Skeletal muscle triacylglycerol in the rat: methods for sampling and measurement, and studies of biological variability

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Summary Previously reported concentrations of triacylglycerol in skeletal muscle have shown high coefficients of variation, and there have been large differences between mean concentrations reported in a given muscle. Conditions for sampling and measurement were therefore investigated. Samples were best taken under anesthesia as breakdown of triacylglycerol was rapid after decapitation. Silicic acid was preferable to zeolite for removal of phospholipids although either agent could interfere with the estimation. Even with apparently reliable methods, a high variability was found in any one muscle and there were large differences between muscles. It is unlikely that the variability was due to contamination with adipose tissue. Concentrations of glycogen and phospholipid were much less variable. Although the store of triacylglycerol in skeletal muscle in caloric terms was found to be 2–18 times greater than that of glycogen, the variability found is likely to hamper studies of its metabolic role.—**Frayn, K. N., and P. F. Maycock.** Skeletal muscle triacylglycerol in the rat: methods

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Although the triacylglycerol in skeletal muscle constitutes a large potential source of energy (1), its metabolic role is not clear. One problem in interpreting studies of muscle triacylglycerol is that previous measurements of its concentration have shown this to be very variable, both within any particular study and from study to study. Coefficients of variation for measurements in a given muscle by a given author are typically 20–50% and the mean concentrations reported for one particular muscle (e.g. rat soleus) differ almost 20-fold (2, 3).

This high variability suggested that conditions for sampling and measurement might be critical, and this report presents studies on this question. Despite the discovery of some pitfalls in methodology, this study confirmed the high variability of concentrations of skeletal muscle triacylglycerol.

MATERIALS AND METHODS

Animals

The rats (229–264 g males) were of albino Porton-Wistar strain. They were kept at 18–22°C with 12 hr light per day and fed ad libitum on P.M. Diet (Oakes Millers Ltd., Congleton, Cheshire, U.K.). Abdominal muscle samples were obtained from decapitated or anesthetized animals by removing the skin and subcutaneous adipose tissue before freeze-clamping (4) in situ. The external oblique muscle was separated from the rectus abdominis in some experiments by scoring the line dividing them with a scalpel before freeze-clamping. Soleus muscles were removed from anesthetized rats either by exposing the Achilles tendon, separating and tying the plantaris, gastrocnemius and soleus tendons, then cutting these tendons distal to the tie, lifting the distal end of the muscle away from the leg, and rapidly freeze-clamping, or by removal of the entire leg before dissection. The latter method was also used for removal of plantaris muscles.

Analytical methods

For triacylglycerol estimation the frozen muscle sample was powdered under liquid nitrogen with a porcelain pestle and mortar. Thereafter the method was based on that of Denton and Randle (5). The powder (100–200 mg) was weighed into a glass tube, 3 ml of chloroform–methanol 2:1 (v/v) (6) was added, and the tube was left at 4°C for at least 16 hr. $MgCl_2$ (3 ml; 4 mmol/l) was then added and the tube was centrifuged at 1000 *g* for 1 hr at 4°C. The muscle powder formed a firm 'cake' at the interface between the two phases. The volume of the organic phase at this stage was assumed to be 2 ml. The upper (aqueous) phase was aspirated and discarded, and as much as possible of the infranatant phase was aspirated and weighed into a fresh glass tube, evaporated to dryness, and the residue redissolved in chloroform (5 ml). This stage is referred to below as the 'post-extraction' stage. The remaining muscle powder in the extraction tube, together with the end of the Pasteur pipette used for aspiration (to which some muscle was always adherent), was dried and dissolved in NaOH (10 ml; 1 mol/l) before protein assay as below. A portion of the lipid extract was taken at this stage for phospholipid determination if required. Phospholipids were then removed (see below) by adding silicic acid (400–500 mg Sil-R, Sigma London Chemical Co. Ltd., Poole, Dorset, U.K.; heated to 120°C for at least 2 hr before use). After mixing and brief centrifugation, portions of the supernatant solution for saponification and for phospholipid determination (to check completeness of removal) were evaporated to dryness. Saponification with ethanolic KOH (500 μl; 0.5 mol/l) was carried out at 75°C for 20 min; $MgSO_4$ (1.0 ml; 0.15 mol/l) was added and after centrifugation glycerol was assayed in a portion of the supernatant solution by the method of Garland and Randle (7). The overall precision, measured by estimating the triacylglycerol concentration of 24 replicate samples from a pool of powdered muscle, was 7% (coefficient of variation). Recovery of glycerol from a tripalmitoylglycerol standard (Sigma London Chemical Co. Ltd.) was 101 ± 3% (mean ± SEM; $n = 34$), and that from a tripalmitoylglycerol standard added to samples from a pool of powdered muscle was 99 ± 6% (mean ± SEM; $n = 6$).

To determine phospholipid, the samples in chloroform were dried and digested with perchloric and sulfuric acids and the resultant inorganic phosphate was estimated (8). A phospholipid standard (L-α-phosphatidylcholine; Sigma London Chemical Co. Ltd.) was used and was digested in the same way as the samples. The overall precision of the assay, estimated on replicate samples from a pool of muscle powder, was 12–13% (coefficient of variation).

Protein was determined by the method of Lowry et al. (9). Glycogen was estimated in separate pieces of muscle by the method of Good, Kramer, and Somogyi (10).

Removal of phospholipids

The choice of agent and its stage of addition for optimal removal of phospholipids were investigated before adopting the above procedure. Zeolite has been added to the initial extraction mixture for this purpose (11), as in the estimation of serum triacylglycerol (e.g. 12), and this represents an apparent simplification. However, it was found that zeolite (Sigma London Chemical Co. Ltd.; activated as for silicic acid) so added formed a loose pellet as well as an interfacial cake after addition of the $MgCl_2$ solution, making aspiration of the organic phase very difficult. Silicic acid under these conditions was found only at the interface, but, as found by Denton and Randle (5), its capacity for adsorption of phospholipids was reduced by the presence of methanol. It was also observed that both zeolite and silicic acid could reduce the recovery of a triacylglycerol standard, depending on the stage at which they were added (**Table 1**); the presence of a small amount of either during saponification had a particularly marked effect. It was thus essential that the aliquot of the post-silicic acid solution used for saponification was free from any visible silicic acid; recentrifugation was sometimes necessary to achieve this.

Silicic acid, when used as described, removed 99.2 ± 0.2% (mean ± SEM; $n = 6$) of the phospholipid present. However, despite the finding of Denton and Randle (5) that phospholipids may yield glycerol on saponification, only 7 ± 2% (mean ± SEM; $n = 6$) (mol/mol) of the muscle phospholipid yielded glycerol under the above conditions. Although removal with silicic acid was always carried out, the effect on the final triacylglycerol measurement of omitting this treatment would have been small (e.g. about 2% in the case of rectus abdominis).

TABLE 1. Recovery of triacylglycerol-glycerol with different methods of removal of phospholipid

Stage of Addition	% Recovery of Triacylglycerol Glycerol	
	Zeolite	Silicic Acid
Extraction mixture	85.0 ± 2.9	91.6 ± 5.4
Post-extraction	59.7 ± 1.5	99.7 ± 1.9
Saponification	19.2 ± 1.4	5.9 ± 0.9

A solution of tripalmitoylglycerol in chloroform–methanol 2:1 (v/v) was analyzed as described in the text. Either zeolite or silicic acid (each activated by heating to 120°C for at least 2 hr before use) was added at the stage indicated; these stages are defined in the text. For each ml of initial solution, 200 mg of the agent was used, except for addition during saponification when only 20 mg (approx.) was added. The results show percentage final recoveries of glycerol relative to untreated samples, as means ± SEM; $n = 4$ in each case.

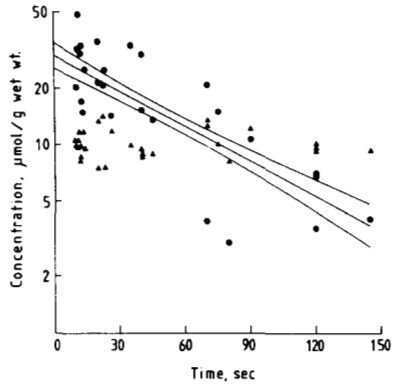

Fig. 1. Breakdown of muscle triacylglycerol after decapitation. Rats were decapitated at time zero. Samples of abdominal muscle (mainly rectus abdominis) were freeze-clamped in situ at intervals thereafter. Triacylglycerol (●) and phospholipid (▲) concentrations were estimated in these samples as described in the text, and are plotted on a logarithmic scale. The regression line of the log of triacylglyerol concentration on time, with its 95% confidence limits, is shown, and has been extrapolated to zero time for prediction of the concentration during life as shown in Table 2. The correlation was significant ($r = -0.77$, $P < 0.001$), whereas that between phospholipid and time was not.

Expression of results

Concentrations of triacylglycerol and phospholipid were expressed initially per unit wet weight determined on the muscle powder in liquid nitrogen; in later experiments concentrations per unit weight of protein were calculated since this measurement was found to be more reliable. In several experiments it was found that the measured phospholipid concentration in samples from a single pool of powdered muscle was significantly less variable when expressed relative to protein rather than to wet weight (F test).

Statistical methods were based on Snedecor and Cochran (13).

RESULTS

Investigation of methods of sampling

In initial experiments abdominal muscle (either mainly rectus abdominis or mainly external oblique) was used because of its accessibility and relative thinness, allowing rapid freezing (14). The triacylglycerol concentration in rectus abdominis fell rapidly after decapitation (**Fig. 1**), less than 20% remaining after 2 min. This rapid breakdown was probably caused by post-decapitation convulsions in view of the results below. The phospholipid concentration did not change with time after decapitation (Fig. 1). Sampling under anesthesia produced values close to that obtained by extrapolation of the data in Fig. 1 to zero time (**Table 2**). The concentration of muscle

TABLE 2. Concentrations of triacylglycerol in abdominal muscle sampled under different conditions of treatment of rats

Method	Triacylglycerol Concentration
	μmol/g wet wt
Ether	28.7 ± 2.3 (13)
Pentobarbitone	27.8 ± 3.9 (6)
Decapitation	25.0 ± 2.8 (14)
Decapitation with extrapolation	29.6; 25.5–34.3

Muscle samples (mainly rectus abdominis) were obtained by freeze-clamping in situ in fed rats, anesthetized either with diethyl ether (1.5–4 min after entry into anesthetic chamber) or pentobarbitone (Sagatal; May and Baker Ltd., Dagenham, Essex, U.K.: 1 ml/kg), or shortly (10–26 sec) after decapitation. The figure for 'Decapitation with extrapolation' is the mean with standard error range estimated by extrapolation of the regression line shown in Fig. 1 to zero time. Other results are means ± SEM with numbers of observations in parentheses.

triacylglycerol was not affected even by prolonged diethyl ether anesthesia (**Table 3**), nor, in confirmation of the results of Fröberg (15), by delay between removal and extraction (Table 3). Triacylglycerol concentrations were compared in soleus muscles freeze-clamped in situ in pentobarbitone-anesthetized rats (see Materials and Methods) and in muscles dissected out from legs removed from anesthetized rats. Concentrations determined by the former method were 30.7 ± 5.6 μmol/g protein (mean ± SEM; n = 8), not significantly different from those obtained with the latter method (shown in Table 4).

It was concluded from the above experiments that muscle sampling was best carried out under anesthesia to avoid contractions, and that freeze-clamping was unnecessary. Before dissecting out, e.g., specific leg muscles, the relevant part of the body could be removed with no significant loss of triacyglycerol.

Some authors have attempted to remove blood from the muscle (5, 16, 17). The concentration of triacylglycerol in blood from these rats, measured by the method used for muscle, was 1.1 ± 0.2 μmol/ml (mean ± SEM; n = 3), and since the blood content of such muscle samples is small (≤5% v/w (15)) this would have little effect on the results (e.g. ≤5% for rectus abdominis).

TABLE 3. Stability of muscle triacylglycerol in isolated muscle and in vivo during prolonged anesthesia

Treatment	Triacylglycerol Concentration
	μmol/g protein
Sampling in situ	
Ether for 2–4 min	66.6 ± 8.0 (12)
15 min	65.1 ± 10.4 (3)
30 min	64.7 ± 17.0 (7)
Isolated muscle	
Left for 10 min	50.8 ± 5.3 (4)
20 min	70.5 ± 17.3 (5)

External oblique muscle was obtained by freeze-clamping in situ from rats under diethyl ether anesthesia for various periods, or was removed from rats after brief ether anesthesia and left at room temperature (19–22°C) for the periods shown before freezing. The triacylglycerol concentration was then measured. Results are means ± SEM with number of observations in parentheses; no results are significantly different from those in the top line (66.6 μmol/g). The results are expressed relative to muscle protein rather than to wet weight for reasons given in the text.

Variability of results

The concentrations of triacylglycerol found in various muscles are shown in **Table 4.** Although there were large differences between the different muscles, there were also relatively large coefficients of variation for each muscle. The source of these variations was investigated as follows.

Samples of rectus abdominis were taken from 6 rats; the combined bilateral muscles from each were divided transversely into two pieces each of about 100 mg wet weight and their triacylglycerol concentrations compared. In a similar experiment using external oblique muscles from each of 9 rats, the left and right muscles were compared. In both cases variance in triacylglycerol concentration between muscle samples from one animal was not significantly different (F test) from variance between animals; each was considerably greater (F test, $P < 0.01$) than analytical variance. For the plantaris muscle, similarly, variance between legs from one animal was not greater than between-animals variance (F test), although the latter was much less in this muscle than in the other muscles studied (Table 4). In the soleus, on the other hand, variance between bilateral muscles was significantly less than between-animals variance (9 rats; F test, $P < 0.025$) and the mean triacylglycerol con-

TABLE 4. Triacylglycerol concentration in various rat muscles

Muscle	Triacylglycerol Concentration	CV
	μmol/g protein	%
Rectus abdominis	123.5 ± 9.3 (33)	43
External oblique	59.8 ± 4.7 (27)	41
Soleus	35.4 ± 4.2 (17)	49
Plantaris	15.9 ± 0.7 (9)	14

Muscle samples were taken from fed rats under diethyl ether anesthesia and their triacylglycerol concentrations measured as described in the text. For removal of soleus and plantaris, the entire leg was removed before dissection; the time from removal of the leg to freezing of the last muscle was 4–6 min. Results are means ± SEM with the number of animals in parentheses, and the percentage coefficient of variation (CV, = SD × 100/mean). The results for soleus and plantaris, compared within rats using a paired t-test, were significantly different ($P < 0.001$).

centration of the soleus muscles was significantly correlated with that of the plantaris muscles from the same animal (**Fig. 2**), showing that real differences do exist between animals in their overall muscle triacylglycerol concentrations.

The variability in triacylglycerol concentration was not due to gross differences in the nature of the samples (e.g., inclusion of large amounts of other tissues) since it was not found in either phospholipid or glycogen concentrations. Variance in phospholipid concentration in 12 samples of rectus abdominis (coefficient of variation 12%) was not greater than analytical variance; that in glycogen in rectus abdominis (coefficient of variation 12%, 16 samples) was also considerably less than that in triacylglycerol (Table 4).

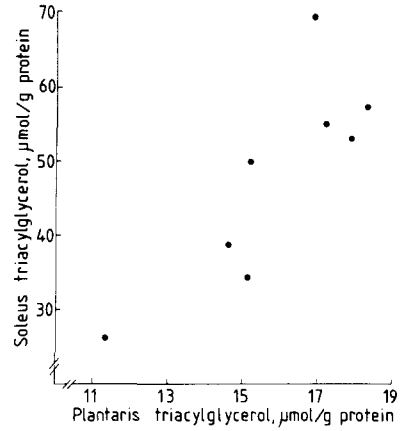

Fig. 2. Concentrations of triacylglycerol in rat soleus and plantaris muscles. Muscles were isolated from legs removed from eight rats under diethyl ether anaesthesia, and their triacylglycerol concentrations estimated as described in the text. The figure shows the results in individual animals; the mean concentration in the two soleus muscles is plotted against the mean concentration in the two plantaris muscles. The correlation is significant ($r = 0.83$, $P < 0.02$).

DISCUSSION

Previous estimations of the triacylglycerol concentration in various mammalian voluntary skeletal muscles have differed considerably, from 1–2 μmol/g wet wt (2, 15, 18–20) in rat gastrocnemius and soleus to 50 μmol/g wet wt (21) in human isolated red fibres. The present results of about 4 μmol/g wet wt (plantaris) to 30 μmol/g wet wt (rectus abdominis) fit into this range. Few authors, however, have commented on the marked variability in any particular muscle, although this is noticeable in many earlier reports. A wide range of methods has been employed for these investigations and apart from the work of Fröberg (15), little attention has been paid to the possible importance of methodological factors. Even with the apparently reliable methods developed in this study, however, biological variability was marked, and of a degree which appears to be unparalleled in other fuel stores or structural components (e.g., concentrations of both glycogen and phospholipid, as above, showing coefficients of variation amongst similar animals of around 12%).

It is unlikely that this variability was caused by contamination with adipose tissue. The abdominal muscles can be easily cleaned of subcutaneous adipose tissue, and there was none visibly adherent to the undersurface of the muscle except along the midline (especially near the xyphoid process), and this region was avoided. Some degree of contamination might explain why the rectus abdominis had the highest concentration of triacylglycerol amongst the muscles studied, but there are two reasons for thinking that this was not so: a particular liability to contamination with adipose tissue might be expected both to increase the observed variability, and to introduce a positive (i.e., rightward) skewness into the observed distribution. Neither of these features was found; the coefficient of variation in the triacylglycerol content of rectus abdominis was no greater than that in other muscles (Table 4), and there was no evidence for any skewness in the distribution (coefficient of skewness 0.17, 33 samples; not significant). Further, even higher values have been found in individual human muscle fibres, and also with a high variability (21). Even in the soleus, which was isolated as an intact muscle and trimmed of its tendinous ends before powdering, the variance between animals was such as to make comparisons between animals very difficult.

The presence of 'interstitial' adipose tissue in the form of cells interleaved with those of the muscle has been suggested as an explanation for the variability of muscle triacylglycerol in the dog (16, 22). Rat muscle, however, contains few such fat cells (5, 20; 1% of the total: 20). In addition, this explanation would raise the question of the definition of muscle triacylglycerol; triacylglycerol stored in adipose cells in such intimate contact with the muscle may well have the same role as that stored in the muscle cells themselves (16, 23).

The concentrations of triacylglycerol found are very high in terms of energy storage. Taking an average muscle glycogen concentration as 3 mg/g wet wt[1] and using caloric values of 17 kJ/g (4 kcal/g) for carbohydrate and 38 kJ/g (9 kcal/g) for fat (24), the energy stores in rectus abdominis (per g wet wt) are equivalent to about 0.05 J as glycogen, and 0.9 J as triacylglycerol. Even the plantaris has 0.1 J/g wet weight as triacyl-

[1] Frayn, K. N., and D. F. Heath. Unpublished observations.

glycerol, about twice the energy available from its glycogen. Despite this potential importance as an energy source, however, the metabolic role of intramuscular triacylglycerol remains unclear.

It is concluded that there is a genuine variability, both between animals and between muscles from a given animal, in the triacylglycerol content of skeletal muscle, and that this variability is probably not due to inclusion of adipose tissue. This variability may have the unfortunate effect of hampering future studies of this interesting fuel reserve.

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