ELSEVIER

Carbohydrate Research 261 (1994) 79–89

CARBOHYDRATE RESEARCH

Glycogen size analysis by mini-gradients

Robert Geddes *, John C.K. Soo Ping Chow

Biochemistry SBS, The University of Auckland, P.B. 92019, Auckland New Zealand

(Received September 24th, 1993; accepted February 28th, 1994)

Abstract

The separation of low (cytosolic) and high (lysosomal) molecular weight glycogen on small induced citrate gradients has been compared with the conventional sucrose gradient separation. The citrate method is shown to be vastly more sensitive and can be used with as little as 0.1 mg of glycogen, compared with 8 mg which is the normal loading on a sucrose gradient. The citrate method has been tested with fractionated and degraded glycogen samples and found to give satisfactory results, although with higher degrees of error, when compared with the conventional method. The success of the citrate method arises from the previously undescribed aggregation of high molecular weight glycogen in the presence of 1.09 M sodium citrate, which has been measured by laser dynamic light-scattering. This phenomenon allows satisfactory separation of the variously sized glycogens to be achieved with centrifugal forces of ca. one-tenth of those used in sucrose density gradient analysis, thereby considerably simplifying the separation methodology.

1. Introduction

Glycogen is a highly polydisperse polysaccharide of high molecular weight ranging between approximately 1×10^6 and 1×10^9 [1–3]. Molecular weight distributions of glycogens have been determined for many species and tissues, mainly by sucrose density gradient analysis [4–7]. When glycogen is extracted from tissue by nondegradative procedures [8–10], the molecular weight distributions show a complex relationship with glycogen metabolism [11–13], providing information which is not otherwise easily accessible. A significant proportion of cellular

* Corresponding author.

SSDI 0008-6215(94)00089-X

glycogen is found to be intimately associated with the lysosomal compartment [11–15], and there is a complex interrelationship between the metabolism of this lysosomal glycogen and its cytosolic counterpart [11–17]. Since the lysosomal glycogen is composed mostly of high molecular weight particles, it has proved convenient to separate, and analyse the glycogen content of, the lysosome and the cytosol by density gradient analysis [14,15].

Unfortunately, the sucrose density gradient method has a number of drawbacks which appear to have inhibited its adoption by international laboratories. It requires relatively sophisticated experimental and analysis methodology, and requires relatively large amounts of sample (> 2 mg of glycogen; 8 mg normally being used for good sensitivity). The latter problem arises from the necessity of utilising a glycogen-specific, but relatively insensitive, chemical analysis system (Krisman [18]), because of the large amounts of sucrose present in all the gradient fractions. The Krisman methodology makes use of the resonance characteristics of complex iodine ions inside a helix of α-(1 → 4)-linked glucose residues [9]. Glycogens isolated from small amounts of muscle [20,21] or yolk sac [10,22], for example, are typically in the microgram range and well below the sensitivity of the Krisman method, especially when the isolated glycogens are fractionated. There was therefore a demonstrable need for a new technique with increased sensitivity and experimental simplicity.

2. Experimental

Rat liver glycogen was extracted as previously described [14]. Alkali-degraded glycogen was prepared by dissolving isolated glycogen, at a concentration of ca. 15 mg/mL, in aq 60% KOH and maintaining this at ca. 100°C for 3 h under anaerobic conditions. The degraded glycogen was then recovered by ethanol precipitation [23]. Reduced glycogen was prepared by treatment with 2-mercaptoethanol followed by iodoacetamide, as described previously [5]. Fractionation of glycogen solutions on sucrose density gradients was performed by standard methodology [1,14] and molecular weights were assigned to fractions as measured previously [1,4,5] (see Table 1). "High" and "low" molecular weight samples of glycogen were made by combining sucrose fractions 1–10 and 11–20, respectively. Dynamic laser light-scattering measurements were made as previously described [1,5].

Citrate "mini"-gradients.—Fractionation of glycogen was achieved using 1.09 M sodium citrate (BDH) in a 3-mL disposable syringe (Monoject, Sherwood Medical), sealed by a simple silicone plug (Fig. 1). The silicone plug was made by cutting an appropriately sized plastic pipette tip and filling it with a silicone sealant (Silastic Marine Sealant, Dow Corning). A 0.1-mL sample of glycogen solution, containing a minimum of 0.1 mg of glycogen, was first taken up into the syringe via its needle. This was followed by 1.5 mL of 1.09 M sodium citrate taken up by slow gentle suction to minimise mixing. 1.09 M Sodium citrate was chosen initially because of its similarity in density and viscosity to the average sucrose concentration in a

Table 1
Molecular weights [a] (ref 4) were assigned to fraction numbers [Fr. no. (suc)] of normal glycogen separated on sucrose density gradients [Mol wt (suc)] and the cumulative glycogen contents [Cum gly (%) (suc)] calculated. The corresponding cumulative values obtained on citrate gradients were then aligned with the sucrose values and, by comparison, imputed molecular weights assigned to the citrate fractions [Imput mol wt (cit)]

Fr. no. (suc)	Fr. no. (cit)	Mol wt [a] (suc)	Cum gly (%) (suc)	Cum gly (%) (cit)	Imput mol wt [a] (cit)
1		1360	2.9		
2	1	1010	6.8	6.3	1010
3	2	858	11.1	11.0	860
4		760	15.6		
	3			16.6	730
5		670	20.2		
	4			22.9	600
6		585	24.6		
7		528	29.1		
8	5	443	33.6	33.6	440
9		380	38.6		
10		318	44.2		
11	6	245	51.3	51.9	250
12		188	59.7		
13		139	69.3		
14		93	78.5		
	7			79.1	80
15		61	86.2		
16		40	91.7		
17		29	95.4		
18		23	97.7		
19	8	10	99.3	100.1	10

[a] All molecular weight values are $\times 10^{-6}$.

sucrose gradient (Table 2). The needle was then discarded and replaced by a silicone plug. The syringe was then centrifuged at 3000*g* (ca. 4500 rpm in an average-sized bench centrifuge) for 1 h. The plug was then discarded and eight 0.2-mL fractions were collected through a fresh needle.

Table 2
Densities and relative viscosities of aqueous solutions of sucrose and citrate

Solution	Concn (% w/w)	Density (g/mL)	Relative viscosity
Sucrose	30	1.127	3.18
Sucrose	40	1.177	6.15
Sucrose	50	1.230	15.40
Citrate	32	1.248	6.53

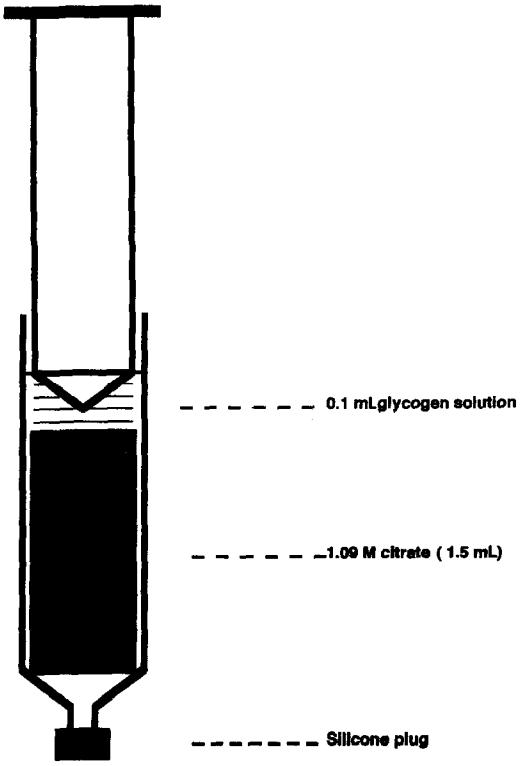


Fig. 1. Diagram of the loading of the disposable syringe utilised in the simple citrate separation method.

Notes: (*a*) Any disposable syringe is potentially suitable, but each type requires separate initial calibration against a sucrose gradient. (See Results and Discussion, and Table 1.) (*b*) 3000*g* was chosen empirically as the best compromise between high centrifugal force (promoting better separation) or leakage through the plug. (*c*) For further simplicity of operation, there is no absolute need to collect 8 samples. Two samples could be taken (say, a first one of 1.2 mL and a second of 0.4 mL) as measures of high and low molecular weight glycogen.

Glycogen concentrations in solutions from gradient fractionations were determined either by the method of Krisman [18] (in the presence of sucrose) or by the phenol–sulfuric acid method [24] (in the presence of citrate).

3. Results and discussion

After experimenting with various noncarbohydrate potential gradient materials, sodium citrate was selected because of (*a*) its noninterference with the highly sensitive, but nonspecific, Hodge and Hofrieter [24] sugar assay; (*b*) its similarity in density and relative viscosity to sucrose solutions of the average concentration used in the density gradient separation of glycogen [4] (Table 1); and (*c*) its availability.

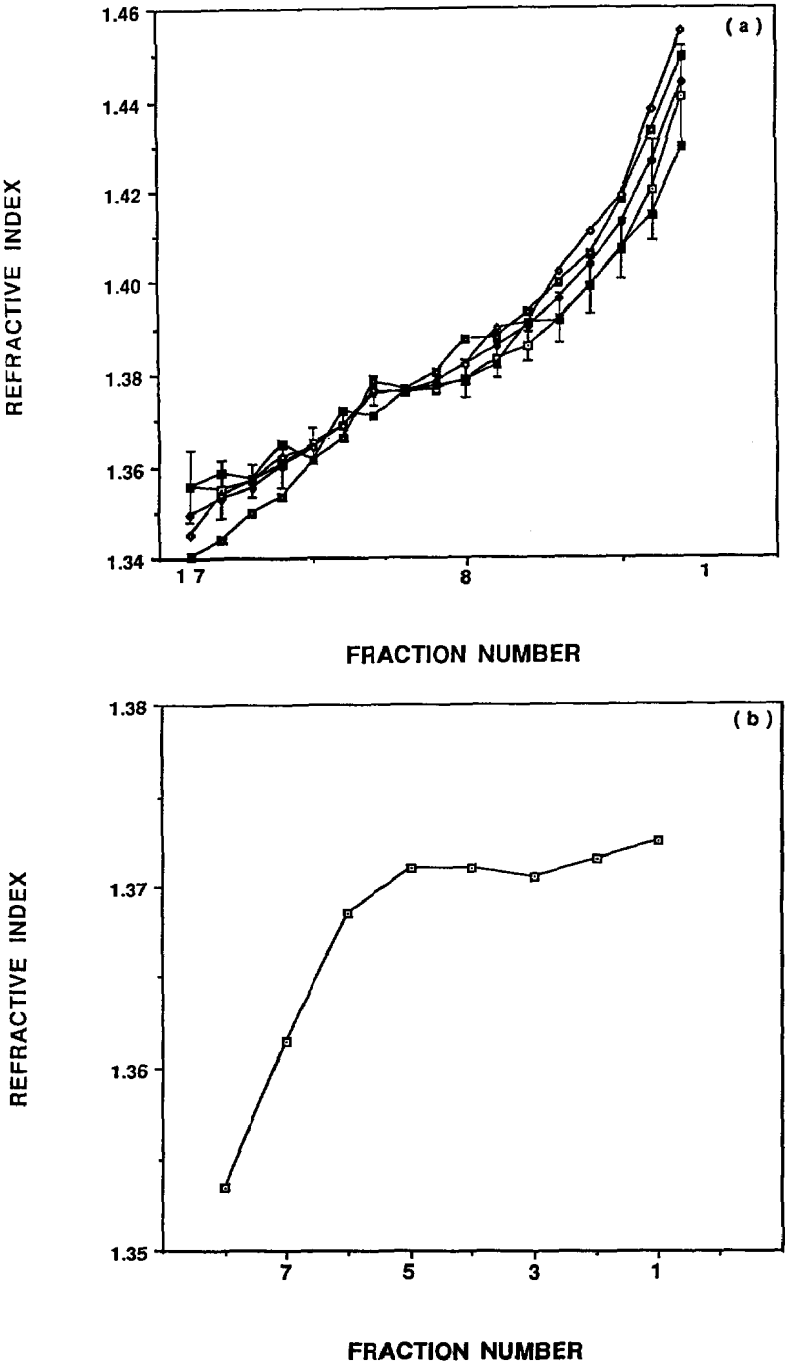
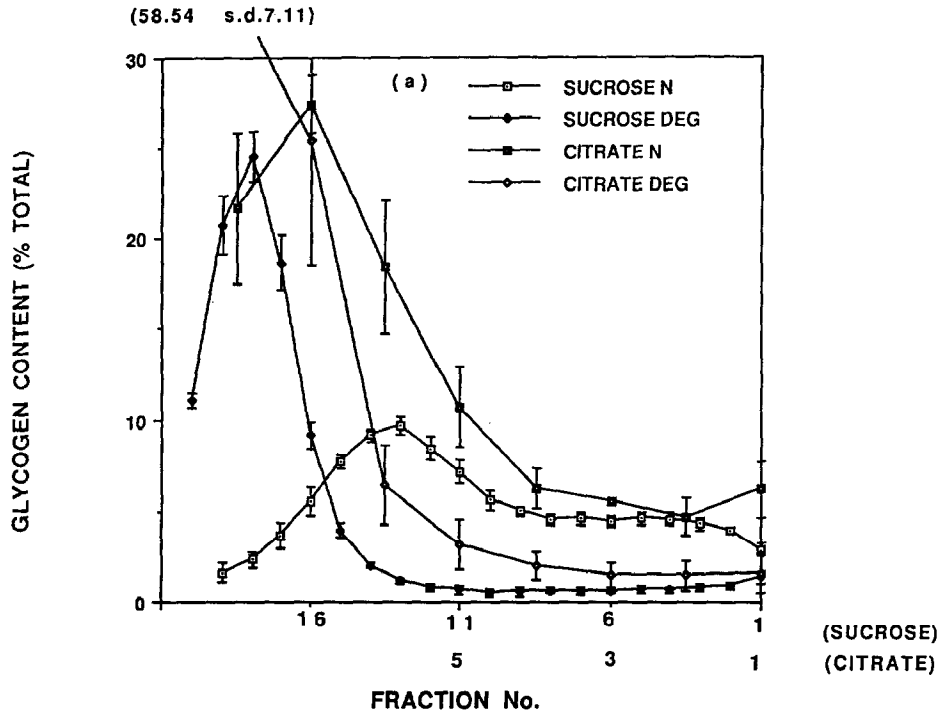
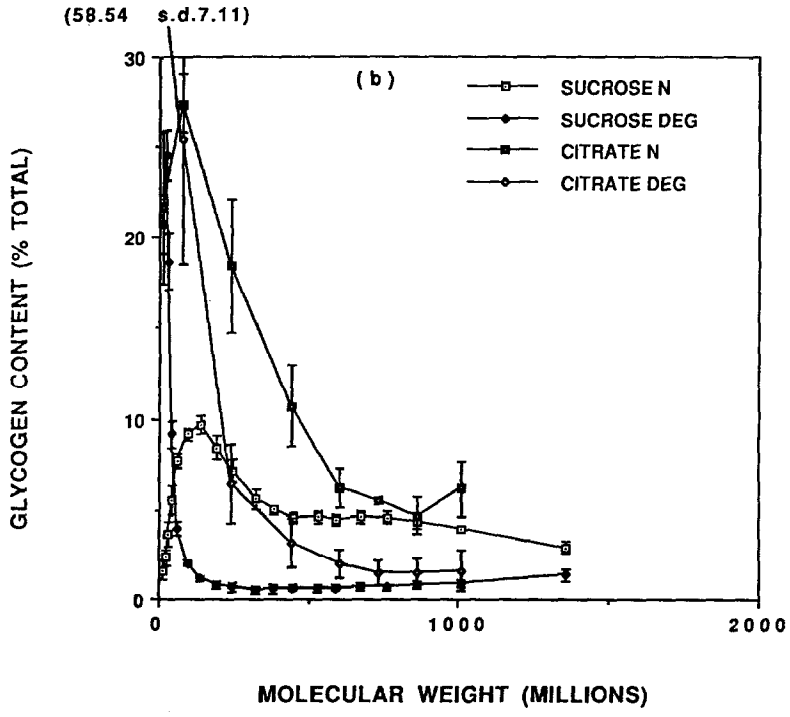


Fig. 2. Density profiles (as measured by refractive indices) of sucrose density gradients (a) and the new citrate method (b), *post* centrifugation. Note that the sucrose results were obtained without the addition of the glycogen sample; hence, the absence of fractions above 17. The sucrose results were obtained from 5 separate gradients and typical standard deviations are shown. The citrate result is a single representative sample.


(58.54 s.d.7.11)
(a)
SUCROSE N
SUCROSE DEG
CITRATE N
CITRATE DEG
GLYCOGEN CONTENT (% TOTAL)
30
20
10
0
16
11
6
1
5
3
1
(SUCROSE)
(CITRATE)
FRACTION No.


(58.54 s.d.7.11)
(b)
SUCROSE N
SUCROSE DEG
CITRATE N
CITRATE DEG
GLYCOGEN CONTENT (% TOTAL)
30
20
10
0
0
1000
2000
MOLECULAR WEIGHT (MILLIONS)

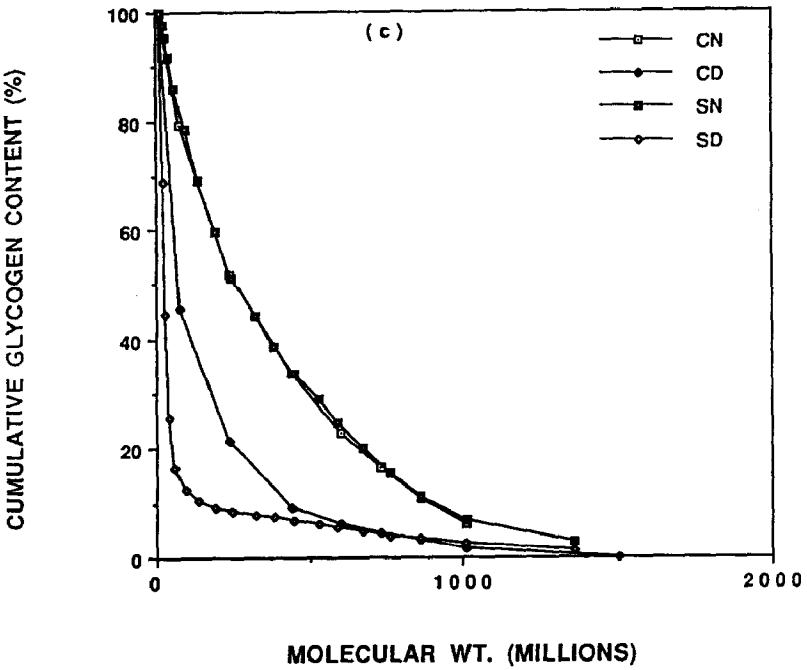


Fig. 3. Separation of whole (N) and alkali-degraded (DEG) glycogen by conventional sucrose density gradient centrifugation (SUCROSE) or by the new citrate method (CITRATE). The results are presented in three ways for comparison: (a) glycogen content (%) vs. fraction number; (b) glycogen content (%) vs. molecular weight; (c) cumulative glycogen content (as measured from fraction 1 (high molecular weight) vs. molecular weight. Obviously, because of the huge differences in the molecular weight ranges covered by the different fractions in the two different methods, only (c) provides a direct comparison between them.

To eliminate the difficulty of preparing, and ensuring the reproducibility of, gradients of small volume, citrate was used throughout the experiments as a uniform 32% (1.09 M) solution. For ease of handling, samples were separated in disposable syringes, as described in the Experimental section. Fig. 2 shows the density profiles of the sucrose and citrate media, post centrifugation. The citrate shows the expected steep increase from the meniscus, declining into a small induced gradient further down the syringe. When the two gradients were used to separate normal glycogen and the same glycogen highly degraded by alkali treatment [4,23] (Fig. 3a), they both gave satisfactory results, at least qualitatively. Since the two gradient centrifugation techniques utilise centrifugal fields that are grossly different (sucrose gradients are typically subjected to ca. 30000g for 30 min [1,4,5,14], and citrate to 3000g for 60 min), this surprising observation arises from some previously unknown characteristic of glycogen in the presence of citrate—see below. However, a quantitative comparison between the two separations is required. In an attempt to provide this comparison, the cumulative glycogen content, from high (fraction 1) to low molecular weight, was tabulated for normal, i.e.,

untreated, glycogen by making use of the well-established relationship between fraction number and molecular weight for sucrose gradients [4] (Table 1). These cumulative contents were then used to assign approximate molecular weights to the fractions of normal glycogen separated on citrate. This enabled the percentage glycogen contents to be plotted against molecular size (Fig. 3b). However, since the molecular weights of the different fractions in the two gradients are substantially different, comparability of the two sets of results is not easily achieved by plots of this type, although, when one type of gradient fractionation is adopted, these plots provide the most accessible information. Comparability of the effectiveness of the two gradient separations is best achieved by plots of the cumulative glycogen content against molecular weight (Fig. 3c). When this is done, there is reasonable similarity in the two sets of results for the alkali-degraded glycogen, although there is a marked discrepancy in the region below 500×10^6.

To test the potential of the citrate gradient separation, further fresh samples of normal glycogens and their products, with altered molecular weight distributions following different experimental treatments, were tested in a similar manner. Firstly, a whole glycogen sample was fractionated conventionally on a sucrose gradient and fractions 1–9 (high) and 10–20 (low) were pooled. The "high" and "low" glycogens were then re-separated on both sucrose and citrate gradients, and the results are shown in Fig. 4. Secondly, since high molecular weight glycogen is built up from glycogen spheres (β-particles) covalently linked to a protein back-

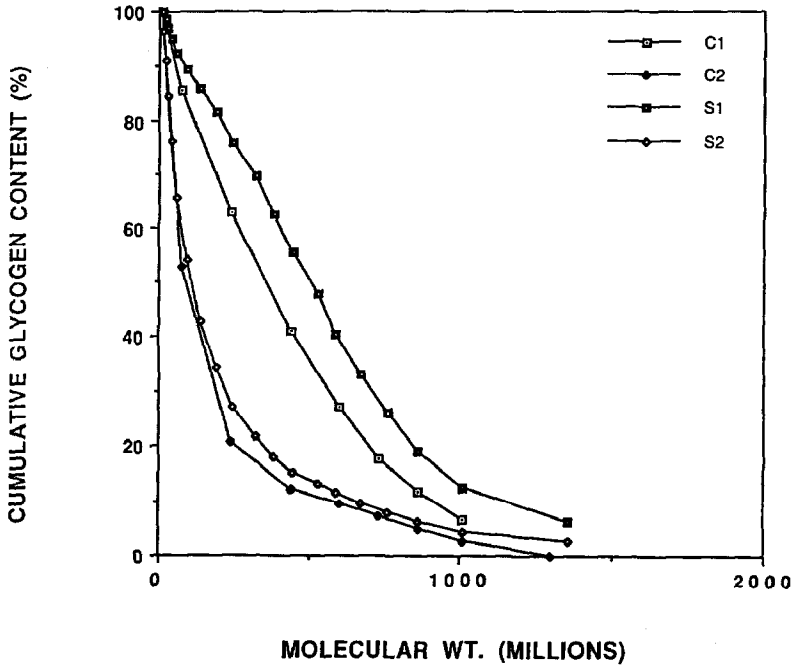


Fig. 4. A comparison [as in Fig. 3(c)] of the separation of two very broad glycogen fractions of "high" (1) and "low" (2) molecular weight (see Experimental section) on sucrose (S) and citrate (C).

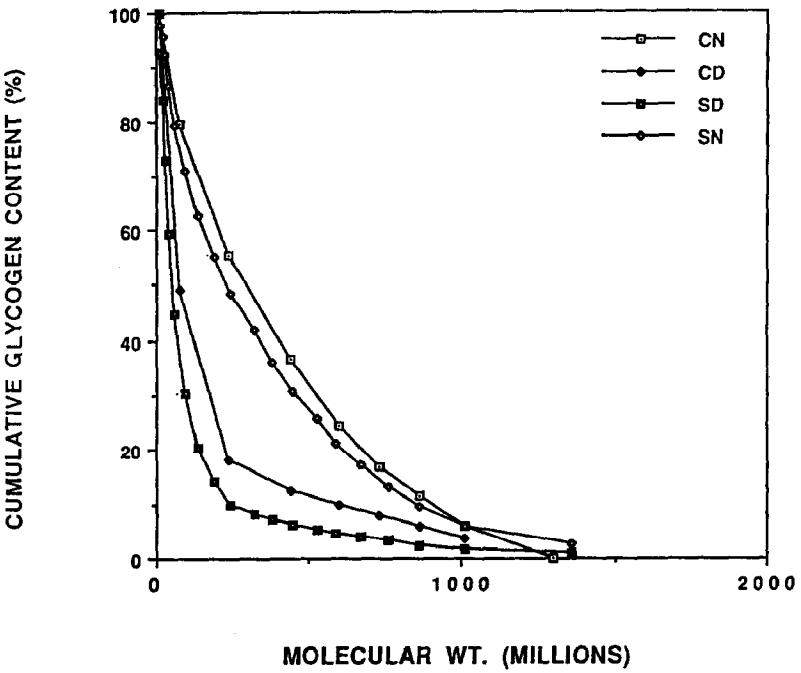


Fig. 5. A comparison [as in Fig. 3(c)] of the separation of whole (N) or disulfide-modified (D) glycogens (see Experimental section) on sucrose (S) or citrate (C).

bone [5] linked to other backbones by disulfide bonds [5,25], another glycogen sample was degraded by treatment with 2-mercaptoethanol followed by iodoacetamide. These samples were then tested on both gradients (Fig. 5).

Table 3
Average radii of samples, in various solvents, of unfractionated (N) glycogen or of two broadly fractionated samples of high or low molecular weight as determined by dynamic laser light-scattering [a]

Sample	Solvent	Radius (nm)
N	Water	68
Low	Water	57
High	Water	68
N	0.1 M Tris	61
Low	0.1 M Tris	53 (49 *)
High	0.1 M Tris	82
N	1.09 M Citrate	154
Low	1.09 M Citrate	64
High	1.09 M Citrate	205
Reduced	Water	69

[a] All samples were at a concentration of 5 mg/mL, except for that marked * which was at 10 mg/mL. The Tris buffer was of pH 7.5. The sample labelled reduced was unfractionated glycogen treated with 2-mercaptoethanol then iodoacetamide. Note that, since all samples are highly polydisperse, the radii, having been derived from the z-average diffusion coefficients [5], can only be used as indicators of gross structural differences.

It is concluded that the new citrate technique described here can provide an easy, rapid method for monitoring changes in the low (cytosolic) and high (lysosomal) molecular weight [11–17] species of glycogen. However, it lacks some of the precision of the sucrose density gradient method, and molecular weights assigned to citrate fractions can only be taken as approximate values.

As mentioned above, the reason for the success of the citrate separation, which utilises only ca. 20% of the centrifugal force of the sucrose gradient method, was not immediately obvious. Clearly, some aggregative phenomenon takes place. However, this aggregation in the presence of citrate must be fairly specific for high molecular weight glycogen as indicated by the results shown in Figs. 3–5. Consequently, the average radii of a number of glycogen samples in citrate, water, and buffer were measured by laser dynamic light-scattering [5], and the results are shown in Table 3. It is clear from Table 3 that 1.09 M citrate does cause high molecular weight glycogen to aggregate while having little or no effect upon the corresponding low molecular weight species. While investigations into the precise reasons for this size-specific aggregation are continuing in this laboratory, it seems possible that it could be related to the relatively high protein content (on a weight-to-weight basis) of high, in comparison to low, molecular weight glycogen [4,16,26]. However, in Fig. 3c, protein-free glycogen (*post*-treatment with aq 30% alkali at 100°C) behaves differently on sucrose and citrate gradients with extra medium-sized material appearing on the latter. This may indicate that factors other than the protein content contribute to the aggregation process.

Nonetheless, the simple citrate method described above should enable any laboratory investigating any aspect of glycogen, or related metabolism, to monitor easily the two pools of cellular glycogen, the cytosolic and the lysosomal [16], during the course of their experiments.

It should be of particular value in studies of the biochemistry and physiology of exercise where, although the role of the lysosome in skeletal glycogen metabolism is well established [7,13,15,21], these studies have been largely confined to one laboratory and require to be extended.

References

[1] R. Geddes, J.D. Harvey, and P.R. Wills, *Biochem. J.*, 163 (1977) 201–209.
[2] S.A. Orrell and E. Bueding, *J. Biol. Chem.*, 239 (1964) 4021–4026.
[3] A.J. Parodi, *Arch. Biochem. Biophys.*, 120 (1967) 547–552.
[4] R. Geddes, in G.O. Aspinall (Ed.), *The Polysaccharides*, Vol. 3, Academic, New York, 1985, pp. 284–336.
[5] R. Geddes, J.D. Harvey, and P.R. Wills, *Eur. J. Biochem.*, 81 (1977) 465–472.
[6] J. Mordoh, C.R. Krisman, and L.F. Leloir, *Arch. Biochem. Biophys.*, 113 (1966) 265–272.
[7] P.C. Calder and R. Geddes, *Int. J. Biochem.*, 24 (1992) 71–77.
[8] R. Geddes, *Int. J. Biochem.*, 2 (1971) 657–660.
[9] P.C. Calder and R. Geddes, *Carbohydr. Res.*, 118 (1983) 233–238.
[10] P.J. Hartfield, K.E. Williams, R. Geddes, and J.B. Lloyd, *Plancenta*, 10 (1989) 45–54.
[11] R. Geddes and K.B. Rapson, *FEBS Lett.*, 31 (1973) 324–326.
[12] R. Geddes and G.C. Stratton, *Carbohydr. Res.*, 57 (1977) 291–299.

[13] P.C. Calder and R. Geddes, *Int. J. Biochem.*, 22 (1990) 847–856.
[14] R. Geddes and G.C. Stratton, *Biochem. J.*, 163 (1977) 201–209.
[15] P.C. Calder, R. Geddes, and H. Leech, *Glyconjugate J.*, 3 (1986) 331–338.
[16] R. Geddes, *Biosci. Rep.*, 6 (1986) 415–428.
[17] D.H. Brown, L.M. Waindle, and B.I. Brown, *J. Biol. Chem.*, 253 (1978) 5005–5011.
[18] C.A. Krisman, *Anal. Biochem.*, 4 (1962) 17–23.
[19] P.Drochmans, *La Morphologie du Glycogene*, Editions Arscia S.A., Brussels, 1966.
[20] P.C. Calder and R. Geddes, *Carbohydr. Res.*, 135 (1985) 249–256.
[21] P.C. Calder and R. Geddes, *Carbohydr. Res.*, 148 (1986) 173–177.
[22] P.J. Hartfield, J.B. Lloyd, K.E. Williams, and R. Geddes, *Proc. Physiol. Soc.*, C29 (1988) 58P.
[23] H.M. Bullivant, R. Geddes, and P.R. Wills, *Biochem. Int.*, 6 (1983) 497–506.
[24] J.E. Hodge and B.T. Hofreiter, *Methods Carbohydr. Chem.*, 2 (1962) 388–389.
[25] R. Geddes, P. Jeyarathan, and J.A. Taylor, *Carbohydr. Res.*, 227 (1992) 339–349.
[26] N.P. Chee and R. Geddes, *FEBS Lett.*, 73 (1977) 164–166.