Protein–Polysaccharide Biosynthesis

MEMBRANE-BOUND SACCHARIDES

By JEFFERY G. DERGE* and E. A. DAVIDSON

*Department of Biological Chemistry, The Milton S. Hershey Medical Center
of The Pennsylvania State University, Hershey, Pa. 17033, U.S.A.*

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Membrane-bound saccharides obtained from ear cartilage from rabbits recovering from papain injection were examined for glycosyl acceptor properties, alkali lability, molecular size and degree of sulphation. Although considerable glucuronosyl transfer from UDP-glucuronic acid could be demonstrated, less than one-third could be identified as representing chondroitin linkage formation; there was little or no effect of molecular size on acceptor properties. Approximately one-half of the membrane-associated chondroitin-like saccharide chains are solubilized by alkali, whereas one-half require proteolysis for solubilization. These fractions are analytically indistinguishable and both contain xylose and galactose as well as uronic acid and sulphated *N*-acetyl-galactosamine. All fragments that were examined contained stoichiometric amounts of axial sulphate ester, strongly suggesting a close relationship between chain extension and sulphation.

The assembly of the chondroitin sulphate–protein complexes characteristically found in cartilaginous tissues is generally considered to proceed by the sequential addition of saccharides to a preformed protein core (Marler & Davidson, 1965). The site of glycosyl transfer has been identified as the Golgi apparatus or rough endoplasmic reticulum, based on both differential-centrifugation analyses (Horwitz & Dorfman, 1968) and electron-microscopic radio-autography (Godman & Lane, 1964; Neutra & Leblond, 1966). A number of facets of the biosynthesis of this class of macromolecules remain unknown, including the position of receptive serine groups on the polypeptide chain, the role of the neutral trisaccharide linkage group, the mechanism of the glycosyltransferase reaction and the chronology and site of sulphate ester formation. The present study is concerned with the structure and reactions of membrane-bound glycopeptides associated with such a system, in an attempt to characterize the chronology of the sulphation step and the presence of alkali-stable protein–saccharide linkages.

Materials and Methods

Preliminary studies were done on cartilage obtained from the long bones of 14-day chick embryos by the procedure of Perlman *et al.* (1964). Subsequently ear-plate cartilage, obtained from eight 10-week-old rabbits treated by papain injection 48 h before killing,

was employed. Rabbits of the indicated age group were injected in the ear artery with 1 ml of 0.85% NaCl containing 15 mg of papain. Crude papain, obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A., was found to give reliable results after overnight dialysis. Failure to dialyse the preparation resulted in high toxicity, apparently caused by low-molecular-weight contaminants, since this problem was not encountered when crystalline enzyme was employed. Injection of papain has been shown to cause depletion of the protein–polysaccharide matrix and subsequent collapse of the ear structure (Thomas, 1956; McCluskey & Thomas, 1958). Recovery took 2–4 days and was associated with the rapid reappearance of matrix polysaccharide, arising from *de novo* synthesis by the cellular elements of the tissue (Bryant *et al.*, 1958). After the recovery period, the animals were killed and the ear-plate cartilage isolated. The tissue was freed of adhering membranes and minced with scissors before homogenization. In some experiments, cartilage from several animals was pooled to provide the required amount of starting material.

Microsomal-membrane preparations were isolated by centrifugation after disruption of the tissue in a motor-driven glass homogenizer in 50 mM-tris buffer, pH 7.8, containing 125 mM-KCl, 10 mM-MgCl₂, 10 mM-EDTA, 6 mM-L-cysteine and 0.25 M-sucrose (3 ml/g wet wt. of tissue). The resulting slurry was centrifuged at 2000 g for 5 min to remove debris; the supernatant was centrifuged for 15 min at 10000 g, yielding a 'low-speed pellet'. The remaining supernatant was centrifuged for 90 min at 144000 g to

* Present address: National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

yield a clear supernatant and a 'high-speed pellet' (microsomal fraction).

Enzyme incubations were for 2 h at 37°C and contained the following: 0.01 ml of 10mM-ATP; 0.01 ml of 1.0mM-UTP; 0.01 ml of 0.1mM-NAD⁺; 0.01 ml of 0.1mM-UDP-*N*-acetylgalactosamine; 0.01 ml of 0.1mM-UDP-[U-¹⁴C]glucuronic acid (10⁵ c.p.m.); 0.06 ml of homogenizing buffer without sucrose; 0.025 ml of enzyme suspension (pellets suspended in buffer). Reactions were assayed by descending chromatography on Whatman no. 1 paper in ethanol-1M-ammonium acetate (7:3, v/v) for 24 h (Palladini & Leloir, 1952). Radioactivity remaining at the origin represented incorporation into macromolecular material (Silbert, 1964).

The nature of endogenous membrane-bound fractions that could serve as acceptor substrate for the formation of chondroitin linkages was examined by the isolation of anionic saccharides from large-scale membrane preparations derived from rabbit ear cartilage by the above procedures, and treated subsequently as indicated in the text. Preparations employed for the isolation of membrane-associated saccharides were assayed for glycosyltransferase activity by using either UDP-*N*-[³H]acetylgalactosamine or UDP-[¹⁴C]glucuronic acid as labelled donor (Silbert, 1964; Perlman *et al.*, 1964). In general, saccharide transfer was significantly higher if sucrose washing of the membrane fractions was omitted, but the analytical procedures required prior removal of the sucrose. The reason for this decrease in activity is not clear but may be related to the physical organization of the membrane structure, reflecting the loss of a readily solubilized component or cofactor.

UDP-*N*-[³H]acetylgalactosamine was prepared by Dr. H. Schulte-Holthausen as described by Roseman *et al.* (1961). Authentic chondrosine was prepared from cartilage chondroitin 4-sulphate as described by Davidson & Meyer (1954). All other materials were the best commercially available.

Amino acids and amino sugars were determined with an amino acid analyser after hydrolysis for 18 h *in vacuo* with 4M-HCl at 100°C. Neutral sugars were released by hydrolysis with a suspension of Dowex 50 (H⁺ form) in 0.5M-HCl for 4 h (Anastassiadis & Common, 1958). After neutralization, samples were prepared for g.l.c. analysis by conversion into the trimethylsilyl derivatives (Sweeley *et al.*, 1963). The presence of xylose was confirmed by paper-chromatographic examination of the deionized hydrolysates in ethyl acetate - pyridine - water (2:1:2, by vol.; upper phase).

G.l.c. of the trimethylsilyl-sugar derivatives was performed by using either 2% SE-30 on Diataport (80-100 mesh) or 1% OV-255 on Chrom G (80-100 mesh) as column packings. Columns were operated at 170°C with N₂ as carrier gas at a flow rate such

that the α -D-glucopyranose derivative had a retention time of about 10 min. Identifications were based on both absolute and relative retention times by using trimethylsilyl sugar standards from Pierce Chemical Co., Rockford, Ill., U.S.A.

I.r. spectra were obtained with a Perkin-Elmer 337 spectrophotometer by using KBr pellets for all samples.

Micro-zone electrophoresis was performed in pyridine-formic acid buffer, pH 3.0 (Herd, 1968). Sugars were located by the silver nitrate stain and sulphated fractions by the use of Alcian Blue. Ninhydrin was used to detect chondrosine or free amino sugars.

Protein was determined by the method of Lowry *et al.* (1951), uronic acid by either the orcinol (Brown, 1946) or carbazole (Dische, 1947) procedures, and hexosamine by the method of Boas (1953) or directly from the amino acid analyser chromatograms.

The identification of chondrosine was undertaken since neither the incorporation of radioactive label from nucleotidyl sugars into polymeric structures nor the analytical identification of uronic acid or amino sugar are sufficient assurance that the appropriate linkages have been synthesized. Chondrosine was liberated from polysaccharide or oligosaccharide fractions by hydrolysis with 0.5M-H₂SO₄ for 3 h at 100°C; all hydrolyses contained 1 mg of chondroitin 4-sulphate carrier. The disaccharide was resolved from free amino sugar and anionic uronides by ion-exchange chromatography and its identity confirmed by paper electrophoresis both at pH 1.9 and at pH 4.75. Chondrosine is positively charged at the former pH and neutral at the latter pH and is thus readily separated from neutral sugars and non-amino sugar-containing uronides. Mobilities were related to standards in every case.

Results

The results in Table 1 demonstrate the glycosyl transferase capacity of the fractions used; the activity is generally in accordance with that observed in other laboratories (Silbert, 1964; Perlman *et al.*, 1964). The approximate size distribution of the labelled compounds formed under typical conditions was examined by gel chromatography on a column (1.8 cm $\times$ 110 cm) of Sephadex G-50 (fine grade) with water as eluting solvent. A large-scale incubation was done by using the microsomal pellet obtained from the long-bone cartilage of ten 14-day chick embryos, 5 $\times$ 10⁶ c.p.m. of UDP-[¹⁴C]glucuronic acid and the appropriate cofactors in a total volume of 0.5 ml. After incubation for 2 h at 37°C the mixture was made 0.1M with NaOH and kept at 0°C for 12 h. The resulting solution was incubated for 24 h at 37°C with 0.5 mg of Pronase in 0.1M-tris-

Table 1. *Glycosyltransferase activity of microsomal fractions*

The radioactivity at the origin of paper chromatograms was determined by liquid-scintillation counting. The percentages are the proportions of the UDP-sugar substrate incorporated; UDP-glucuronic acid was the labelled nucleotide employed except where indicated for rabbit cartilage. In Expt. 1 pellets were washed by suspension in 0.25M-sucrose and recentrifugation. The rabbit cartilage experiments were done with the high-speed pellet obtained from 24g of material (Expt. 1) or the indicated fractions obtained from 27.5g of material (Expt. 2).

Sample	Radioactivity (c.p.m.)
Chick cartilage	
Unwashed high-speed pellet	3100 (3.1%)
Washed high-speed pellet	2200 (2.2%)
Unwashed high-speed pellet, stored 3 months in liquid N ₂	1500 (1.5%)
Rabbit cartilage	
Expt. 1	
UDP-[¹⁴ C]glucuronic acid	9733 (3.5%)
UDP-N-[³ H]acetylgalactosamine	750 (1.0%)
Expt. 2	
Cell debris	1094 (0.4%)
Low-speed pellet	10700 (4.0%)
High-speed pellet	14580 (5.4%)

HCl buffer, pH 8, before transfer to the column. The results are shown in Fig. 1. A broad radioactive peak of uronic acid-containing material was observed, suggesting that compounds of a wide size range can apparently function as glucuronosyl acceptors under these conditions. The proportion of label present in chondrosine was generally the same in all fractions, suggesting little, if any, difference in the size specificity of the acceptor substrate. Under these same column conditions, radioactivity eluted from the origins of the paper chromatograms run in ethanol-ammonium acetate was eluted at the void volume, along with the chondroitin sulphate standard (tubes 1-6).

To assess the amount of glucuronosyl transfer resulting in chondroitin chain elongation, an estimate was made of that proportion of the incorporated glucuronic acid that was identifiable as the repeating disaccharide, chondrosine. The results are summarized in Table 2 and indicate that the amount of transfer resulting in the formation of chondrosine linkage was between 10 and 30% of the incorporated radioactivity. This relatively low value may reflect either the presence of alternative acceptor loci for uronic acid in the enzyme preparation, or decarboxylation and xylose transfer.

Fig. 1. *Chromatography on a column (1.8 cm × 110 cm) of Sephadex G-50 of labelled products formed after incubation of chick cartilage microsomal pellet*

Experimental details are described in the text. Standard chondroitin sulphate emerged in tubes 1-6, UDP-glucuronic acid in tubes 56-60, and free glucuronic acid after tube 70. Fractions of volume 3 ml were collected.

The neutral radioactive material liberated by acid hydrolysis was identified as xylose by electrophoresis and paper chromatography. In a large-scale incubation employing the high-speed pellet obtained from 24g of rabbit ear-plate cartilage, the ¹⁴CO₂ released from UDP-[¹⁴C]glucuronic acid was trapped on a paper wick soaked in benzyltrimethylammonium hydroxide and corresponded exactly to the amount of xylose incorporated into macromolecules. In the same experiment, approximately one-third of the incorporated radioactivity could be identified as free glucuronic acid after acid hydrolysis, a portion of which may represent the formation of glucuronosylgalactose saccharides in the protein-polysaccharide linkage region. Free glucuronic acid liberated from chondroitin sulphate under the hydrolytic conditions employed represented 10-15% of the total; no decarboxylation was noted.

In view of observations on the presence of an alkali-stable carbohydrate-peptide linkage in a cartilage chondromucoprotein fraction (Katsura & Davidson, 1966a), a direct quantitative assessment of alkali-stable and alkali-labile membrane-bound saccharides was undertaken. In the first experiment, the microsomal fractions obtained from 24.5g of rabbit ear cartilage were treated with 0.1M-NaOH for 12h at 0°C. The mixture was centrifuged, and after removal of the supernatant (alkali-soluble fraction) the resulting pellet was digested with Pronase for 24h at 37°C. Separately, the low-speed

Table 2. *Distribution of label in products after Sephadex chromatography*

'Sample source' for chick cartilage refers to the gel chromatography depicted in Fig. 1. Rabbit cartilage is from a similar incubation from which the crude supernatant obtained after Pronase treatment was used for analysis. Values represent the radioactivity found in the areas corresponding to the standards after electrophoresis at pH 1.9 and 4.75. The proportion of label present as chondrosine ranged from 8–14% for the chick sample and was somewhat higher for the rabbit sample.

Sample source	Radioactivity in chondrosine (c.p.m.)	Radioactivity in xylose (c.p.m.)	Radioactivity in glucuronic acid (c.p.m.)
Chick cartilage			
Tube 11	500	1300	1800
Tube 18	100	715*	—
Tube 56	237	1420	898
Tube 57	262	3374*	
Rabbit cartilage			
Pronase-solubilized	7888	8382	9693

* Values represent xylose and glucuronic acid combined.

pellet was treated directly with Pronase for 24h at 37°C. The alkali- or Pronase-solubilized saccharides were precipitated quantitatively by 70% (v/v) ethanol, but not by a 1% solution of cetyltrimethylammonium bromide. The total yield of saccharides from this preparation was approx. 2.5mg. The analytical results for these fractions are presented in Table 3.

In a second experiment, the high-speed and the low-speed pellets obtained from 101g of rabbit ear cartilage were separately digested with Pronase. The digests were placed on a column (2.5cm × 25cm) of DEAE-Sephadex A-25 and eluted with a linear gradient of NaCl. Tests for neutral sugar and uronic acid showed only one carbohydrate peak, which was eluted at an ionic strength of 1.5; the peak tubes were pooled, and the material precipitated by ethanol was examined for molecular-size distribution on Sephadex G-50. The results are illustrated in Fig. 2. The total yield of polysaccharide from this preparation was 10mg. Selected adjacent fractions were combined and examined for electrophoretic mobility (see Fig. 3), i.r. spectra and xylose and galactose content. All fractions examined (e.g. 6–9, 10–12, etc.) had identical electrophoretic mobilities and contained xylose and galactose in a 1:2 ratio, consistent with the known structure of the linkage region. Because of lack of material and the experimental procedure employed, it was not possible to measure quantitatively the amounts of xylose and serine in the same fractions. The i.r. spectra of all fractions were identical and showed the presence of axial sulphate ester groupings (Orr, 1954) in relative amounts equivalent to those of a fully sulphated chondroitin 4-sulphate standard. Samples of both preparations were tested for glucuronosyltransferase

activity and proved to be normal (3–5% of added UDP-sugar). The molecular-weight estimates for these fractions, based on the hexosamine:serine ratio, range from 17000 to 5000, and are consistent with the Sephadex elution pattern.

Discussion

Saccharide transfer with the microsomal preparations was comparable with that previously reported (Silbert, 1964; Robinson *et al.*, 1966). The lower incorporation of radioactivity when labelled *N*-acetylhexosamine is used can be attributed to the fact that it is incorporated only into the polysaccharide backbone, and is not converted into other sugars, which are then incorporated. Attempts to stimulate the incorporation of UDP-sugar into polymeric linkage by addition of a sulphate-activating system were generally unsuccessful. Rabbit cartilage was employed as source material to permit the isolation of sufficient quantities of membrane preparations to allow the analysis of bound acceptor molecules. The papain treatment results in extensive biosynthetic activity at 24–72h after the depletion of the matrix protein-polysaccharide, thus maximizing the quantity of membrane-associated intermediates.

The distinction between alkali-stable and alkali-labile membrane-bound saccharide fragments may reflect an alternative pathway for polymer assembly. By analogy with lipid intermediates, which appear to function as glycosyl carriers in bacterial polysaccharide biosynthesis (Wright *et al.*, 1967), the alkali-resistant material may represent saccharides stably bound to a peptide carrier that functions as an intermediate between nucleotidyl sugars and the

Table 3. *Analyses of membrane-associated polysaccharides*

The recovery of material (on a weight basis) was 85% or better in each case. Material for analysis was derived from 24.5g of rabbit ear cartilage. MI-OH refers to polysaccharide solubilized from the microsomal pellet by alkali treatment, MI-PR refers to polysaccharide solubilized from alkali-treated microsomal fractions by subsequent Pronase digestion and LP-PR refers to polysaccharide released from the low-speed pellet by direct Pronase digestion.

Sample	Residue ...	Composition (residues/1000 residues)							
		Asp	Thr	Ser	Glu	Gly	Gln	GalN	Uronic acid
MI-OH		10.6	6.8	8.0	13	16.1	39.3	410	437
MI-PR		10.1	7.5	22	18.3	25.5	7.7	414	466
LP-PR		8.5	4.0	16.6	9.0	20.0	6.5	449	470

Fig. 2. *Chromatography of polysaccharide from Pronase digests of rabbit ear cartilage high-speed and low-speed pellets*

A column (1.3cm×50cm) of Sephadex G-50 was employed and 2.5ml fractions were collected; chondroitin 4-sulphate standard appears in tubes 6–7. Peak I represents high-speed and peak II low-speed pellet material.

finished protein-polysaccharide. However, there is at present no direct experimental evidence to support this hypothesis, and other explanations are possible.

The presence of xylose and galactose together with 4-sulphated chondroitin chains bound to membrane fractions argues for the close relationship of these saccharides with the serine-linked macrostructure that comprises most of the extracellular matrix. The possibility remains that the alkali-stable fraction still contains saccharide chains attached to peptide via the β -xylosyl-serine linkage, but that the serine residue is at the terminus of a polypeptide chain and thus does not readily undergo the characteristic β -elimination reaction (Derevitskaya *et al.*, 1967). However, this seems unlikely in view of the amount present and the stoichiometry of the complex structure, which requires approx. five polysaccharide chains per polypeptide chain (Marler & Davidson, 1965). But not all *O*-glycosidic linkages

involving serine residues in which both the α -amino and α -carboxyl groups are blocked are readily split by alkali (Derevitskaya *et al.*, 1967). A second possibility is that the polypeptide core is still intimately associated with the membrane structure such that its interaction with alkali does not result in the characteristic β -elimination reaction. Finally, it must be considered that polysaccharide chains may be covalently linked to other types of residues. Attempts to define possible precursor-product relationships between alkali-stable and alkali-labile fractions based on differential incorporation of label were not conclusive. However, previous studies have shown that an alkali-stable fraction derived from chondromucoprotein of porcine articular cartilage incorporated both sulphate and carbon label more rapidly than did serine-linked material (Katsura & Davidson, 1966b, 1969; Bentley & Rokosova, 1970). The relationship of these observations to those made on the membrane fragments remains uncertain.

Most of the material transferred in these experiments, as well as in previously described experiments where protein-bound acceptors were employed, probably does not represent extensive chondroitin chain elongation. This conclusion is based on the relatively low yield of chondrosine compared with the amount of labelled precursor saccharide incorporated into high-molecular-weight fractions.

Based on the assumption that each membrane-associated saccharide chain is capable of further elongation, it can be estimated that there are approx. 12n-equiv. of acceptor/g of rabbit ear cartilage. Typical experiments showed the incorporation of 0.2nmol of glucuronic acid, much of which is not involved with chondroitin chain elongation, in the presence of approx. 6n-equiv. of acceptor. Thus, the incorporation system is utilizing *in vitro* only a small fraction of the acceptor that is apparently available *in vivo*.

Although the absolute extent of saccharide incorporation observed was small, macromolecular

Fig. 3. Micro-zone electrophoresis of selected samples from the experiment illustrated in Fig. 2

Sample numbers refer to specific tubes from the Sephadex chromatography illustrated in Fig. 2. The origin is designated as 0 and the shadings represent material staining with Alcian Blue. Rabbit PPL is a sample of protein-chondroitin 4-sulphate isolated from ear cartilage by the procedure of Gerber *et al.* (1960); desulphated CSA is standard chondroitin 4-sulphate, desulphated by the procedure of Kantor & Schubert (1957). PPL migrates approx. 20mm under the conditions employed.

assembly systems generally require intact multi-enzyme complexes or organelles, and systems studied under these conditions may have been deficient in some essential structural or enzymic feature. The presence of significant activity in the low-speed pellet probably reflects the presence of Golgi membranes. The physical disruption of these organelles in this and the microsomal pellet may well be a factor causing the low incorporation generally observed.

The presence of completely sulphated oligosaccharides of a variety of molecular weights that contain xylose, galactose and 4-sulphated *N*-acetyl-galactosamine residues suggests a close inter-relationship between chain assembly and sulphation. The failure to detect grossly undersulphated fractions of any molecular size argues strongly for an obligatory coupling between chain extension and sulphation (Kleine *et al.*, 1968). As the lowest-

molecular-weight membrane-bound saccharides examined were fully sulphated, proposals involving extensive chain assembly before sulphation seem untenable, and it seems reasonable to speculate that sulphation may be a prerequisite for further saccharide transfer.

Other investigators have shown the highest specific activity in sulphating systems to correspond to those fractions with the highest degree of sulphation (Kleine *et al.*, 1968; Greiling & Stuhlsatz, 1966; Greiling *et al.*, 1967). This result would not be expected if the acceptor molecule were an unsulphated polymer. The explanation for this may be the initial dependence on molecular weight of the cetylpyridinium chloride separation procedure, and to a lesser extent the Dowex separation procedure (Bentley & Rokosova, 1970; Scott, 1960). The critical electrolyte concentration for the solubility of these complexes is inversely proportional to the log of the molecular weight, and the small oligosaccharides found in this work would appear to be undersulphated molecules if examined by the cetylpyridinium chloride technique. The similarity in the profiles of Figs. 1 and 2 shows that compounds of all molecular weights are approximately of equal acceptor activity in this system.

The isolation of UDP-*N*-acetylgalactosamine 4-*O*-sulphate from the isthmus of the hen oviduct (Strominger, 1955) and rat epiphyseal cartilage (Picard *et al.*, 1964) has prompted suggestions that this amino sugar derivative functions as an intermediate in chondroitin sulphate biosynthesis. The failure to demonstrate such a role (Hardingham & Phelps, 1970) should not, however, be taken as conclusive, especially as detailed mechanisms for the sulphation of the chondroitin chains cannot yet be written. The ready solubilization of sulphotransferase activity may provide a continual obstacle to reconstruction of the natural biosynthetic system (Meezan & Davidson, 1967).

The absence of significant quantities of newly synthesized 6-sulphated material is in accord with the composition of the rabbit ear-plate cartilage from the age group employed. The effect of the age of the animal on the biosynthetic specificity for mucopolysaccharide formation has not been studied nor have tissues containing chondroitin 6-sulphate been examined in a comparable manner.

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References

- Anastassiadis, P. A. & Common, R. H. (1958) *Can. J. Biochem. Physiol.* **36**, 413
- Bentley, J. P. & Rokosova, B. (1970) *Biochem. J.* **116**, 329

- Boas, N. F. (1953) *J. Biol. Chem.* **204**, 553
- Brown, A. H. (1946) *Arch. Biochem.* **11**, 269
- Bryant, J. H., Leder, I. G. & Stetten, D. (1958) *Arch. Biochem. Biophys.* **76**, 122
- Davidson, E. A. & Meyer, K. (1954) *J. Amer. Chem. Soc.* **76**, 5686
- Derevitskaya, V. A., Vatina, M. G. & Kochetkov, N. K. (1967) *Carbohydr. Res.* **3**, 377
- Dische, Z. (1947) *J. Biol. Chem.* **167**, 189
- Gerber, B. R., Franklin, E. C. & Schubert, M. (1960) *J. Biol. Chem.* **235**, 2870
- Godman, G. C. & Lane, N. (1964) *J. Cell Biol.* **21**, 353
- Greiling, H. & Stuhlsatz, H. W. (1966) *Hoppe-Seyler's Z. Physiol. Chem.* **345**, 236
- Greiling, H., Stuhlsatz, H. W. & Plagemann, L. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 121
- Hardingham, T. E. & Phelps, C. F. (1970) *Biochem. J.* **119**, 885
- Herd, J. K. (1968) *Anal. Biochem.* **23**, 117
- Horwitz, A. L. & Dorfman, A. (1968) *J. Cell Biol.* **38**, 358
- Kantor, T. G. & Schubert, M. (1957) *J. Amer. Chem. Soc.* **79**, 152
- Katsura, N. & Davidson, E. A. (1966a) *Biochim. Biophys. Acta* **121**, 128
- Katsura, N. & Davidson, E. A. (1966b) *Biochim. Biophys. Acta* **121**, 120
- Katsura, N. & Davidson, E. A. (1969) *Biochim. Biophys. Acta* **184**, 503
- Kleine, T. O., Kirsig, H.-J. & Hilz, H. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1037
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
- Marler, E. & Davidson, E. A. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 648
- McCluskey, R. T. & Thomas, L. (1958) *J. Exp. Med.* **108**, 371
- Meezan, E. & Davidson, E. A. (1967) *J. Biol. Chem.* **242**, 1685
- Neutra, M. & Leblond, C. P. (1966) *J. Cell Biol.* **30**, 137
- Orr, S. F. D. (1954) *Biochim. Biophys. Acta* **14**, 173
- Palladini, A. C. & Leloir, L. F. (1952) *Biochem. J.* **51**, 426
- Perlman, R. L., Telser, A. & Dorfman, A. (1964) *J. Biol. Chem.* **239**, 3623
- Picard, J., Gardais, A. & Dubernard, J. (1964) *Nature (London)* **202**, 1213
- Robinson, H. C., Telser, A. & Dorfman, A. (1966) *Proc. Nat. Acad. Sci. U.S.* **56**, 1859
- Roseman, S., Distler, J. J., Moffatt, J. G. & Khorana, H. G. (1961) *J. Amer. Chem. Soc.* **83**, 659
- Scott, J. E. (1960) *Methods Biochem. Anal.* **8**, 145
- Silbert, J. E. (1964) *J. Biol. Chem.* **239**, 1310
- Strominger, J. L. (1955) *Biochim. Biophys. Acta* **17**, 283
- Sweeley, C. C., Bentley, R., Makita, M. & Wells, W. W. (1963) *J. Amer. Chem. Soc.* **85**, 2497
- Thomas, L. (1956) *J. Exp. Med.* **104**, 245
- Wright, A., Dankert, M., Fennessey, P. & Robbins, P. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 1798