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α -1:4-Glucosans

10. GLYCOGEN STRUCTURE AND *RIGOR MORTIS* IN MAMMALIAN MUSCLES*

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Numerous chemical studies have demonstrated that glycogens from various sources may differ in such characteristics as molecular weight, average chain length and in the relative number of D-glucose residues in external and internal chains (Manners, 1957). That constitutional differences in the glycogen molecule have metabolic significance has been indicated by Stetten, Katzen & Stetten (1956), who showed that in the rat and rabbit the larger molecules of muscle glycogen are more reactive, and by Stetten & Stetten (1955), who found that D-glucose residues located peripherally in the chains have a higher rate of turnover than those more centrally situated. Another aspect of the apparent physiological inhomogeneity of

mammalian-muscle glycogen has arisen during investigations on post-mortem glycolysis, and on the concomitant onset of *rigor mortis*. In general, provided that pre-slaughter stress has not seriously depleted glycogen reserves, post-mortem glycolysis will continue until pH 5.4–5.5 is reached in the muscles, at which the enzyme systems producing lactic acid are inactivated and some residual glycogen may be found. At a final pH of 5.9, 0.6–1 % of glycogen frequently remains in the psoas and diaphragm muscles of the horse (Lawrie, 1955), and 0.6 % at a final pH of 6.1 in the sternocephalic muscle of the ox (Howard & Lawrie, 1957). This could signify that the glycogens remaining in such muscles after the completion of *rigor mortis* were either inaccessible or insusceptible to attack during post-mortem glycolysis, or that they might differ in

* Part 9, Eddy, Fleming & Manners (1958).

constitution from the glycogens which predominate in the pre-rigor state. The analytical methods employed in previous papers of this series have therefore been applied to glycogens prepared from some mammalian muscles during the pre- and post-rigor phases.

MATERIALS AND METHODS

Muscles. Samples (0.5–1 kg.) from 7–8-year-old draught horses and 3–4-year-old steers were excised 15–20 min. after death. The following locations were studied: heart (left ventricular wall); diaphragm (junction of pars sternalis with pars costalis); psoas (portion of psoas major at level of sacral vertebrae); longissimus dorsi (l. dorsi; portion posterior to first, second and third lumbar vertebrae); sternocephalicus (entire muscle).

Definition of pre-rigor and post-rigor samples. General features of the time course of *rigor mortis*, and of its associated biochemical changes, in the horse (Lawrie, 1953) and the ox (Howard & Lawrie, 1956, 1957), indicate that muscles from these species, with the exception of horse heart, if sampled when at a pH greater than 6.8 (horse muscles, ox sternocephalicus) or than 6.4 (ox psoas), will then be in the pre-rigor condition provided that they eventually attain a pH of 6.1 (horse muscles, ox sternocephalicus) or 5.7 (ox psoas). Samples of glycogen characteristic of the pre-rigor condition were accordingly prepared from these muscles at a standard time of 1 hr. *post mortem*, when the pH was above the prescribed limit.

Horse heart, however, goes into *rigor mortis* within an hour of death and thus, for practical purposes, yields only post-rigor glycogen, unless special techniques are used.

Samples of glycogen characteristic of the post-rigor phase were prepared from muscle samples held in moist nitrogen at 37° for 5 hr. and thereafter stored for 17 hr. at 0°. The high temperature ensures that conversion of glycogen into lactic acid will occur with the minimum accumulation of hexose phosphate intermediates (Bendall & Davey, 1957); the elapse of 24 hr. from the time of death ensures that the ultimate pH has been attained when the muscle is processed.

pH was determined by glass electrode on samples of muscle, 0.5–1.0 g. being homogenized in 10 ml. of 5 mM-sodium iodoacetate (Bate-Smith & Bendall, 1947).

Preparations of glycogen for analysis. Small pieces cut from the intact muscle were quickly added to hot 30% (w/v) NaOH held in a boiling-water bath (1 g. of muscle to 9 ml. of alkali) and heated until homogeneous. On cooling, ethanol was added to give a final concentration of approximately 50% (v/v). The impure glycogen was centrifuged down after settling overnight and washed with a mixture of 1 vol. of ethanol with 2 vol. of 20% (w/v) NaOH. From this point the procedure followed that described by Somogyi (1957) for the preparation of glycogen free from nitrogen and phosphorus. Each glycogen sample used for structural analysis represented the bulked yields from the muscles of several animals.

Electrophoresis of glycogen. This was kindly examined by Dr D. H. Northcote, using the method of Fuller & Northcote (1956).

Analytical methods

Glucose content. Glycogen was hydrolysed in 2N-sulphuric acid (0.1%) at 100° for 2 hr. and the glucose content of the hydrolysate determined by the Somogyi (1952) reagent. Hydrolysis of glycogen under the conditions devised by Pirt & Whelan (1951) for the analysis of starch (with 1.5N-sulphuric acid) gave values 1–2% lower than the above. The slightly stronger acid is necessitated by the greater proportion of α -1:6-glucosidic linkages. A control experiment showed that no destruction of glucose occurred (reducing-power measurements).

Estimation of glycogen. The procedure of Good, Kramer & Somogyi (1933) was employed.

Enzymic degradation and iodine-staining. These analyses were carried out as described by Liddle & Manners (1957), except that, in the α -amylolysis digests, 3.7 units of enzyme/mg. of polysaccharide was used. P_M denotes apparent percentage conversion into maltose. E_{max} represents the extinction (absorption value) of the iodine-stained polysaccharide at the λ_{max} .

Glycogen value. The interaction of glycogen and concanavalin-A prepared from jack-bean meal was examined under the conditions of Cifonelli, Montgomery & Smith (1956), except that extinctions were measured on a Unicam SP. 500 spectrophotometer. The extinction given under these conditions by 1 mg. of glycogen was compared with that of a standard sample of rabbit-liver glycogen (glycogen value 1.00) kindly provided by Professor F. Smith. Amylopectins did not react under these conditions (see Calderbank, Kent, Lorber, Manners & Wright, 1960).

Periodate oxidation. The horse l. dorsi (post-rigor) muscle glycogen (about 250 mg.) was oxidized with a mixture of 20 ml. of 8% (w/v) sodium metaperiodate solution and 80 ml. of 5% (w/v) potassium chloride solution at room temperature, as described by Bell & Manners (1952). A second sample (about 100 mg.) was oxidized with sodium metaperiodate at 2° under the conditions of Manners & Archibald (1957).

The remaining glycogens (about 75 mg.) were oxidized with a suspension of potassium metaperiodate (5 ml. of sodium metaperiodate and 20 ml. of potassium chloride solutions) at room temperature. The oxidation was completed after 9–10 days, and 10 ml. samples were removed for analysis of formic acid after 10 and 12 days. A reagent control was also prepared and analysed.

RESULTS

Glycolytic criteria of horse and ox muscles

Characteristic pre- and post-rigor values for the pH and glycogen concentration in the muscles studied are given in Table 1. There is a considerable concentration of residual (or post-rigor) glycogen in all the muscles except horse heart. In horse l. dorsi and ox psoas, where the final pH is about 5.5, inactivation of glycolytic enzymes presumably accounts for the unattacked glycogen. This reason cannot automatically be adduced for horse psoas and diaphragm and ox sternocephalicus muscles, since the final pH is 5.8–6.0.

Table 1. *Characteristic pH values and glycogen contents of muscles investigated*

Values in parenthesis indicate the number of specimens examined. Initial pH and initial glycogen values were obtained 1 hr. *post mortem*.

Muscle	Initial pH	Final pH	Initial glycogen (pre-rigor) (mg./100 g.)	Residual glycogen (post-rigor) (mg./100 g.)
Horse heart	6.14±0.07 (4)	5.86±0.06 (4)	723±64 (4)	79±45 (4)
Horse psoas*	6.77±0.04 (6)	5.85±0.06 (6)	1229±190 (6)	606±143 (6)
Horse diaphragm	6.92±0.03 (6)	5.86±0.04 (6)	1883±127 (6)	1184±43 (6)
Horse l. dorsi	6.87±0.05 (6)	5.45±0.08 (6)	2216±125 (6)	1179±101 (6)
Ox psoas†	6.41±0.07 (12)	5.53±0.07 (12)	772±125 (12)	205±43 (9)
Ox sternocephalicus	7.11±0.04 (3)	6.01±0.15 (3)	1333±350 (3)	492±110 (3)

* From Lawrie (1955).

† From Howard & Lawrie (1956).

Table 2. *Properties of horse- and ox-muscle glycogens*

$E_{\max}$ represents the extinction at $\lambda_{\max}$ after iodine treatment. ECL represents the average length of the exterior chains (no. of glucose residues removed by β -amylase + 2.5).

Sample	Chain length (average)	Glycogen value	$\lambda_{\max}$ (m μ)	$E_{\max}$	P_M	β -Amylo- lysis limit (%)	ECL
Horse, l. dorsi { pre-rigor post-rigor	17	0.87	480	0.34	83	53	11-12
	17	0.82	490	0.36	78	47	10
Horse, diaphragm { pre-rigor post-rigor	17	0.81	475	0.31	80	53	11-12
	17	0.85	480	0.32	80	51	11
Horse, psoas { pre-rigor post-rigor	16-17	—	490	0.32	79	48	10-11
	17	0.76	490	0.35	76	46	10
Horse, heart, post-rigor	16-17	0.83	470	0.29	78	48	10-11
Ox, psoas { pre-rigor post-rigor	16-17	0.86	490	0.34	82	50	11
	15	—	480	0.30	79	50	10
Ox, sternocephalicus { pre-rigor post-rigor	19	0.78	475	0.27	83	51	12
	15	0.89	485	0.21	79	44	9

Characterization of purified glycogens

Glucose contents. The glycogen samples had glucose contents in the range 96-100%, and the following analyses are based on these figures.

Iodine staining. The glycogens stained deep red-brown with iodine, and the spectra showed maximum absorption in the range 470-490 m μ . The $\lambda_{\max}$ values in Table 2 represent the mid-point of a wide absorption peak covering 20-30 m μ . Mammalian-liver glycogens under similar conditions show maximum absorption over the range 450-470 m μ ($E_{\max}$ 0.2-0.3), whereas amylopectins have $\lambda_{\max}$ values of 530-540 m μ and $E_{\max}$ > 0.6.

Glycogen values. The glycogen values of the muscle glycogens varied between 0.76 and 0.89, indicating a slightly lower degree of branching than usual. There was no appreciable difference between pre- and post-rigor samples.

Average chain length. Potassium metaperiodate oxidation of horse l. dorsi (post-rigor) muscle glycogen (252 mg.) gave formic acid corresponding to chain lengths of 16.9, 16.6 and 16.6 glucose residues after 9, 10 and 11 days. In a duplicate analysis, the production of formic acid from 251 mg. of this glycogen corresponded to an average chain length of 16.6 glucose residues.

Sodium metaperiodate oxidation of the same glycogen (105 mg.) at 2° for 20 days indicated an average chain length of 16.8 glucose residues.

The remaining glycogens were analysed after 10-12 days' oxidation; the sodium hydroxide titres after 10 and 12 days were identical. The production of formic acid indicated average chain length values as follows: Pre-rigor samples: horse l. dorsi, 16.8; horse diaphragm, 16.8; horse psoas, 16.5; ox sternocephalicus, 18.6; ox psoas, 16.5. Post-rigor samples: horse diaphragm, 16.6; horse psoas, 17.1; horse heart, 16.5; ox sternocephalicus, 14.7 and 15.0 in duplicate analyses; ox psoas, 16.5. These values are considered to be accurate to within ± 0.5 glucose residue. These results, to the nearest whole number, are summarized in Table 2.

β -Amylolysis limits. Incubation of the glycogens at pH 4.6 and 35° with barley β -amylase resulted in 44-53% conversion into maltose.

α -Amylolysis limits. On incubation with purified salivary α -amylase at pH 7.0 and 35° for 24 hr., P_M values in the range 76-83 were obtained. The majority of these figures are significantly higher than those observed (approx. 75%) with mammalian-liver glycogens (average chain length 13-14), and indicate a slightly lower degree of branching.

Electrophoresis. On zone electrophoresis (glass-fibre paper) in 0.1M-sodium borate buffer, pH 9.3, at 2000V and 140 mA for 90 min., the glycogens moved 6–8 cm. as one component. The apparent homogeneity of the muscle glycogens may be contrasted with the studies of Lewis & Smith (1957), who found that a variety of glycogens gave two distinct components on electrophoresis in 2N-sodium hydroxide solution. There were small differences between the mobilities of the pre- and post-rigor samples examined, but these were not in a consistent direction.

The properties of the mammalian-muscle glycogens are summarized in Table 2.

DISCUSSION

Extraction and isolation of glycogen from animal tissues

The conventional solvents for the extraction of glycogen from animal tissues are hot water, cold dilute aqueous (5–10%) trichloroacetic acid and hot concentrated aqueous (30%) potassium or sodium hydroxide (Pflüger method). The last-named method has been criticized by many workers (e.g. Meyer & Jeanloz, 1943; Stetten, Katzen & Stetten, 1958; Bryce, Greenwood & Jones, 1958) as causing considerable degradation of the glycogen molecules. There is ample evidence to show that glycogen is degraded by hot dilute alkali (e.g. Greenwood & Manners, 1957; Stetten *et al.* 1958), but the available data indicate that hot concentrated alkali does not cause a progressive reduction in molecular weight. Thus Staudinger (1948) found that treatment of guinea-pig liver and muscle glycogen with 15 or 30% potassium hydroxide solution at 100° for 1 hr. did not alter the molecular weight (light-scattering measurements). Bryce *et al.* (1958) showed that the sedimentation constant of rabbit-liver glycogen was not progressively reduced after treatment for 3 hr. with hot 30% potassium hydroxide. Furthermore, Cori & Cori (1958) reported that the distribution of sedimentation constants of glycogen was unaffected after

treatment with hot concentrated alkali. This marked difference in the degradative action of dilute and concentrated alkali may be related to the low solubility of oxygen in the latter.

The Pflüger method has therefore been used throughout the present study since it gives a high yield of protein-free glycogen (extraction of muscle tissue with hot water or trichloroacetic acid is extremely inefficient), and does not appear to cause appreciable degradation of the constituent glucopyranosidic linkages.

Previous studies on muscle glycogen

Although the molecular structures of mammalian liver and invertebrate glycogens have been extensively studied (Abdel-Akher & Smith, 1951; Manners, 1957), considerably less attention has been paid to mammalian-muscle glycogens. This may be attributed to the relative difficulty in obtaining adequate samples of tissue, and their low glycogen contents (usually < 1%). The presence of glycogen in muscle tissue was first noted by Sanson (1857), but some 80 years elapsed before the first chemical investigations were reported (Young, 1937; Bell, 1937). These indicated that rabbit-muscle and liver glycogens had similar properties, and that a sample of horse-muscle glycogen (pooled tissues) had an average chain length of 11–12 by methylation. A summary of the results of other end-group assays of muscle glycogens is given in Table 3. These differ markedly from those reported in Table 2. Furthermore, the β -amylolysis limits ($42 \pm 4\%$) are significantly lower than those of our present analyses.

Molecular structure of pre- and post-rigor muscle glycogens

Although glycogens have been isolated with average chain length values ranging from 6 to 18, the majority of samples have values of 12 ± 2 (Manners, 1957). The most noticeable feature of the mammalian-muscle samples as a whole (and, in particular, of the sternocephalicus pre-rigor glycogen) is

Table 3. *Properties of some mammalian-muscle glycogens*

Methods of assay are indicated by: (m) methylation; (p) periodate oxidation; (e) enzymic.

Sample	Chain length (average)	β -Amylolysis limit (%)	References
Horse	11–12 (m, p)	42	Bell (1937); Bell & Manners (1952)
Human	11 (p)	—	Halsall, Hirst & Jones (1947)
	12 (p)	41	Bell & Manners (1952)
	11 (p)	40	Liddle & Manners (1957)
Rabbit	13 (p)	—	Halsall <i>et al.</i> (1947)
	11–13 (m, p)	45	Bell (1948a); Bell & Manners (1952)
	11 (p)	39	Liddle & Manners (1957)
	15 (e)	—	Illingworth, Larner & Cori (1952)
	13 (p)	46	Manners & Wright (unpublished work)

therefore the significantly lower degree of branching, as determined by potassium periodate oxidation. This method is well established in carbohydrate chemistry for the analysis of non-reducing polysaccharides, and the possibility of any inherent error is reduced by the close agreement reported by other workers between methylation, enzymic and periodate-oxidation analyses of the same samples.

The potassium periodate-oxidation results in Table 2 are supported by the glycogen and P_M values.

The interaction of concanavalin-A and glycogen was first examined in detail by Cifonelli *et al.* (1956), who noted that the glycogen values of various glycogens were increased by β -amylolysis. They concluded that the reaction mainly involved the interior chains. However, it seems probable that other factors are also significant since measurement of the glycogen values of various human glycogens (from cases of glycogen-storage disease) have indicated an approximate relationship with the degree of branching (Calderbank *et al.* 1960). The glycogen values of the horse-muscle glycogens are significantly lower than those of 12–14 unit glycogens and are therefore consistent with the lower degree of branching and the relatively longer exterior chains.

The extent of degradation by α -amylase may, to a first approximation, be related to the degree of branching in the polysaccharide, since the inter-chain linkages and certain adjacent α -1:4-glucosidic linkages are resistant to enzyme action (Whelan & Bines, 1955). The observed trend of P_M values for the muscle glycogens is fully in accord with the significantly lower degree of branching, as the values are intermediate between those of a normal glycogen (average chain length 14, P_M 75) and an amylopectin (average chain length 22, P_M 89).

The iodine-staining power of mammalian-muscle glycogen is appreciably greater than that of liver or invertebrate glycogens (Manners, 1957). However, this feature of muscle glycogens, which has been noted by other workers (Young, 1937; Bell, 1948*b*), is not apparently directly related to the molecular structure. The iodine-staining data in Table 2 serve to characterize the muscle polysaccharides as 'glycogens' rather than 'amylopectins'. For example, the amylopectin component of malted barley starch has an average chain length of only 18 (Aspinall, Hirst & McArthur, 1955) but has λ_{max} 540 m μ and E_{max} 0.77 (unpublished data).

The results recorded in Tables 1 and 2 show that the glycogens remaining after the development of *rigor mortis* in horse muscles, and in ox psoas, do not differ significantly in molecular structure from the initial pre-rigor polysaccharides. On the other hand, in ox sternocephalicus muscle there is a significant shortening of the outer chains, from 12

to 9 glucose residues, during post-mortem glycolysis.

Biochemical and physiological implications

The biochemically significant quantity of glycogen which remains unattacked by the glycolysis system at a final pH of 6.0 in ox-sternocephalicus muscle appears to represent a molecular species having a shorter average external chain length than that initially present. In this instance, the presence of residual glycogen at a high final pH may be rationalized at the chemical level. Although in horse psoas and diaphragm residual glycogen at a high final pH is found, this does not apparently differ from the pre-rigor glycogens in these muscles. The pre- and post-rigor glycogens in these muscles need not be physiologically equivalent, however, since fractionation of a given glycogen sample can yield species of different molecular weight but similar branching characteristics (Stetten & Stetten, 1958). This may have physiological importance as glycogen of high molecular weight appears to be preferentially degraded *in vivo* and *in vitro* by muscle phosphorylase (Stetten *et al.* 1958; Larnier, Ray & Crandall, 1956) and to exchange its glucose units more vigorously with the environment than that of low molecular weight (Stetten *et al.* 1956). In general, post-rigor glycogens would thus be expected to have a low molecular weight and a more compact molecular-weight distribution. If this is so, there would seem to be no relationship between the degree of branching and molecular size in the horse-muscle glycogens examined.

Dr W. A. J. Bryce has kindly determined the sedimentation constants of the l. dorsi muscle glycogens; the polysaccharides were polymolecular, values of 96 and 80×10^{-13} c.g.s. unit being obtained for the major components of the pre- and post-rigor samples, corresponding to molecular weights of 4.4 and 3.7×10^6 [assuming a diffusion constant of 1.50×10^{-7} (Bell, Gutfreund, Cecil & Ogston, 1948)]. These results are qualitatively in agreement with the above suggestion.

In view of the similar average chain length values of the pre- and post-rigor glycogens from horse muscle and ox psoas, and assuming that the major pathway of glycogen breakdown involves phosphorylase, it is clear that the relative activity of phosphorylase and amylo-1:6-glucosidase is largely unaltered during post-mortem glycolysis. This may be contrasted with other metabolic conditions, e.g. glycogenesis, in which an alteration in activity is manifested by the presence of glycogen with relatively short exterior chains and a higher degree of branching (Manners, 1957). On the other hand, with ox-sternocephalicus muscle, the significant shortening of the outer chains from 12 to 9 glucose residues may indicate either a partial inactivation

of the debranching enzyme, or *in vivo* heterogeneity with respect to branching characteristics.

The concentration of glycogen in muscle has hitherto been recognized as an aspect of functional specialization, since it tends to be high in so-called white muscles, which derive energy for short bursts of activity by anaerobic glycolysis, and low in so-called red muscles, which are equipped for sustained energy production by respiration. That this relatively simple concept might be inadequate was indicated by the work of Bloom, Lewis, Schumpert & Shen (1950) and Kits van Heyningen & Kemp (1955). It now appears that distinct molecular species of glycogen, exhibiting different susceptibilities to glycogen breakdown, may occur in a given muscle. This presumably reflects some further aspect of functional specialization.

SUMMARY

1. The molecular structure of glycogen isolated by the Pflüger method from some mammalian muscles during the pre- and post-phases of *rigor mortis* has been examined by chemical and enzymic methods.

2. Glycogen from pre- and post-rigor horse-muscle tissues has an average chain length of approximately 17 glucose residues. This indicates a significantly lower degree of branching than is present in other samples of mammalian and invertebrate glycogen.

3. The glycogen isolated from ox-sternocephalicus muscle after the onset of *rigor mortis* has significantly shorter exterior chains than that isolated from the pre-rigor muscle. This could indicate either partial inactivation of the enzyme system catalysing the glycogen $\rightleftharpoons$ glucose 1-phosphate interconversion or a true heterogeneity of the glycogen in this muscle *in vivo*.

4. The chemical and physiological significance of these observations is discussed.

5. The possibility of the degradation of glycogen during alkaline extraction is considered.

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