CLXXX. METABOLITES OF CONTRACTING MUSCLE. UTILIZATION OF FUMARATE

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In opening his Harvey Lecture, Lundsgaard [1938] remarked: "It is an assumption frequently encountered in physiological literature, that the metabolism of the aerobically working muscle is a pure carbohydrate metabolism. That assumption cannot have originated from the experience gained in metabolism determinations during muscular exercise. It is a familiar fact that even heavy muscular exercise can be performed on a pure fat diet, and with a respiratory quotient which indicates a combustion almost entirely of fat....Of course, the fact that hard muscular work can be performed under conditions in which the R.Q. is low, does not disprove the belief that working muscles oxidize carbohydrate only; for the possibility certainly exists that in the organism—presumably in the liver—there is a process of carbohydrate formation from fat, so that the organism as a whole is metabolizing fat alone. However, this purely hypothetical possibility can scarcely explain why the conception of a pure carbohydrate metabolism in the aerobically working muscles is so hard to get rid of, as actually seems to be the case. From this remark perhaps you will anticipate that I think that this conception ought to be discarded."

Lundsgaard's statement applies with particular force to mammalian tissues. Hitherto amphibian muscle has been the stronghold of those who support predominant, if not exclusive, carbohydrate oxidation, and therefore the finding of R.Q. lower than unity in the exercising aerobic frog muscle is all the more remarkable. Gemmill [1934] observed that the R.Q. of the contracting sartorius was 0·90 by a manometric and 0·94 by a volumetric method. Later [1936], using improved instruments, the range found was 0·86–0·98, with an average of 0·90. The same author [1935], working also with aerobic frog muscle, found that the average disappearance of sugar accounted for only 42 % of the total energy exchange as calculated from the oxygen consumption. It is clear that these facts cannot be squared with exclusive carbohydrate combustion. The problem then arises, what is the nature of the metabolites concerned?

This question cannot be answered at once. Nothing is known about fat metabolism in the exercising frog, and the first experiments must of necessity be conducted by trial and error. The simplest approach is to find out what substances are removed from solution by a working muscle, and it is important that the tissue chosen for experiment should exercise under completely aerobic conditions, as in the living animal when the steady state has been reached. Hill [1928] has discussed the factors which influence the penetration of oxygen into amphibian muscle, and the criteria laid down by him have been carefully observed.

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Experimental

(1) *Physiological details*

Sartorii from English frogs have been used throughout. A cylindrical glass vessel, holding not more than 5 ml., contains bicarbonate-Ringer, and has sealed through the bottom a platinum hook, to which the distal end of the sartorius is fixed by a small **S**-shaped piece of platinum wire passing through the muscle. The thicker (pelvic) end of the tissue is fastened with a nickel clip, and is hooked by means of a copper stirrup and wire to an isometric lever. Stimulation takes place via the platinum hook and the isometric lever. The control muscle (from the opposite limb) goes into a similar vessel containing an identical volume (4 ml. usually) of Ringer. Through each vessel is passed a stream of oxygen which has traversed a wash-bottle fitted with a Jena glass filter and filled with Ringer; hence the gas is saturated with water vapour at the temperature of the laboratory, and evaporation cannot occur. In addition, the rate of oxygen flow is regulated by screw clips which ensure that the volume passing through each vessel is the same. The Ringer was composed as follows:

NaCl, 0·6 %; $NaHCO_3$, 0·1 %; KCl, 0·01 %; $CaCl_2$, 0·01 %; the phosphate buffer was 0·028 % and had *p*H 7·6; the gas mixture contained 97·5 % O_2 and 2·5 % CO_2. A standard concentration of $M/200$ was employed for the metabolites.

After dissection the muscles are placed in a watch-glass containing a few ml. of the $M/200$ substrate-Ringer, and left for an hour; then the solution is drained off and replaced, and the muscles soaked for another hour. The muscle to be stimulated is now drained on filter paper and put into the appropriate vessel; after passing the gas mixture for a few minutes, stimulation is begun. The control is treated in a similar fashion. Experiments last for 3–5 hr., during which time the muscle is caused to twitch every 5 sec.: under these conditions, with the tissue exposed to oxygen on both sides, it is in a fully aerobic condition [Hill, 1928]. At the end of the allotted period the stimulated muscle is removed from the solution, drained on bibulous paper, dried at 110° and weighed. Meanwhile, 2 ml. of the liquid are taken from each vessel, and the amount of substrate determined. In this way we arrive at the mg. of fatty acid consumed, over and above the control, per g. dry weight per hour, when the sartorius is stimulated in oxygen at twelve shocks per minute.

(2) *Sources of material*

The bulk of the material was of commercial origin, and carefully purified by several recrystallizations from appropriate solvents. Sorbic acid was synthesized by the method of Doebner [1900]; maleic acid purified by conversion into the anhydride [Kempf, 1906]; acetoacetic acid prepared as sodium salt according to Ljunggren [1924]. The glyceride, α-monocrotonin, appears not to have been made before. Epichlorohydrin on hydrolysis furnishes pure α-monochlorohydrin, which is heated with sodium crotonate in a sealed tube. The resulting oil, which could not be crystallized, boiled at 120°/0·25 mm. (Found (Weiler): C, 52·8; H, 7·12 %; mol. wt. by hydrogenation, 154·5. $C_7H_{12}O_4$ requires C, 52·5; H, 7·5 %; mol. wt. 160.)

(3) *Methods of estimation*

Unsaturated compounds. These were hydrogenated in Barcroft-Warburg manometers, using colloidal palladium as catalyst. The main vessel contains 2 ml. of the fluid to be analysed, made acid with 1 ml. N H_2SO_4. The catalyst

is prepared by dissolving 0·2 g. gum arabic in 100 ml. water, and adding 0·2 g. $PdCl_2$ [cf. Skita & Franck, 1911]; 0·5 ml. of this solution goes into the side cup of the manometer. Hydrogen is passed through for 15 min., shaking the while, thus driving out most of the air and saturating the catalyst; 15 min. further equilibration are then allowed. The determinations are carried out in a bath at room temperature.

Formic acid. The usual chemical procedures are not nearly sensitive enough when applied to small quantities of this acid. Woods [1936] has, however, shown that *Bact. coli* grown in formate broth develops a powerful enzyme, formic hydrogenlyase, which in the absence of oxygen liberates hydrogen quantitatively from formates. The method is very specific; but as the enzyme is inhibited by CO_2, bicarbonate was omitted from the Ringer and the amount of phosphate raised to 0·035 %. Dr Woods was kind enough to grow the organism and supply details of his method.

Succinic acid. Estimated with succinoxidase, prepared according to Ogston & Green [1935]. This is the least sensitive of the methods used, and a good deal of practice was required in order to obtain consistent results.

Acetoacetic acid. Steam distillation in the presence of N H_2SO_4 breaks down the acid to acetone, which is then trapped in alkaline hypoiodite; the excess iodine is titrated, after acidification, with $N/200$ thiosulphate.

Control experiments have been performed to show that no interfering substances diffused out of the muscle, whether stimulated or not. In all cases about 1 mg. of substrate is present in 2 ml. of the fluid to be analysed. The difference in substrate content to be expected between "stimulated" and "control" solutions is about 0·1 mg., or 10 % of the whole. Obviously, then, the experimental error of the analysis must not exceed ± 1 %, for work in which the error is greater than 20 % of the change to be anticipated is not likely to carry much conviction. Muscles larger and thicker than the sartorius cannot be employed, because they become anaerobic when stimulated [Hill, 1928], thus defeating an essential purpose of this investigation.

Table I

No. of exps.	Substrate	mg./g. dry wt./hr.
4	Formic acid H.COOH	—
—	*Oxalic acid COOH.COOH	—
2	Acrylic acid CH_2:CH.COOH	—
6	Crotonic acid CH_3.CH:CH.COOH	—
2	Tiglic acid CH_3.CH:C(CH_3).COOH	—
3	Sorbic acid CH_3.CH:CH.CH:CH.COOH	—
2	Muconic acid COOH.CH:CH.CH:CH.COOH	—
6	Succinic acid COOH.CH_2.CH_2.COOH	—
3	Acetoacetic acid CH_3.CO.CH_2.COOH	—
2	Maleic acid COOH.CH:CH.COOH (*cis*)	—
7	Fumaric acid COOH.CH:CH.COOH (*trans*)	8·6
2	α-Monocrotonin CH_2OH.CHOH.CH_2.O.CO.CH:CH.CH_3	—

* Oxalic acid, even when supplied as a saturated solution of the calcium salt, is toxic to frog muscle.

Discussion

A critical eye, glancing down the list of compounds studied (Table I), might remark the odd choice of material. This is not deliberate; it is simply due to lack of precise methods for estimating the fatty acids and their allies. The requirements are severe: quantities of the order of 1 mg. must be determined with a maximum error of ± 1 %. Very few biochemical methods of any kind reach this

standard. The case of β-hydroxybutyric acid may serve as an illustration. Hubbard [1921], oxidizing small amounts of the acid with dichromate, recovered between 84 and 88 % as acetone. These results are the best that have been obtained; and it is doubtful whether the yield of acetone is really so consistent as Hubbard claims. In my own hands dichromate oxidation has given quantities of acetone varying from 80 to 90 % of the theoretical, and in a recent paper by Shipley & Long [1938] the figures range from 63 to 77 %. In connexion with the present study, oxidation has been carried out using persulphate, permanganate, hydrogen peroxide and a ferrous salt, selenium dioxide, perchlorate, colloidal MnO_2, bromine and silver oxide: attempts have been made also to dehydrate the acid, after evaporating the solution to dryness, with conc. H_2SO_4, fuming H_2SO_4, 100 % orthophosphoric acid and zinc chloride. None of these methods represented an improvement over dichromate oxidation, and this is rather unfortunate, as β-hydroxybutyric acid is quite likely to be a fuel of exercise. A satisfactory procedure for β-hydroxybutyric acid has yet to be found, and the same may be said of all the saturated fatty acids.

That the negative results are truly negative, and not spuriously so, appears to be indicated by the following considerations. First, the sartorius is permeable to some, at least, of the substrates employed. By soaking a pair of muscles in $M/200$ solutions of acrylate, crotonate, sorbate and succinate, it has been found that after 3 hr. these substances pass into the tissue in such proportions as to imply that simple osmotic equilibrium has been attained. In the case of succinate, indeed, rather more goes in than can be accounted for by the 80 % of water which the muscles contain—perhaps in order to satisfy the needs of respiration. Secondly, the unsaturated acids are not merely oxidized to the corresponding keto-acids (which would be reduced by hydrogen and palladium); crotonic acid, for example, does not yield acetoacetic acid, since neither Rothera's test nor distillation into hypoiodite yield the faintest trace of acetone. Thirdly, it is unlikely that the acids are oxidized at the terminal methyl group, giving carboxyl; if this were the case, crotonate would change into fumarate, which would disappear in the usual way, and acrylate would also be destroyed. Fourthly, the failure to oxidize α-monocrotonin indicates that preliminary esterification with glycerol is not an essential step in the combustion.

The negative results are not due to seasonal influence, for experiments have been carried out on "summer" and "winter" frogs with consistent lack of success. Nor can they be due to competition by the glycogen-lactic acid system: intraperitoneal injections of 30–40 units of insulin were given to some of the frogs 24 hr. before killing, and although the glycogen (extracted by the method of Kerly [1930], and estimated as sugar "after hydrolysis" with the reagent proposed by Somogyi [1937]) was depleted by this procedure, the sartorii from another batch of frogs treated in the same way were unable to use any of the substrates (except fumarate, of course; but even here no gain in fumarate disappearance was observed with the insulinized muscles). The concentration employed likewise cannot be held responsible, for no disappearance was noted when $M/100$ solutions of crotonate, succinate and acetoacetate were supplied: in any case the utilization of $M/200$ fumarate suggests that this concentration is adequate. The failure to remove these fatty acids means, therefore, that frog muscle is unable to initiate their combustion; and this finding must be regarded as significant.

Nor does the positive result with fumarate appear to be false. The acid is not functioning as a hydrogen acceptor; were this so, the succinate formed would diffuse out of the muscle (since it is not burnt, and the tissue is permeable to it),

but tests of the solution with succinoxidase have proved that no succinate is present at the end of the period of exercise. Moreover, the amount of fumarate metabolized is on a smaller scale than can be conveniently explained on the hypothesis of partial combustion, for calculation shows that the disappearance is about what would be expected on the basis of complete oxidation. Hill [1928] has found that a single maximal twitch of a frog muscle liberates 7×10^{-3} cal. per ml. (i.e. per g. wet wt. roughly). With 720 twitches an hour the heat liberated would be 5·04 cal. per g. wet wt., or, multiplying by 5 (since the muscle contains 80 % water), 25·2 cal. per g. dry wt. Now when fumaric acid is burnt [Kharasch, 1928] it yields 320 kg. cal. per mol., or 2·76 cal. per mg. Hence the amount of heat which might have come from the acid in these experiments is $8{\cdot}6 \times 2{\cdot}76 = 23{\cdot}6$ cal., or 94 % of the total. Too much reliance must not be placed on these figures, and work is now in progress to determine the relation between fumarate disappearance and energy output.

It would be premature to base any wide conclusions upon the limited findings of the work here described. The disappearance of fumarate indicates that frog muscle can use other sources of energy than carbohydrate, but the mystery surrounding the low R.Q. of the exercising sartorius has not been dispelled. Until precise methods are available, we shall remain ignorant of the part played by fats in the economy of muscular exercise.

Summary

1. A number of fatty acids and a glyceride have been supplied to frog sartorii stimulated twelve times a minute under completely aerobic conditions.

2. Formic, oxalic, acrylic, crotonic, tiglic, sorbic, muconic, succinic, maleic and acetoacetic acids, together with α-monocrotonin, were not used in significant amounts during exercise.

3. Fumaric acid disappears at the rate of 8·6 mg. per g. dry wt. per hr.; a rough calculation indicates that the amount of fumarate consumed, if completely oxidized, would account for 94 % of the total heat developed by the muscle. The calculation is not considered to be very reliable, and further experiments are in progress.

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