Free and Fixed Glycogen in Rat Muscle

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(*Received* 27 *September* 1954)

It has been known for many years that part of the tissue glycogen remains undissolved when tissues are extracted with trichloroacetic acid (TCA). This fact led Nerking (1900) and Willstätter & Rhodewald (1934) to conclude that tissue glycogen appears in two forms: an acid-extractable or free form and an acid-non-extractable or protein-fixed form. According to Pflüger (1903) and Loeschke (1904) this protein-fixed fraction is produced by the enclosure of part of the glycogen by the proteins in the course of their precipitation, and should be considered an artifact. It has never been settled satisfactorily since, whether the glycogen–protein complexes are artifacts or whether they constitute a physiological entity.

In a previous paper (Kemp & Kits van Heijningen, 1954) on a new micro-method for the determination of glycogen it was shown that the fixed glycogen can be extracted from tissues with TCA at 100°, and it was pointed out that the method could be easily adapted to the study of these two glycogen fractions in tissues. In the present work we have used this method to approach in two ways the question of the artificial or physiological nature of the protein-fixed glycogen in muscle. (i) It was tested whether a physiological process, i.e. the performance of work by a muscle, would affect the two glycogen fractions differently, thus altering their ratio. (ii) It was investigated whether different methods of glycogen extraction would yield the same values for both fractions respectively.

EXPERIMENTAL

Determination of free and fixed glycogen. Glycogen was extracted with a 5% (w/v) TCA solution containing 0·1% of silver sulphate and determined by means of a colour reaction (Mendel & Bauch, 1926) which occurs when glycogen is heated with concentrated sulphuric acid (Mendel, Kemp & Myers, 1954; Kemp & Kits van Heijningen, 1954). In order to separate the two fractions the extraction of tissue samples with the TCA reagent was performed in two stages:

Stage 1. The tissue sample was homogenized in the TCA reagent and subsequently centrifuged at 3000 rev./min. The clear supernatant contains all the free glycogen plus glucose and glucose 1-phosphate. The glucose and glucose 1-phosphate were determined in the 80% (v/v) methanol extract of a separate sample and the value found was subtracted from the glycogen plus glucose value (for details see Kemp & Kits van Heijningen, 1954).

Stage 2. The tissue residue was then washed once with the TCA reagent, heated for 15 min. at 100° with another 5 ml. of the reagent and centrifuged. This supernatant contains the fixed glycogen.

Muscular work. Female rats of a cross-bred Wistar strain, weighing 250–300 g. were used. The animals were anaesthetized with sodium pentobarbitone 45 mg./kg. intraperitoneally. If necessary another 2 or 3 mg. of pentobarbitone were given in the course of the experiment. One gastrocnemius muscle was freed from surrounding tissues, the achilles tendon was cut and connected to a lever weighted with 10 g. The sciatic nerve was freed, dissected and, with the aid of a silk thread attached to the end, placed over a pair of needle electrodes from a square wave stimulation apparatus. Stimuli of 0·8 mA strength and 20 msec. duration were applied every second. At the end of the stimulation period the glycogen in both gastrocnemius muscles was determined, the unstimulated one serving as control.

Extraction experiments. Two different methods of extraction were compared, namely the TCA extraction described above and an extraction with distilled water containing 0·02 M ethylenediaminetetraacetic acid (EDTA) (Versene) to prevent glycogenolysis. According to Gross (1953) the enzymic function of myosin-ATPase can be inhibited by removing Ca ions with the aid of EDTA, a chelating agent. ATPase activity is essential to provide the inorganic phosphate required for the phosphorylysis of glycogen. It was expected, therefore, that EDTA, in a concentration sufficient to inhibit ATPase activity, would inhibit glycogenolysis. In preliminary experiments a satisfactory inhibition of glycogenolysis in muscle was obtained by using a concentration of 0·02 M EDTA. The animals were killed by a blow on the head, and one gastrocnemius muscle was removed immediately.

Muscle samples of 250–500 mg. were weighed quickly on a torsion balance and ground with chloride-free quartz sand in mortars containing 5 ml. of the EDTA solution. The brei was transferred quantitatively into a centrifuge tube with the aid of another 3 ml. of EDTA solution and centrifuged subsequently at 3000 rev./min. for 5 min. The clear supernatant was decanted into another centrifuge tube and deproteinized by adding 2 ml. of a 20% (w/v) solution of metaphosphoric acid containing 400 mg./100 ml. of silver sulphate or by adding 2 ml. of 20% (w/v) TCA containing 400 mg./100 ml. of silver sulphate. The precipitate thus formed in the EDTA extract (VE precipitate) was centrifuged down, the clear supernatant decanted and its glycogen content determined. Up to this point the experiments were carried out in the cold room. The tissue residue and the VE precipitate were washed once with the ordinary TCA reagent, and the fixed glycogen was extracted and determined as described above.

RESULTS

As can be seen from Fig. 1, computed from control determinations over a period of a year, the values for both fractions as well as their ratios vary greatly from one animal to another. Even in a litter of seven animals, which were kept for 3 months under carefully controlled constant conditions, a great variation in the free: fixed glycogen ratio was found (Table 1).

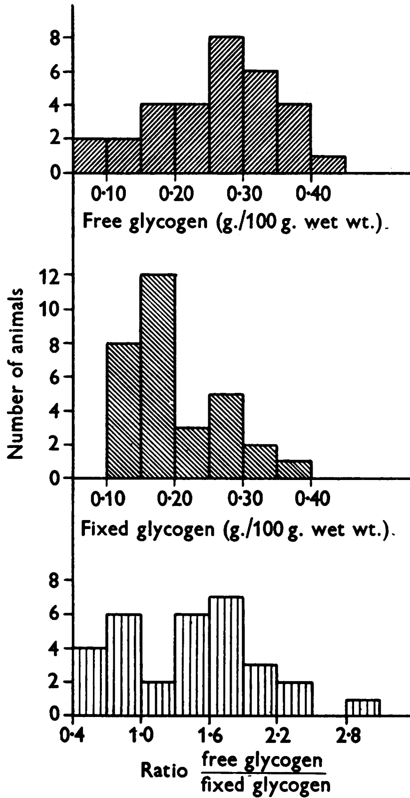

Fig. 1. Frequency distribution of free and fixed glycogen contents, and the ratio free: fixed glycogen in the gastrocnemius muscles of thirty-one normally fed rats.

In Table 2 a comparison is made between the values for both glycogen fractions in the left and right gastrocnemius muscle of the same animal. As could be anticipated from the results found by Cramer (1888) in other animal species, the differences do not exceed those between different pieces of the same muscle. From Table 3 it appears that a lapse of time of 3 hr. under anaesthesia, between the removal of left and right gastrocnemius muscle, does not influence the glycogen content of the muscle which is removed last. Experiments were therefore designed in such a way that each animal could serve as its own control.

In Table 4 the results of the stimulation experiments are given. In the last two experiments, stimulation was carried on until the muscle was no longer able to lift the weighted lever. To shorten the duration of the experiments the weight on the lever was increased as indicated in the table.

The glucose and glucose 1-phosphate concentrations were determined in the experimental and in the control muscles. As it appeared that the content of the experimental and control samples was the same, their mean value was calculated and given in the table.

Table 1. *Free and fixed glycogen content in the gastrocnemius muscles of seven rats of one litter*

The animals were kept for 3 months under constant conditions. Mean values of two determinations are given. Glycogen values are expressed in terms of glucose equivalents.

Rat no.	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Ratio: free glycogen / fixed glycogen
1	0·20	0·16	1·25
2	0·23	0·14	1·64
3	0·25	0·16	1·56
4	0·32	0·17	1·88
5	0·26	0·13	2·0
6	0·28	0·13	2·15
7	0·22	0·13	1·69
		Mean value ± S.E.	1·74 ± 0·26

Table 2. *Comparison of free and fixed glycogen content in left and right gastrocnemius muscles of single rats*

Mean values are given ± S.E. Glycogen values are expressed in terms of glucose equivalents.

Samples of left muscle				Samples of right muscle			
Wt. of muscle samples (mg.)	No. of determinations	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Wt. of muscle samples (mg.)	No. of determinations	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)
207–230	5	0·35 ± 0·03	0·19 ± 0·02	186–252	5	0·39 ± 0·04	0·18 ± 0·02
179–211	4	0·33 ± 0·03	0·17 ± 0·00	186–270	4	0·29 ± 0·02	0·19 ± 0·01
171–229	4	0·33 ± 0·02	0·11 ± 0·01	139–182	4	0·33 ± 0·02	0·13 ± 0·01
144–206	4	0·34 ± 0·01	0·19 ± 0·01	154–254	3	0·35 ± 0·01	0·19 ± 0·03
26–42	3	0·21 ± 0·01	0·30 ± 0·01	24–38	3	0·21 ± 0·02	0·30 ± 0·01
182–239	4	0·34 ± 0·01	0·18 ± 0·01	180–211	4	0·39 ± 0·01	0·18 ± 0·01
172–198	4	0·29 ± 0·01	0·16 ± 0·01	173–204	4	0·31 ± 0·01	0·16 ± 0·01

Table 3. *Comparison of free and fixed glycogen content of left and right gastrocnemius muscles of single rats*

The animals were anaesthetized with sodium pentobarbitone. The left muscle was removed after the onset of anaesthesia and the right one 3 hr. later. Mean values of three determinations are given ± S.E. Glycogen values are expressed in terms of glucose equivalents.

Samples of left muscle			Samples of right muscle		
Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)
59–74	0·42±0·03	0·21±0·004	51–88	0·42±0·02	0·28±0·02
63–106	0·43±0·02	0·23±0·02	74–86	0·49±0·02	0·23±0·01
54–100	0·52±0·003	0·23±0·01	76–95	0·56±0·03	0·24±0·01
53–86	0·35±0·002	0·12±0·01	45–71	0·30±0·003	0·11±0·02
39–83	0·42±0·02	0·17±0·02	44–56	0·35±0·01	0·20±0·02
47–101	0·45±0·04	0·17±0·01	59–86	0·48±0·02	0·20±0·02

In several experiments blood sugar values were determined according to Mendel *et al.* (1954) (cf. Mendel & Hoogland, 1950), before the onset of anaesthesia, after the preparation of the muscle for stimulation, and at the end of the stimulation period. Anaesthesia and preparing the muscle for stimulation did not affect the blood sugar markedly. Only in the last experiment was a considerable decrease in blood sugar found at the end of the stimulation period, namely from 72 mg./100 ml. before anaesthesia to 47 mg./100 ml. at the end of the experiment. In this experiment, liver samples were taken at the end of the stimulation period, and their glycogen content determined. The values were 0·11 and 0·09 g./100 g. wet wt. for the free fraction, and 0·08 and 0·06 g./100 g. wet wt. for the fixed glycogen. This very low glycogen content might explain the marked fall in blood sugar found at the end of this experiment.

In Table 5 the results of the extraction with water containing EDTA are compared with those of the TCA extraction. In some of the experiments the EDTA extracts were deproteinized with metaphosphoric acid instead of TCA. It appears that a small part of the fixed glycogen dissolves in the distilled water. This part is recovered by the hot TCA extraction of the VE precipitate. The extractions with cold TCA and with distilled water yield the same values for the free glycogen fraction. The sum of the values found by hot extraction with TCA of the VE precipitate and the residue of the aqueous extracts is in good agreement with the values found for the fixed fraction by the TCA extraction method.

DISCUSSION

No constant ratio could be demonstrated between the free and fixed glycogen in the gastrocnemius muscle of the rat. On this point our results are at variance with those of Bloom, Lewis, Schumpert & Shen (1951), who postulate a constant ratio of 1·22. However, the free and fixed glycogen contents of the right and left gastrocnemius muscle of the same rat were found to be identical.

As appears from Table 4 the two fractions behave differently under the influence of work performance, and consequently their ratio changes. This is in agreement with the results found by Bloom & Knowlton (1953) in similar stimulation experiments, though their methods of stimulation and estimation are essentially different.

On the other hand, two different methods of extraction, one of which is not likely to precipitate proteins that might enclose the glycogen, yield the same values for the free and fixed glycogen fractions respectively. It seems therefore that the fixed glycogen fraction which is found in muscle as well as in liver and other tissues is not an artifact, but a physiological entity. Presumably one of the two fractions represents the stored glycogen, whereas the other constitutes the 'active' form. However, our experiments do not answer the question which function should be ascribed to which fraction.

SUMMARY

1. The occurrence of free and fixed glycogen in the gastrocnemius muscle of the rat was studied.

2. Considerable individual differences were observed in the ratio, free:fixed glycogen. Identical values, however, were found for free and fixed glycogen respectively, in the right and left gastrocnemius muscle of the rat.

3. Performance of work alters the ratio free: fixed glycogen in the gastrocnemius muscle.

4. Two different methods of extraction, i.e. with trichloroacetic acid and with water containing ethylenediaminetetraacetic acid to prevent glycogenolysis, yield the same values for the two glycogen fractions respectively.

5. It is concluded that the fixed glycogen is probably a physiological entity and not an artifact.

The authors are indebted to Professor B. Mendel for much valuable discussion.

Table 4. *Influence of electrical stimulation on free and fixed glycogen in gastrocnemius muscles of rats*

In each animal one muscle was stimulated via the nerve with a square wave current of 0·8 mA for 20 msec. every second. Mean values of three determinations are given ± S.E. In all cases except Expt. 3, free sugar was determined in the control and stimulated muscles, and the mean values were subtracted from those for free glycogen. Glycogen values are expressed in terms of glucose equivalents.

	Control muscles					Stimulated muscles					
Expt. no.	Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Ratio: free/fixed glycogen	Free sugar (%)	Weight on lever (g.)	Duration of stimulation (min.)	Wt. of muscle (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Ratio: free/fixed glycogen
1	61–236	0·31 ± 0·01	0·15 ± 0·01	2·07	0·06 ± 0·02	10	2	40–226	0·23 ± 0·04	0·16 ± 0·02	1·44
2	55–236	0·29 ± 0·03	0·14 ± 0·004	2·07	0·06 ± 0·01	10	2	62–231	0·21 ± 0·04	0·13 ± 0·01	1·61
3	153–252	0·31 ± 0·04	0·19 ± 0·02	1·63	—	10	4½	144–248	0·14 ± 0·02	0·11 ± 0·02	1·27
4	21–231	0·27 ± 0·01	0·18 ± 0·03	1·50	0·05 ± 0·02	15	4½	28–246	0·16 ± 0·03	0·14 ± 0·02	1·14
5	58–240	0·19 ± 0·01	0·16 ± 0·01	1·19	0·09 ± 0·01	10	9½	78–254	0·05 ± 0·02	0·10 ± 0·00	0·50
6	59–212	0·21 ± 0·01	0·16 ± 0·01	1·31	0·05 ± 0·01	10	9½	98–180	0·06 ± 0·02	0·05 ± 0·01	1·20
7	66–260	0·31 ± 0·01	0·17 ± 0·01	1·82	0·04 ± 0·01	10 20 30	93 10 } 113 10	32–235	0·06 ± 0·01	0·07 ± 0·003	0·86
8	39–275	0·33 ± 0·005	0·17 ± 0·004	1·94	0·00	10 20 30	90 10 } 132 32	58–243	0·04 ± 0·01	0·05 ± 0·01	0·80

Table 5. *Comparison of free and fixed glycogen content in the same rats' gastrocnemius muscle as obtained by extraction with TCA and 0·02* M *EDTA*

The EDTA extracts were deproteinized with metaphosphoric acid (HPO_3) or TCA. Fixed glycogen was determined in the VE precipitate thus obtained, and in the tissue residues of TCA and EDTA extracts. The glycogen was determined colorimetrically as described by Kemp & Kits van Heijningen (1954). Mean values of three determinations are given ± S.E. When only two determinations were made the individual results are given. Glycogen values are expressed in terms of glucose equivalents.

	TCA extractions			Extraction with 0·02 N EDTA					
						Fixed glycogen content (%) in VE precipitate, precipitated with			
Rat no.	Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	TCA	HPO_3	Fixed glycogen content in residue (g./100 g. wet wt.)	Total fixed glycogen (g./100 g. wet wt.)
1	130–174	0·20 ± 0·01	0·16 ± 0·01	389; 361	0·23; 0·22	0·05; 0·09	—	0·22; 0·19	0·27; 0·28
2	143–178	0·16 ± 0·01	0·11 ± 0·01	365–468	0·17 ± 0·003	0·03 ± 0·003	—	0·08 ± 0·01	0·11 ± 0·01
3	124–159	0·30; 0·27	0·19 ± 0·02	283–494	0·26 ± 0·03	0·06 ± 0·01	—	0·11 ± 0·01	0·17 ± 0·01
4	137–175	0·28 ± 0·01	0·21 ± 0·01	445–493	0·29 ± 0·02	0·08 ± 0·003	—	0·20; 0·14	0·25
5	94–119	0·30 ± 0·02	0·20 ± 0·003	424–499	0·25 ± 0·02	0·08; 0·08	—	0·16 ± 0·02	0·24
6	109–162	0·23; 0·25	0·17 ± 0·003	278–330	0·22 ± 0·01	—	0·06 ± 0·003	0·08 ± 0·01	0·14 ± 0·01
7	81–122	0·29 ± 0·003	0·24 ± 0·01	285–313	0·23 ± 0·01	—	0·06 ± 0·01	0·15 ± 0·01	0·21 ± 0·01
8	77–95	0·25 ± 0·01	0·18 ± 0·01	320–357	0·23 ± 0·01	—	0·04 ± 0·01	0·11 ± 0·01	0·15 ± 0·01
9	102–128	0·25 ± 0·02	0·19 ± 0·01	368–497	0·29 ± 0·02	—	0·06 ± 0·02	0·11 ± 0·02	0·17 ± 0·03
10	68–108	0·26 ± 0·01	0·16 ± 0·01	299–451	0·23 ± 0·01	—	0·08 ± 0·01	0·09 ± 0·03	0·17 ± 0·03

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Studies in Carotenogenesis

14. CAROTENOID SYNTHESIS IN THE PHOTOSYNTHETIC BACTERIUM *RHODOPSEUDOMONAS SPHEROIDES*

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(*Received* 17 *August* 1954)

The general features of the non-sulphur photosynthetic bacterium *Rhodopseudomonas spheroides* have been described by van Niel (1941). Under anaerobic conditions in the light, cultures are brown but, as noted by French (1940), on allowing them access to air their colour changes to purplish red. This is also the colour obtained in cultures grown aerobically from the start. French further observed that this colour change could not be brought about by cell-free extracts, nor could it be accelerated by the addition of oxidizing agents; furthermore, the reverse process, the conversion of red cultures into brown by changing from aerobic to anaerobic conditions, was not possible. From a study of the absorption spectra of the pigment extracts, French concluded that the colour change from brown to red was mainly due to the formation of a pigment with an absorption maximum at 550 mμ. (in carbon disulphide); he did not consider this pigment to be a carotenoid.

A more detailed study of the pigments of *R. spheroides* was later carried out by van Niel (1947) (the abbreviation *R.* will be used in this paper for *Rhodopseudomonas*). On chromatography of the pigments remaining after bacteriochlorophyll had been removed, two main carotenoid pigments, one yellow (*Y*) and one red (*R*) were obtained, neither of which could be identified with previously described carotenoids. Associated with *R* and *Y* were a number of minor components, most of which appeared to be *cis* isomers of the parent pigments. Van Niel (1947) also demonstrated that the brown → red (anaerobic → aerobic) change was characterized by the disappearance of *Y* accompanied by the appearance of an equivalent amount of *R*. This change also took place in the dark, although not to the same extent as in the light, but, in agreement with the observations of French (1940), it did not occur in cell-free extracts. Furthermore, van Niel (1947) confirmed that the reverse change red → brown could not be brought about.

The present investigation was undertaken to extend the observations of van Niel on carotenoid synthesis by *R. spheroides* in the light of present knowledge of carotenogenesis in general (Goodwin, 1953), and in another photosynthetic bacterium, *Rhodospirillum rubrum* (*Rsp. rubrum*) in particular (Goodwin & Osman, 1953, 1954).

EXPERIMENTAL

Cultures. The *R. spheroides* used throughout this investigation was a strain kindly supplied by Dr R. Y. Stanier, Department of Bacteriology, University of California, Berkeley. It was maintained in stab cultures on an agar medium. The liquid culture medium used was almost identical with that recommended by van Niel (1947): $(NH_4)_2SO_4$ (1·0 g.), $MgCl_2$ (0·5 g.), K_2HPO_4 (3·0 g.), sodium malate (3·0 g.), yeast extract (Oxoid) (1·0 g.), water 1000 ml. The final solution was adjusted to pH 6·8–7·0 with 6N-NaOH. The only difference from van Niel's medium is that 'Yeast autolysate (1·0 ml.)' (source unstated) has been replaced by the yeast extract. Inoculation of the liquid media was carried out with 1 ml. of a 5- to 6-day-old liquid culture. Anaerobic cultures were grown as previously described for *Rsp. rubrum* (Goodwin & Osman, 1953, 1954) in completely filled Pyrex bottles (100 ml.) fitted with ground-glass stoppers. Aerobic cultures (100 ml.) were grown in Erlenmeyer flasks (250 ml.). All cultures were shaken vigorously by hand twice a day. They were incubated at 30° either in the dark in a conventional electrically heated incubator or in the light in an all glass incubator illuminated on two sides with two banks of 4 × 60 W incandescent lamps (Goodwin & Osman, 1953).