Biochem. J. (1977) **164**, 179–183
Printed in Great Britain

Heterogeneity of Proteoglycans in Developing Chick Limb Cartilage

By NAGASWAMI S. VASAN and JAMES W. LASH
Department of Anatomy, School of Medicine/G3, University of Pennsylvania, Philadelphia, PA 19104, *U.S.A.*

(*Received* 26 *October* 1976)

Proteoglycan heterogeneity was studied during the maturation of embryonic-chick limb cartilage *in vivo*. The results suggest that during the differentiation of limb-bud cartilage the aggregated forms of proteoglycans increase between stages 24 and 35, whereas the non-aggregated or monomeric forms decrease. Only one link protein is found in stage-24 limb buds, whereas two are present at stage 35. Evidence suggests that the synthesis of link proteins may be a regulatory factor in limb chondrogenesis.

Changes in the types of proteoglycans synthesized during development have been reported by Kleine (1973, 1974), Goetinck *et al.* (1974) and Levitt & Dorfman (1974). These studies were based on changes taking place in embryonic-limb primordia or cells while in culture. In the present report we have studied the heterogeneity of proteoglycans and the changes that occur during the development of the chick limb-bud cartilages *in vivo*.

A method has been described by Sajdera & Hascall (1969) which makes it possible to extract proteoglycans (protein-bound acid mucopolysaccarides) from cartilage matrix by using mild dissociative solvents. By using dissociative solvents such as guanidinium chloride, $CaCl_2$ or $MgCl_2$, it has been shown that cartilage from a variety of vertebrates (bird, ox, dog, mouse, pig) contain proteoglycans as both aggregated and non-aggregated molecules (Hardingham *et al.*, 1972; Hardingham & Muir, 1973; Bjelle *et al.*, 1974; Hascall & Heinegård, 1974; Shipp & Bowness, 1975; Strider *et al.*, 1975; Hascall *et al.*, 1976). These solvents extract 85–90% of the uronic acid- and hexosamine-containing materials (Sajdera *et al.*, 1970; Bjelle *et al.*, 1974; Hascall *et al.*, 1976). The chemical and metabolic heterogeneity of cartilage proteoglycans extracted with dissociative solvent has been reported previously (Muir & Jacobs, 1967; Campo *et al.*, 1972; Kleine, 1973, 1974). These proteoglycans are in a more native state than those obtained by using other extraction methods.

Aggregation of the proteoglycans requires the interaction between three types of molecules, the proteoglycan monomer, the smaller link proteins and hyaluronic acid (Hascall & Heinegård, 1975). The results of the present study suggest that during the maturation of limb cartilage the aggregated forms of proteoglycan increase, whereas the non-aggregated or monomeric forms decrease. Thus one of the regulatory factors in limb chondrogenesis may be the synthesis of an aggregation factor, the hyaluronic acid-binding region of the proteoglycan molecule, or the link proteins.

Materials and Methods

Limb buds of stages 18, 24 and 35 were obtained from White-Leghorn-chick embryos, and the stages of development were determined with the staging series of Hamburger & Hamilton (1951). Precartilaginous stage-18 limb buds were cut free of the embryos and analysed without further dissection. The cartilages of the later stages were cleaned free of adhering tissues, weighed and cut into small (1 mm^3) pieces. The proteoglycans were labelled by incubating them in F12X medium (Marzullo & Lash, 1970) containing 2μCi of carrier-free $Na_2{}^{35}SO_4$ (Amersham/Searle, Arlington Heights, IL, U.S.A.)/ml. After 12h incubation in a humidified incubator in air/CO_2 (19:1), the tissues were rinsed several times with Simms' balanced salt solution (Simms & Sanders, 1942).

Proteoglycans were extracted with 0.4M-guanidinium chloride (Ultra Pure grade, Schwarz/Mann, Orangeburg, NY, U.S.A.), buffered at pH5.8 with 0.05M-sodium acetate (Hascall & Heinegård, 1975). Some samples of tissues were extracted in guanidinium chloride/acetate solvent also containing 0.01 M-EDTA (disodium salt; Sigma Chemical Co., St. Louis, MO, U.S.A.), 0.1M-6-aminohexanoic acid (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) and 0.005M-benzamidine hydrochloride (Eastman Kodak, Rochester, NY, U.S.A.) to inhibit proteolysis (Oegema *et al.*, 1975). The tissues were extracted in the guanidinium chloride/sodium acetate solvent by gentle agitation on a rotary shaker for 36h at 4°C. There was no detectable difference in the extraction products obtained with or without the use of proteolytic inhibitors if the extracts were analysed shortly

after extraction. The products of the 0.4M-guanidinium chloride extraction constitute Fraction 1, which are obtained under 'associative' conditions, as described by Heinegård (1972). At this concentration of guanidinium chloride the proteoglycans remain associated and the easily diffusible proteoglycan monomer is primarily extracted along with some aggregates. The tissues were subjected to a further extraction as above, except that guanidinium chloride concentration was raised to 4.0M (the 'dissociative' conditions of Heinegård, 1972). Under these conditions, the proteoglycan aggregates remaining in the cartilage matrix are dissociated and go into solution as monomeric proteoglycans. This second extract, which represents primarily proteoglycan which was in the aggregated (or associated) form, is termed Fraction 2. Both fractions were dialysed against 0.04M-Na_2SO_4 for 24h at 4°C, followed by 48h against cold running tap water (4–8°C), and then 24h against water (4°C). This procedure causes the proteoglycans in the 4.0M-guanidinium chloride extract to re-aggregate. The fractions were centrifuged at 20850*g* for 30min and the supernatants were freeze-dried. The resulting material was dissolved in 0.5ml of deionized water. One portion (0.1ml) was used for uronic acid assay (Bitter & Muir, 1962), and another 0.1ml was used for determining incorporation of [^{35}S]sulphate in an Intertechnique liquid-scintillation counter. The samples were dissolved in toluene containing 6.0g of PPO/litre (2,5-diphenyloxazole), 0.3g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre (Packard Instruments, Downers Grove, IL, U.S.A.) and 5% (v/v) Biosolve (Beckman Instruments, Fullerton, CA, U.S.A.).

Gel chromatography was performed on columns (0.4cm×110cm) of Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden). The [^{35}S]sulphated proteoglycans were extracted from the limb buds for 36h. The extracts were dialysed against cold water for 48h and freeze-dried. The proteoglycan sample dissolved in the elution buffer was applied to the column. The column was eluted with 0.5M-sodium acetate buffer (pH7.0), which was boiled (to deaerate) before use. Fractions (0.4ml) were collected and portions (0.1ml) were counted for radioactivity by using the toluene mixture described above, containing 5% Biosolve. The void volume was determined by using bovine nasal-cartilage proteoglycan (kindly supplied by Dr. V. C. Hascall).

Link proteins were isolated after the proteoglycans were extracted under 'dissociative' conditions. Limb buds of stages 24 and 35 were incubated in F12X medium containing 5μCi of [^{3}H]leucine (specific radioactivity 70–100Ci/mmol; New England Nuclear, Boston, MA, U.S.A.)/ml. After 12h of incubation the tissues were rinsed several times with Simm's balanced salt solution.

Proteoglycans were extracted for 36h at 4°C with 4.0M-guanidinium chloride buffered at pH5.8 with 0.05M-sodium acetate containing the proteolytic inhibitors. The extracts were cleared by centrifugation at 13500*g* for 15min at 4°C and dialysed overnight against 100vol. of 0.5M-guanidinium chloride (containing the reagent mixture described above). The non-diffusible material was adjusted to a density of 1.6g/ml by the addition of CsCl (1.1g/g of solution). The samples were then centrifuged at 105000*g* (Beckman type 40 rotor) at 10–12°C for 44h. The gradients were partitioned into thirds. The lower third was diluted with an equal volume of buffered 7.5M-guanidinium chloride and the density was adjusted to 1.50g/ml and centrifuged as before. The gradients were partitioned into three fractions (lower two-fifths, middle two-fifths and upper one-fifth). The upper fifth, containing the link proteins, was dialysed extensively against water and freeze-dried.

Electrophoresis of the link proteins was performed on slab gels, in an apparatus designed by Reid & Bieleski (1968) as modified by Vandenburgh (1976). The chemicals for the gel were purchased from Bio-Rad Laboratories, Rockville Center, NY, U.S.A. Portions of the dried sample were dissolved by the method of Laemmli (1970) and electrophoresed on sodium dodecyl sulphate/polyacrylamide slab gels as described by Ames (1974).

Results

Uronic acid determinations

Uronic acid is first easily detected in stage-24 limb buds, and there is a fourfold increase in the uronic acid-containing extractable material between stages 24 and 35 (Table 1). As shown in Table 1, the actual amount of Fraction 1, as determined by uronic acid content of the extractable proteoglycan, increases from stage 24 to 35, whereas the amount relative to Fraction 2 decreases from stage 24 to 35 (65 to 32%). There is an increase in both the absolute and relative amount of Fraction 2 that is extracted (from 35 to 68%). This shift in the ratio (w/w) of Fraction 1 to Fraction 2 from 1.86 (stage 24) to 0.47 (stage 35) suggests that with age there is an increase in the amount of extractable proteoglycan aggregate (Fraction 2) and a decrease in the amount of extractable proteoglycan monomer (Fraction 1).

The extracted tissues were dialysed against water to remove the extracting solvent, and analysed for residual glycosaminoglycans. After Pronase digestion the remaining tissues were precipitated with trichloroacetic acid and the glycosaminoglycans in the supernatant were extensively dialysed and then freeze-dried (Daniel *et al.*, 1973). The freeze-dried material was reconstituted with water and assayed for uronic acid

by the method of Bitter & Muir (1962). The amount of uronic acid remaining in the tissue at stage 24 was 3.5–6.1% ($n = 3$), and at stage 35, 8.2–11.8% ($n = 3$).

Incorporation of [^{35}S]sulphate

There was not enough of the stage-18 material on which to measure uronic acid content accurately, so the proteoglycan labelled with [^{35}S]sulphate was related to wet tissue weight (20mg). Fraction 1 of the stage-18 material contained 34d.p.m. of sulphate-labelled proteoglycan/mg of tissue (72%) and Fraction 2 contained 13d.p.m./mg of tissue (28%). These values are shown in Table 2. There is a continuing decrease in the amount of [^{35}S]sulphate incorporated (per mg of tissue) into Fraction 1, from stage 18 (72%), to stage 24 (62%), to stage 35 (10%), and a corresponding increase in the amount into Fraction 2, from stage 18 (28%), to stage 24 (38%), to stage 35 (90%) (see Table 2).

[^{35}S]Sulphate incorporation per mg of tissue may give a distorted view of the relationship between Fractions 1 and 2, because these measurements include non-cartilaginous tissues, particularly in the stage-18 material. [^{35}S]Sulphate incorporation related to uronic acid content is a measure of the rate of proteoglycan synthesis (e.g., Hardingham & Muir, 1973). Incorporation of [^{35}S]sulphate was thus related to uronic acid content of the limb cartilages (which could be measured only for stages 24 and 35), to obtain a closer approximation to the changes occurring during development. These changes are seen in Table 3. Fraction 1 decreases in proportion from stage 24 (81%) to stage 35 (40%), whereas Fraction 2 shows a corresponding increase from stage 24 (19%) to stage 35 (60%). Regardless of whether [^{35}S]sulphate incorporation is related to weight or to uronic acid content, the trend is the same, i.e. Fraction 1 (proteoglycan monomer) decreases, whereas Fraction 2 (proteoglycan aggregate) increases with age (Tables 1, 2 and 3).

Although the results on [^{35}S]sulphate incorporation are consistent with our interpretations within each age group tested, the 'specific radioactivity' (d.p.m. of ^{35}S/unit of uronic acid) of stage-24 material (130×10^5) is much higher than that of stage-35 material (70×10^5). Fractions 1 and 2 from the same age group (stage 24 or 35) had variable specific radioactivities. We cannot, however, rule out the possibility that within any fraction there might be microheterogeneities with respect to specific radioactivity. Such microheterogeneities would be contributed to by the presence of keratan sulphate within the proteoglycan molecules.

The residual tissues were analysed for non-extracted radioactive materials by the same procedure used for non-extracted uronic acid; 3.2 ± 0.5% (S.D.) ($n = 3$) of the radioactivity remained in the stage-24 tissues, and 9.5 ± 0.6% ($n = 3$) remained in the stage-35 tissues.

Fig. 1 is the chromatographic profile of [^{35}S]-sulphated proteoglycan observed on Sepharose 2B. Proteoglycan from stage-24 limb bud shows a small peak (16%) at the void volume and a broad included peak (84%). Proteoglycan from stage-35 limb bud shows a large peak (90%) at the void volume and a small broad included peak (10%). This pattern further confirms the heterogeneity of proteoglycans

Table 1. *Amount of uronic acid-containing proteoglycans at stages* 24 *and* 35

The ratio of Fraction 1 to Fraction 2 is given in the last column. Each value is the average of three determinations.

	Uronic acid (μmol/mg of wet tissue)			
Stage	(*a*) Fraction 1	(*b*) Fraction 2	Total (*a*+*b*)	Ratio (*a*)/(*b*)
24	0.011	0.006	0.017	1.83:1
35	0.021	0.045	0.066	0.46:1

Table 2. *Amount of ^{35}S label in proteoglycan fractions from tissue at stages* 24 *and* 35 *in terms of mg wet weight of tissue*

The ratio of Fraction 1 to Fraction 2 is given in the last column. The data represent a single experiment of three similar experiments. Because of the low values for the youngest (stage-18) material, these data are not included in the Table.

	$10^{-3}\times$ [^{35}S]Sulphate radioactivity (d.p.m./mg of wet tissue)			
Stage	(*a*) Fraction 1	(*b*) Fraction 2	Total (*a*+*b*)	Ratio (*a*)/(*b*)
24	128	78	206	1.64:1
35	16	148	164	0.11:1

Table 3. *Amount of ^{35}S label in proteoglycan fractions from stage-*24 *and* -35 *tissues in terms of the uronic acid content of each fraction*

This is a representative experiment of three similar experiments.

	$10^{-5}\times$ [^{35}S]Sulphate radioactivity (d.p.m./μg of uronic acid)			
Stage	(*a*) Fraction 1	(*b*) Fraction 2	Total (*a*+*b*)	Ratio (*a*)/(*b*)
24	114	27	141	4.22:1
35	30	45	75	0.66:1

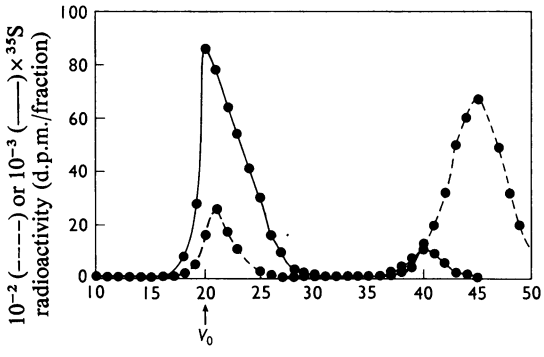

Fig. 1. *Elution profile of the two peaks of* [^{35}S]*sulphated proteoglycan obtained from stages-24 and -35 limb buds on Sepharose 2B chromatography*

The column size was 0.4cm×110cm. For further details, see the Materials and Methods section. ----, Stage 24; ——, stage 35.

synthesized in early embryonic limb as well as the profiles observed in Tables 1, 2 and 3.

Link-protein analysis

The sodium dodecyl sulphate/polyacrylamide-gel analysis of the link-protein fraction recovered in the upper fifth of the dissociative gradient indicate the presence of only one link-protein band in gels from stage 24, whereas two protein bands are present in gels for extracts of stage-35 limbs (Fig. 2). The protein bands migrated into the polyacrylamide gel to a position characteristic of a globular protein with a mol. wt. of approx. 42000 (Fig. 2).

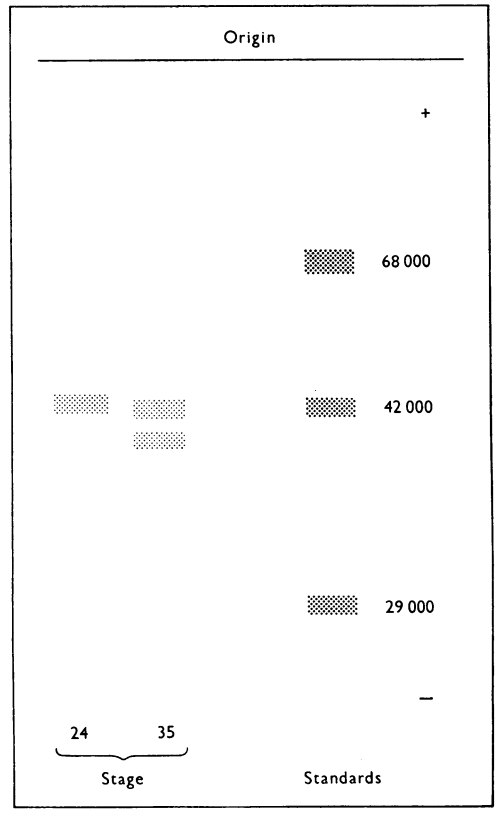

Fig. 2. *Slab-gel-electrophoresis pattern of the link proteins*

Standards of known mol. wt. are shown next to the link proteins [bovine serum albumin, 68000; ovalbumin, 42000–43000; carbonic anhydrase, 29000 (Sigma Chemical Co.)]. The electrophoresis was carried out as described by Ames (1974). Stage 24 shows one link protein; stage 35 shows two bands.

Discussion

Sajdera *et al.* (1970) have reported that a 0.4M-guanidinium chloride extract (associative conditions) of bovine nasal cartilage contains more proteoglycan monomer (subunit) (56%) than the proteoglycan aggregate (44%). A further extraction with 4.0M-guanidinium chloride (dissociative conditions) gives more aggregate (71%) than monomer (29%). In the present study, Fraction 1 contains more of the proteoglycans in the non-aggregated (monomeric) form (cf. Hascall & Heinegård, 1974), whereas Fraction 2 contains most of the remaining proteoglycans, which have to be removed under dissociative conditions. The fact that the proteoglycans extracted with 4.0M-guanidinium chloride increase in both absolute and relative amounts during chondrogenic differentiation supports the view that proteoglycans are present in differentiated cartilage, primarily in the aggregated form.

The decrease in the proportion of proteoglycans extracted with 0.4M-guanidinium chloride between stages 24 and 35 is not due to the development of a less permeable matrix, since the subsequent extraction with 4.0M-guanidinium chloride (which dissociates and removes the remaining proteoglycans) does not produce any of the molecules of the type removed with the 0.4M-guanidinium chloride extraction. This was determined by sucrose-linear-density-gradient centrifugation and polyacrylamide-gel electrophoresis. Fraction 1 proteoglycans have a distinctly different mobility (i.e. smaller molecular weight) from the Fraction 2 proteoglycans (cf. Fig. 1).

It is noteworthy that the proteoglycans synthesized at different ages appear to have different specific radioactivities (^{35}S/uronic acid). It was not feasible to measure this for the stage-18 material, but it is clear that the stage-24 limb bud synthesizes proteo-

glycans with much higher specific radioactivity than does stage-35 material. The lower specific radioactivity in the older material could be due to a lower degree of sulphation in the chondroitin sulphate molecules and/or an increased amount of keratan sulphate (which does not contain uronic acid, but is sulphated).

Previous studies have shown that there is an increase in the amount of chondroitin sulphate synthesized during embryonic development of the chick limb (Searls, 1965; Medoff, 1967; Huffner, 1970; Levitt & Dorfman, 1974). More recent studies focus on the synthesis of the parent molecules, the proteoglycans. Dorfman (1974) and Goetinck *et al.* (1974) have shown with cultured embryonic-chick limb cells that there are heterogeneities in the proteoglycans synthesized, and that there may also exist a cartilage-specific proteoglycan. Goetinck & Royal (1976) have presented evidence that the proteoglycans from pre-cartilaginous limb buds are similar to the proteoglycans of differentiated limb cartilage. The results in the present paper suggest that proteoglycan synthesis by early embryonic limb buds is cartilage-specific in the sense that it is correlated with the appearance of cartilage, and that the molecules become more aggregated with later stages of development. This aggregation can be mediated either by the simple appearance of the aggregation factor(s), or by changes in conformation leading to the aggregation of the monomeric forms.

The synthesis of small quantities of extractable proteoglycans by the pre-cartilaginous limb buds (stage 18) suggests that the tissue is programmed for the production of the parent proteoglycan molecule during the very early stages of development. The predominance of the monomeric forms (i.e. those molecules released by extraction with 0.4M-guanidinium chloride) in the younger limb buds, and the augmentation of the aggregated forms at stage 35, implicate the protein link factor(s) as being acquired during differentiation. And indeed, the appearance of two link proteins is correlated with both the increase in the absolute and relative amounts of proteoglycans extracted in a manner which indicates that they exist in the matrix in the aggregated forms. The increases are correlated with the onset of vigorous chondrogenesis. Similar transitions from a predominance of proteoglycan monomeric forms to a predominance of proteoglycan aggregates have also been observed in explants of embryonic-chick somites (J. W. Lash & N. S. Vasan, unpublished work) and pig articular cartilage (Šimůnek & Muir, 1972). The synthesis of the link protein may be a regulating factor in the aggregation of proteoglycan monomers during chondrogenesis.

We gratefully acknowledge the technical assistance of Mrs. Gladys Treon, and the assistance of Dr. Vincent C. Hascall in preparing the manuscript. This work was supported by grants HD-00380 and T32 HD-07067 from the United States Public Health Service.

References

Ames, G. F. (1974) *J. Biol. Chem.* **249**, 634–644
Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
Bjelle, A., Gardell, S. & Heinegård, D. (1974) *Connect. Tissue Res.* **2**, 111–116
Campo, R. D., Bielen, R. J. & Hetherington, J. (1972) *Biochim. Biophys. Acta.* **261**, 136–142
Daniel, J. C., Kosher, R. A., Lash, J. W. & Hertz, J. (1973) *Cell Differ.* **2**, 285–298
Dorfman, A. (1974) *Mol. Cell. Biochem.* **4**, 45–65
Goetinck, P. F. & Royal, P. D. (1976) *J. Gen. Physiol.* **68**, 6a
Goetinck, P. F., Pennypacker, J. P. & Royal, P. D. (1974) *Exp. Cell Res.* **87**, 241–248
Hamburger, V. & Hamilton, H. L. (1951) *J. Morphol.* **88**, 49–92
Hardingham, T. E. & Muir, H. (1973) *Biochem. Soc. Trans.* **1**, 282–284
Hardingham, T. E., Fitton-Jackson, S. & Muir, H. (1972) *Biochem. J.* **129**, 101–112
Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4232–4241
Hascall, V. C. & Heinegård, D. (1975) in *Extracellular Matrix Influences on Gene Expression* (Slavkin, H. C. & Greulich, R. C., eds.), pp. 423–434, Academic Press, New York
Hascall, V. C., Oegema, T. R., Brown, M. & Caplan, A. I. (1976) *J. Biol. Chem.* **251**, 3511–3519
Heinegård, D. (1972) *Biochim. Biophys. Acta* **285**, 181–192
Huffner, E. S. (1970) *Calcif. Tissue Res.* **6**, 55–69
Kleine, T. O. (1973) *FEBS Lett.* **31**, 170–174
Kleine, T. O. (1974) *FEBS Lett.* **39**, 225–248
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Levitt, D. & Dorfman, A. (1974) *Curr. Top. Dev. Biol.* **8**, 103–151
Marzullo, G. & Lash, J. W. (1970) *Exp. Biol. Med.* **1**, 213–219
Medoff, J. (1967) *Dev. Biol.* **16**, 118–143
Muir, H. & Jacobs, S. (1967) *Biochem. J.* **103**, 367–374
Oegema, R. T., Jr., Hascall, V. C. & Dziewiatkowski, D. D. (1975) *J. Biol. Chem.* **250**, 6151–6159
Reid, M. S. & Bieleski, R. L. (1968) *Anal. Biochem.* **22**, 374–381
Sajdera, S. W. & Hascall, V. C. (1969) *J. Biol. Chem.* **244**, 77–87
Sajdera, S. W., Hascall, V. C., Gregory, J. D. & Dziewiatkowski, D. D. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 2, pp. 851–858, Academic Press, New York
Searls, R. L. (1965) *Dev. Biol.* **11**, 155–168
Shipp, D. W. & Bowness, J. M. (1975) *Biochim. Biophys. Acta* **379**, 282–294
Simms, H. S. & Sanders, M. (1942) *Arch. Pathol.* **33**, 619–635
Šimůnek, Z. & Muir, H. (1972) *Biochem. J.* **126**, 515–523
Strider, W., Subhash, P. & Rosenberg, L. (1975) *Biochim. Biophys. Acta* **379**, 271–281
Vandenburgh, H. (1976) Ph.D. Dissertation, University of Pennsylvania