FFA and it follows that working muscles (skeletal muscle and heart) may contain a triglyceride lipase which may be metabolically or hormonally regulated. The presence of such an enzyme, however, has not been definitively demonstrated. The actual contribution of "endogenous" triglycerides as fuel for muscles has been controversial. Carlson (1967) has presented studies which indicate that exercise in rats reduces the content of esterified fatty acids, mainly triglycerides in muscle tissue. Havel *et*

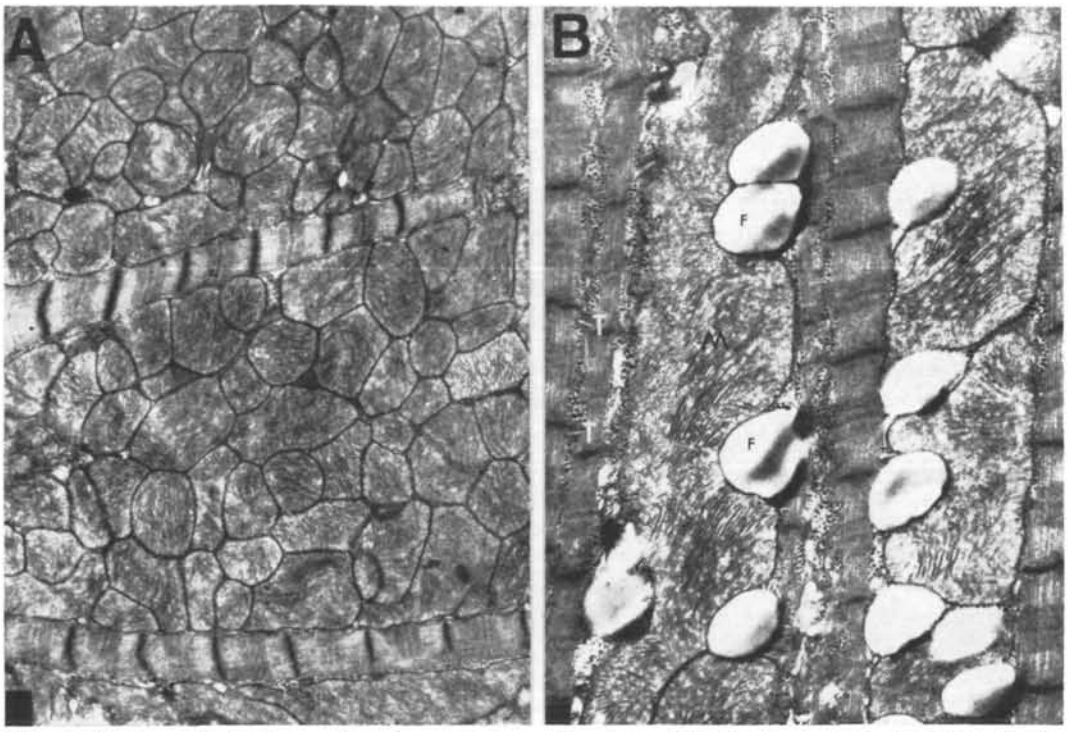

FIG. 8. Electron photomicrographs of supracoracoideus muscle of the ruby throated hummingbird. (Courtesy of J. C. George and the National Research Council of Canada).

al. (1964) have suggested that intramuscular fat constitutes a major fuel for muscular exercise in men, and Issekutz *et al.* (1966) from their studies in dogs exercised on a treadmill concluded that the intracellular fat pool constitutes an important energy source. In contrast, Masoro *et al.* (1966) and Masoro, Rowell, and McDonald (1966) found that when monkey and rat gastrocnemius and soleus muscles were stimulated electrically for 5 hours no decrease in tissue triglycerides occurred, and in rat gastrocnemius they actually increased. They concluded that intracellular lipids are not used as a net source of fuel for the increased energy of contracting muscle. It is apparent from what has previously been said, that an examination of muscle lipids in flying birds might contribute valuable information to our understanding of this problem. Such studies have just recently been performed. George and associates (Vallyathan and George, 1969; Vallyathan, Grinyer, and George, 1970) have used histochemical and biochemical methods to examine the effect of fasting and exercise on lipids in the pectoralis muscle of the pigeon. Their data (both histochemical and direct lipid determination) have revealed that a considerable *increase* in both intracellular and extracellular lipids actually occurs when flight muscles are made to contract by electrical stimulation. Surprisingly, similar increases also occurred in the contralateral, control, quiescent muscles. The inflow of lipids was selective; they were deposited only in the red lipid-utilizing fibers and not in the glycogen-loaded white type. They have concluded that intracellular muscle lipids are not immediately drawn upon for energy, either during exercise or fasting, as long as there is a ready supply of lipids transported through the blood from adipose tissue. Mobilization seems to be regulated so that the supply exceeds the demand, at least under these experimental circumstances, so that lipids are actually deposited in contracting fibers. These intracellular stores could be drawn upon

when muscular activity is prolonged to the extent that external sources of supply are depleted.

We thus see that mobilization and utilization of glycogen and lipids are precisely regulated by a variety of mechanisms which permit an organism to readily adapt to changing physiological circumstances. The picture is not yet complete, but our understanding is expanding rapidly. A discussion of glycolytic processes and oxidative reactions which permit the complete oxidation of these fuels to CO_2 and water with the ultimate entrapment of energy as ATP has been beyond the scope of this presentation. Suffice it is to say that these processes, too, are under precise physiological control. The area covered and the examples cited should serve to remind us of the wisdom, the unity and beauty which is in nature.

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