Studies of Frog Oviducal Jelly Secretion

I. CHEMICAL ANALYSIS OF SECRETORY PRODUCT [1,2]

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ABSTRACT The jelly secreted by the oviduct of *Rana pipiens* has been analyzed histochemically and biochemically. Histochemical techniques show that the jelly contains neutral carbohydrates and a protein moiety. Colorimetric estimation reveals that the jelly contains protein and also appreciable quantities of hexose and hexosamine. Sialic acid is present. Chromatographic methods were employed to identify the simple sugars, galactose, fucose, mannose, xylose and glucose, and the hexosamines, glucosamine and galactosamine.

Frog ova become coated with three layers of jelly as they traverse the oviduct. Eggs taken from the body cavity, which have not passed through the oviduct or received a coat of jelly, cannot be fertilized (Bataillon, '19). Artificially covering coelomic eggs with jelly capsules dissected from eggs expressed from the cloaca markedly improves the percentage of normal development induced when blastula nuclei are implanted into the eggs (Subtelny and Bradt, '61). The jelly may affect maturation of the egg; somehow interacts with sperm in fertilization; and appears to have mechanical and possibly nutritive significance during cleavage (Humphries, '66). An understanding of the chemical nature of this secretion product of the oviduct is meaningful in the interpretation of the events of early development.

Amphibian egg jelly harvested from egg masses collected in ponds contains galactosamine (Schulz and Ditthorn, '00); glucosamine, glucose, and galactose (Schulz and Becker, '35); fucose (Bray and James, '49); mannose and xylose (Folkes, Grant and Jones, '50). Concentrations calculated for the various carbohydrates include: total hexosamines, 40.4%; fucose, 10.4%; galactose and mannose, 11.4% (Minganti, '55). The present study is concerned with the chemical analysis of jelly dissected from eggs in distilled water immediately after spawning, and with a histochemical analysis of sections of frog oviducts. Samples were analyzed histochemically and biochemically, with greatest emphasis on carbohydrates. In a subsequent paper (part II), the morphological details of synthesis of secretory granules of oviducal cells will be described.

MATERIALS AND METHODS

Histochemical analyses

Histochemical analyses were performed on oviducts of females of *Rana pipiens* collected by the author in the vicinity of Ann Arbor, Michigan, or the University of Michigan Biological Station during the summer months of 1962 and 1963 as well as upon animals purchased from a commercial supplier in Wisconsin. Biochemical studies were made with jelly collected from eggs obtained directly from oviducts or after stripping them.

Tests for protein. Mercury bromphenol blue (Pearse, '60) was used as a general protein stain. Special tests for reactive components of proteins included the ninhydrin-Schiff method for protein-bound amino groups (Pearse, '60), Millon's reaction for tyrosine (Pearse, '60), and Barrnett and Seligman's ('56) method for sulfydryl and disulfide protein.

Tests for carbohydrate. The periodic acid-Schiff reaction was used to identify 1–2 glycol linkages (Lillie, '54), if staining was abolished by acetylating sections with

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two parts acetic anhydride and three parts pyridine. Diastase incubations followed by the PAS reaction and Best's carmine stain were used to test for glycogen. Deacetylation of sections was accomplished with 0.1 N HCl at room temperature. The presence of acidic polysaccharide groupings was tested for by the following methods: methylene blue extinction (Pearse, '60) over a pH range from 2.82 to 8.18; staining with Alcian blue (Conn et al., '60); a carboxyl group reaction (Barrnett and Seligman, '58); toluidine blue metachromasia (Montagna, '51); methods for dialyzed iron reactions (Pearse, '60); reduction of staining by toluidine blue after incubating sections with hyaluronidase (Pearse, '60). Sialic acid was identified by the sialic acid reaction of Shear and Pearse ('63) and by reduction of a positive PAS-Alcian blue reaction after sialidase digestion (Spicer and Warren, '60). Sulfated polysaccharides were indicated by uptake of $Na_2S^{35}O_4$ in oviducts of frogs sacrificed at intervals from ten minutes to 72 hours (Heatley et al., '56) after injection.

Biochemical analyses

Samples of jelly dissected from frog eggs stripped from the cloaca were lyophilized and subjected to analysis by chromatographic and colorimetric methods. Samples were hydrolyzed in 1 N H_2SO_4 at 100°C for six hours then cooled and neutralized with $BaCO_3$ (Flood et al., '48; Forsyth, '50; Binger et al., '54).

Simple sugars were separated by descending chromatography on Whatman No. 1 paper, and by thin-layer chromatography with cellulose as substrate. Solvents used for paper chromatography were: (1) isopropanol : pyridine : acetic acid : water (8:8:1:4), after Gordon et al., '62; (2) water-saturated phenol with 1% NH_4OH (Partridge, '48); (3) water-saturated S-collidine (Taurog et al., '50); (4) n-butanol: acetic acid: water (4:1:5), after Dakshinamurti ('54). Successful solvents for thin-layer chromatography, using one or two dimensions, were: butanol: pyridine: 0.1 N HCl (5:3:2), after Bourrillon and Michon ('59), and the solvents (1) and (2) listed above. Spots were detected by one of the following methods: (1) dried papers or plates were sprayed with aniline-phthalate reagent and placed in an oven at 105°C for ten minutes (Partridge, '49); (2) dried papers or plates were dipped in a silver nitrate-acetone solution, dried, sprayed with 0.5 N alcoholic NaOH (Trevelyan et al., '50; Petronici and Safina, '53), were partially dried, and dipped in sodium thiosulfate solution (Benson et al., '52).

Chemical tests. Four basic chemical tests were carried out. These included: Benedict's test (Benedict, '11), for reducing sugars; Seliwanoff's ketose test, modified by Asnis and Brodie ('53); Bial's test for pentoses (Bial, '03); and the mucic acid test for galactose as modified by Cole (Hawk et al., '49).

Colorimetry. For each colorimetric reaction in samples of frog egg jelly, an absorption curve for a span of wavelengths was plotted to insure no interference with absorption maxima. Colorimetric tests for hexose included methods utilizing anthrone (Dreywood, '46; Scott and Melvin, '53), carbazole (Seibert and Atno, '46), and cysteine-sulfuric acid (Dische, '49, '55). Mannose was measured by a cysteine-sulfuric acid test (Dische et al., '49), fucose by the method of Dische and Shettles, ('48), hexosamines by methods utilizing indole (Dische and Borenfreund, '50) and acetylacetone (Elson and Morgan, '33, modified by Rimington, '40). Sialic acid was measured by perchloric acid methods of Seibert et al. ('48) and the diphenylamine method of Ayala et al. ('51), as used by Winzler ('55). Proteins were tested by the Folin-Ciocalteu method (Lowry et al., '50), albumin by the methyl orange technique of Bracken and Klotz ('53). Phosphate was measured by the Fiske and Subbarow ('25) techniques.

RESULTS

Histochemistry

Staining with mercury bromphenol blue produced an intense reaction for protein (fig. 1). The stain localized specifically in the basophilic, hematoxylin-positive granular component of mature glandular cells of the oviduct and gave a diffuse reaction in the background cytoplasm. Millon's reaction for tyrosine and the alloxan-Schiff reagent produced only diffuse staining of glandular cells in the oviducts fixed at

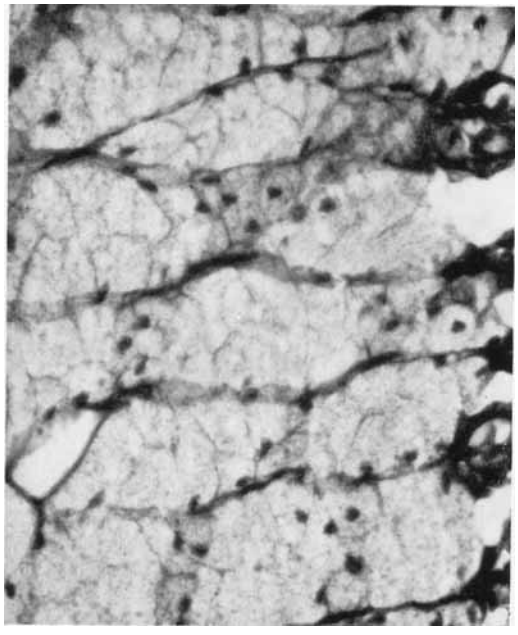

Fig. 1 Section shows secretory cells with nuclei basally displaced in glands of mature oviduct. Fine granules in these cells stain positively with mercury bromphenol blue for detection of protein. × 385.

various stages of growth. The ninhydrin-Schiff method gave a dark stain in cells that had appreciable accumulations of secretory product. Sulfydryl and disulfide groups detected by the technique of Barrnett and Seligman were localized in regions containing secretory granules and, with greatest intensity, in cells loaded with granules.

A strong, positive PAS reaction in secretory cells provided evidence for the presence of carbohydrates (fig. 2). The reaction was most pronounced in cells filled with secretory product. Best's carmine stain for glycogen was negative, and digestion with diastase did not diminish the intensity of PAS-staining. The carbohydrate which stained with PAS was evidently not glycogen. Acetylation to prevent oxidation of 1–2 glycol groups by periodic acid in the PAS reaction greatly reduced the intensity of staining. Deacetylation with 0.1 N HCl nearly restored the intensity of the positive PAS reaction. Methylene blue extinction tests over the pH range 2.82 to 8.18 provided no evidence for the presence of acid mucopolysaccharides. Glandular cells did not stain until the pH reached 6.12. Toluidine blue at pH 4.0 stained glandular cells orthochromatically. The stain was more intense in granules but was also present in the background. Localization in granules was lost at pH 5.0. Other tests for acid mucopolysaccharides were also negative in glandular cells. These were the carboxyl group reaction of Barrnett and Seligman, and staining with colloidal iron or Alcian blue.

Jelly glands of the oviduct contain sialic acid, as indicated by the positive red coloration developed in the Shear and Pearse reaction. After treatment with sialidase, the jelly glands stained purple rather than blue during Alcian blue-PAS staining. Spicer and Warren ('60) consider such a staining reaction a positive identification for sialic acid.

During time intervals up to 72 hours after injection of $Na_2S^{35}O_4$, jelly-secreting glands developed only slight radioactivity. This low level of incorporation of $Na_2S^{35}O_4$ indicates that their secretory product does not contain sulfated mucopolysaccharide.

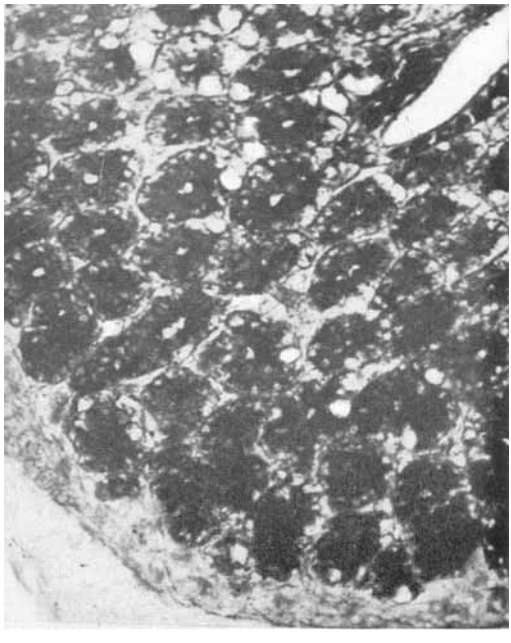

Fig. 2 Oviducal glands stained by the periodic acid-Schiff reaction. Secretory cells give an intense positive reaction. × 194.

Low incorporation could result from uptake into sulfur-containing enzymes or other proteins.

Chemical characterization of jelly

Test tube reactions. When tested with Benedict's solution, hydrolyzed jelly was strongly positive. Bial's test for pentoses and Seliwanoff's test for ketoses did not give distinctly positive reactions. Samples tested for mucic acid yielded crystals presumed to be mucic acid.

Paper chromatography. Samples of jelly for paper chromatography were hydrolyzed six hours at 100°C. The best solvent of those tested was isopropanol:pyridine:acetic acid:water. Galactose, fucose and glucosamine were the major constituents isolated by this solvent; and minor amounts of glucose, mannose and xylose and galactosamine were also identified. Phenol separated sugars into two groups: (1) glucose, galactose, and mannose; and (2) fucose, galactosamine, and glucosamine. Collidine, as a solvent, isolated the amine sugars, galactose, fucose and mannose. N-butanol:acetic acid:water separated three groups: galactosamine and glucosamine; glucose, galactose, and mannose; and fucose. The mixture butanol:methanol:water separated only fuocse. Very heavy application of sample was necessary to produce a separated spot for xylose and glucose, but these sugars could be definitely isolated with isopropanol:pyridine:acetic acid:water. Amine sugars gave the greatest intensity of color in spots separated with this mixture.

Thin layer chromatography. Thin-layer cellulose chromatography was used to determine with greater resolution the content of hexoses and hexosamines in the jelly. Known samples of these carbohydrates migrated and separated into four groups when isopropanol:pyridine:acetic acid:water was used as an eluate: (1) glucosamine and galactosamine; (2) glucose and galactose; (3) mannose; (4) xylose and fucose. A sample of jelly hydrolysate migrated over the span of distances attained by all of the known compounds but spots were most concentrated opposite groups 2 and 4. Butanol:pyridine:0.1 N HCl allowed separation of known samples of the same sugars into the same four groupings. Constituents of jelly hydrolysate spread over the range covered by all four groups. Water-saturated phenol separated jelly hydrolysate into two spots: one corresponding to levels achieved by migrating glucosamine and galactosamine; the other corresponding to the remaining five known compounds.

Two-dimensional chromatography permitted good separation of the seven types of sugars and sugar amines. Using isopropanol:pyridine:water:acetic acid as the first solvent, followed by butanol:pyridine:0.1 N HCl as the second (fig. 3), all these components except glucose and galactose migrated independently to form separate spots. The latter two migrated together as a single spot. Water-saturated phenol as first solvent, followed by butanol:pyridine:0.1 N HCl as second solvent, permitted good separation of the amino sugars and mannose. Glucose and galactose, as well as fucose and xylose, were poorly separated.

Colorimetry. Colorimetric determinations for hexoses (and methylpentoses) gave the following results. The anthrone procedure indicated that jelly contains about 20% hexoses on a weight basis. Values for hexoses measured by the cysteine-sulfuric acid method ranged from 20–25%. Hexoses measured 24.4% by the carbazole procedure.

Differences of optical densities (o.d.) in the range of 350–400 mμ permit one to distinguish galactose, glucose and mannose in the presence of other hexoses. The ratio (o.d. 370 mμ — o.d. 350 mμ)/ (o.d. 370 mμ — o.d. 375 mμ) gives relative values of 1 for galactose, 2 for glucose and about 0.5 for mannose (Dische et al., '49). A ratio less than 1 thus indicates the presence of mannose. In my experiments two standard samples of mannose gave ratios of 0.5 and 0.59; a standard of galactose gave a ratio of 1.0 and standard of glucose gave 2.0. Several samples of jelly hydrolysate gave ratios ranging from 0.8 to 1.2. It appears that the jelly contains much galactose, but mannose and glucose are probably present in only small quantities. Fucose measured by the method of Dische and Shettles ('48) was present in the jelly in a concentration of 10.4 mg/100 ml of lyophilized jelly.

Hexosamines react with indole in the indole-hydrochloric test to form compounds which absorb maximally at 492–494 mμ. Differences in optical density at 492 mμ and at 520 mμ in this test are proportional to the concentration of hexosamine. Results on samples of hydrolyzed jelly indicated that hexosamines comprise 34–45% of the dry weight of dehydrated jelly. Tested by the acetylacetone reaction for hexosamines, samples of jelly gave readings which indicated levels of hexosamines up to 45%.

Two tests for sialic acid by the tryptophan-perchloric acid technique gave values of 15.7% and 19.7%. According to the diphenylamine method, samples of jelly hydrolysate contained 13.3%, 15.2%, and 18.5% in three separate measurements. It thus appears that sialic acid constitutes approximately 13–20% of the dry weight of frog egg jelly.

A colorimetric test for inorganic phosphate by the Fiske and Subbarow technique was negative for a sample of jelly hydrolysate. Quantitative analysis of total phosphate (inorganic and organic) showed no color development for a sample containing 55 mg dehydrated hydrolysate per ml.

Estimation of proteins by the Folin-Ciocalteu method, utilizing a standard of assayed, dehydrated sheep blood serum, showed that frog egg jelly contains 28.7–41.4% protein. Albumin, measured by the methyl orange method, constitutes about 35% of the sample. Table 1 summarizes the results of the colorimetric tests.

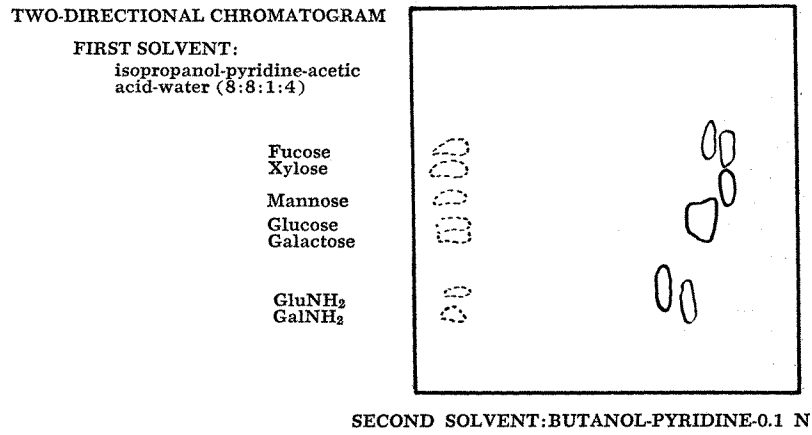

Fig. 3 Tracing of localization of spots from a sample of hydrolysate of frog egg jelly separated by two dimensional thin-layer chromatography on cellulose. Spots have been identified from a corresponding control chromatograph. Glucose and galactose have migrated together, although the other components are well separated. Dashed spots represent chromatogram visualized after migration with first solvent only. First solvent: isopropanol: pyridine: acetic acid: water (8:8:1:4). Second solvent: butanol: pyridine: 0.1 N HCl (5:3:2). Reduced by one-half.

TABLE 1

Calculated percentages of components present in frog egg jelly

Substance	Procedures for measurement	Range of percentages by weight [1]
Hexose and methylpentose	Anthrone, carbazole, cysteine-sulfuric acid	20.0–25.0
Hexosamine	Indole-HCl, Elson-Morgan	28.0–45.0
Sialic acid	Tryptophan-perchloric acid, diphenylamine	13.3–19.7
Protein	Folin-Ciocalteu, methyl orange	28.7–41.4

[1] The range of percentages calculated for various components indicates that the measurements varied in sensitivity and/or specificity. True percentages, totaling 100%, evidently lie somewhere within the reported ranges.

DISCUSSION

Histochemical evidence on sections of the oviduct from female *Rana pipiens* showed that the oviducal secretion contains protein, neutral mucopolysaccharide and sialic acid. There is no evidence that it contains a phospholipid or acid polysaccharide. Increased intensity of staining by the PAS reaction as the oviduct grows indicates that synthesis of carbohydrates is a major process in the development of the secretory product of oviducal glands.

Chemical analysis of hydrolysates of frog egg jelly indicates that jelly contains reducing sugar but no appreciable amounts of pentose or ketose. Galactose is present in abundance. Other hexoses and amino sugars identified in the jelly are fucose, mannose, glucosamine, and galactosamine and traces of xlyose and glucose. The negligible amount of inorganic phosphate in jelly indicates that phospholipids and nucleic acids are absent.

Colorimetric and chromatographic evidence indicates that jelly contains mucopolysaccharide. The lack of appreciable incorporation of $Na_2S^{35}O_4$ into jelly-secreting cells indicates that jelly is not an acidic mucoprotein. Colorimetric data indicate the relative constituents as follows: protein, approximately 28–42%; hexosamines, 28–45%, hexoses 20–25%, and sialic acid 15–18%. Undoubtedly these figures could be refined by more extensive sampling. Average values for protein, hexosamines, and hexoses derived from these data agree reasonably with those reported by Minganti ('55) for the jelly of *Bufo vulgaris*. He reported a variation of $\pm$ 15% of mean values for these constituents. Hexosamines apparently exist naturally in polysaccharide complexes in the form of N-acetyl hexosamines (Meyer, '57). My calculations were based on values for hexosamine hydrochloride, which is a product of hydrolysis of frog egg jelly. Molecular weights of the natural and derived hexosamines were considered sufficiently similar to permit meaningful calculations.

Values obtained for sialic acid in this study seem high; but published values are usually in terms of milligrams per cent of serum. Perhaps the methods used in my study were nonspecific for sialic acid in frog egg jelly, since these methods were designed for plasma or serum.

Chromatography indicates that the most abundant hexoses and hexosamines in jelly of *Rana pipiens* are galactosamine, glucosamine, fucose, and galactose. Colorimetric measurements show fucose to be about 10.4% of total jelly. These four major carbohydrate constituents of jelly were also reported by Bray and James ('49), Folkes, Grant and Jones ('50) and James ('51) for *Rana temporaria*. Hexosamine and galactose are found in the jelly coverings of *Rana japonica* and *Bufo vulgaris* (Hiyama, '49a,b,c). Folkes, Grant and Jones (op. cit.) reported small amounts of mannose and traces of glucose and xylose, and the present study confirms their findings in *Rana pipiens*. Xylose and glucose are separable by chromatography but require application of 100 to 200 mg of sample to make spots visible, in contrast to applications of 20 mg for the other carbohydrate constituents.

Oviducal jelly of *Rana pipiens* (present study), *R. temporaria* (Bray and James, '49; Folkes, Grant and Jones, '50; and James, '51) and *R. esculenta* (Minganti, '55) all possess the same major monosaccharides, namely, galactose and fucose. Jelly of *Bufo vulgaris* contains galactose, mannose, and fucose. *Discoglossus* sp. and the axolotl both lack galactose in their oviducal jelly. *Discoglossus* has predominantly glucose and mannose in its jelly, whereas *Siredon* has principally fucose and mannose (Minganti, '55).

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LITERATURE CITED

Asnis, R. E., and A. F. Brodie 1963 A glycerol dehydrogenase from *Escherichia coli*. J. Biol. Chem., *203:* 153–159.

Ayala, W., L. V. Moore and E. L. Hess 1951 The purple color reaction given by diphenylamine reagent. J. Clin. Invest., *30:* 781–785.

Barrnett, R. J., and A. M. Seligman 1956 Histochemical demonstration of protein-bound sulfhydryl groups. Science, *116:* 323–327.

——— 1958 Histochemical demonstration of protein-bound alpha-acylamide carboxyl groups. J. Biophys. Biochem. Cytol., *4:* 169–176.

Bataillon, E., 1919 Analyse de l'activation par la technique des oeufs nus et la polyspermie expérimentale chez les batrachiens. Ann. Sci. Nat. Zool., *3:* 1–38.

Benedict, S. R. 1911 The detection and estimation of glucose in the urine. J. Am. Med. Assoc., *57:* 1193–1194.

Benson, A. A., J. A. Brasshom, M. Calvin, A. G. Hirsch, S. Kaunguchi, V. Lynch and N. E. Talbert 1952 The path of carbon in photosynthesis. J. Biol. Chem., *196:* 703–716.

Bial, M. 1903 Ueber die Diagnose der Pentosarie mit dem von mir angege benen Reagent. Deut. Med. Wochschr., *29:* 477–478.

Binger, H. P., J. F. Sullivan and C. O. Jensen 1954 The isolation and analysis of hemicellulose from orchard grass. J. Agr. Food Chem., *2:* 696–700.

Bourrillon, R., and J. Michon 1959 Résolution et détermination quantitative par chromatographie sur papier des glucides lies aux proteines. Bull. Soc. Chim. Biol. (Paris), *41:* 267–276.

Bracken, J. S., and I. M. Klotz 1953 A simple method for the rapid determination of serum albumin. Am. J. Clin. Path., *23:* 1055–1058.

Bray, H. G., and S. P. James 1949 Examination of sugars produced by hydrolysis of jelly-like mucin of frog spawn. First Int. Congr. Biochem., Abstr., p. 225.

Conn, H. J., M. A. Darrow and V. M. Emmel 1960 Staining Procedures. Second Edition. Williams and Wilkins, Baltimore.

Dakshinamurti, K. 1954 Current Sci. (India), *23:* 89, Quoted from R. J. Block, E. L. Durrum, and G. Zweig 1958 A Manual of Paper Chromatography and Paper Electrophoresis. Second Edition. Acad. Press., New York.

Dische, Z. 1949 Spectrophotometric method for the determination of free pentose and pentose in nucleotides. J. Biol. Chem., *181:* 379–392.

——— 1955 New color reactions for determination of sugars in polysaccharide. In: Methods of Biochemical Analysis, David Glick, ed. Vol. II, Interscience Publ., New York. pp. 313–358.

Dische, Z., and E. Borenfreund 1950 A spectrophotometric method for the microdetermination of hexosamines. J. Biol. Chem., *184:* 517–522.

Dische, Z., and L. B. Shettles 1948 A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem., *175:* 595–603.

Dische, Z., L. B. Shettles and M. Osnos 1949 New specific color reactions of hexoses and spectrophotometric micromethods for their determination. Arch. Biochem., *22:* 169–184.

Dreywood, R. 1946 Qualitative test for carbohydrate material. Anal. Chem., *18:* 499.

Elson, L. A., and W. T. J. Morgan 1933 A colorimetric method for the determination of glucosamine and chrondrosamine. Biochem. J., *27:* 1824–1828.

Fiske, C. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375–400.

Flood, H. E., E. L. Hirst and J. K. M. Jones 1948 Quantitative analysis of mixtures of sugars by the methods of partitive chromatography. J. Chem. Soc., 1679–1683.

Folkes, B. F., R. A. Grant and J. K. M. Jones 1950 Frog spawn mucin. J. Chem. Soc., *8:* 2136.

Forsyth, W. G. C. 1950 Studies on the more soluble complexes of soil organic matter. Biochem. J., *46:* 141–146.

Gordon, H. T., W. Thornburg and L. N. Werum 1956 Rapid paper chromatography of carbohydrates and related compounds. Anal. Chem., *28:* 849–855.

Hawk, P. B., B. H. Oser and W. H. Summerson 1949 Practical Physiological Chemistry. Blakiston Company, Philadelphia.

Heatley, N. G., D. W. Jerrome, M. A. Jennings and H. W. Florey 1956 On the fixation of mucin and the preparation of autoradiographs. Quart. J. Exp. Physiol., *41:* 124–130.

Hiyama, H. 1949a Biochemical studies on carbohydrates. CII. On the jelly of toad and frog eggs. Tôhoku J. Exp. Med., *50:* 373–378.

——— 1949b Biochemical studies on carbohydrates. CIII. Hexosamine in the toad mucin. Tôhoku J. Exp. Med., *50:* 379–383.

——— 1949c Biochemical studies on carbohydrates. CIV. Hexosamine in the frog mucin. Tôhoku J. Exp. Med., *50:* 385–387.

Humphries, A. A., Jr. 1966 Observations on the deposition, structure, and cytochemistry of the jelly envelopes of the egg of the newt, *Triturus viridescens*. Devel. Biol., *13:* 214–230.

James, S. P. 1951 An examination of frog jelly. Biochem. J., *49:* liv.

Lillie, R. D. 1954 Histopathologic Technic and Practical Histochemistry. The Blakiston Co., New York.

Lowry, O. H., N. J. Rosebrough, A. L. Faro and R. J. Randall 1950 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265–275.

Meyer, K. 1957 The chemistry of the mesodermal ground substances. The Harvey Lectures, *51:* 88–112.

Minganti, A. 1955 Chemical investigations on amphibian egg jellies. Exp. Cell Res., Suppl. *3:* 248–251.

Montagna, W., H. B. Chase and H. P. Melaragno 1951 Histology and cytochemistry of human skin. I. Metachromasia in the mons pubis. J. Natl. Cancer Inst., *12:* 591–597.

Partridge, S. M. 1949 Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature, *164:* 443.

Pearse, A. G. E. 1960 Histochemistry, Theoretical and Applied. Second Edition. Little, Brown, Boston.

Petronici, C., and G. Safina 1953 The technique of chromatographic analysis, in particular the analysis by paper chromatography of the amino acids and simple sugars. Conserve e deriv agrumari, Palermo, 2: 3–11 from 1953 Chem. Abstr., *47:* 11297.

Rimington, C. 1940 Seromucoid and the bound carbohydrate of serum proteins. Biochem. J., *34:* 931–940.

Schulz, F. N., and M. Becker 1935 Uber die Kohlenhydrate der Eiweissdruse von *Rana esculenta.* Biochem. Z., *280:* 217–226.

Schulz, F. M., and F. Ditthorn 1900 Galaktosamin, ein neuer Amidozucker, als Spaltungsprodukt des Glykeproteids der Eiweissdruse des Frosches. Z. Physiol. Chem., *29:* 373–385.

Scott, T. A., and E. H. Melvin 1953 Determination of dextran with anthrone. Anal. Chem., *25:* 1656–1661.

Seibert, F. B., and J. Atno 1946 Determination of polysaccharide in serum. J. Biol. Chem., *163:* 511–522.

Seibert, F. B., M. L. Pfoff and M. V. Seibert 1948 A serum polysaccharide in tuberculosis and carcinoma. Arch. Biochem., *18:* 279–295.

Shear, M., and A. G. E. Pearse 1963 A direct chemical method for the demonstration of sialic acid. Nature, *198:* 1273–1275.

Spicer, S. S., and L. Warren 1960 The histochemistry of sialic acid containing mucoproteins. J. Histochem. Cytochem., *8:* 135–137.

Subtelny, S., and C. Bradt 1961 Transplantations of blastula nuclei into activated eggs from the body and from the uterus of *Rana pipiens II.* Devel. Biol., *3:* 96–114.

Taurog, H., W. Tong and I. L. Chaikoff 1950 The monoiodotyrosine content of the thyroid gland. J. Biol. Chem., *184:* 83–97.

Trevelyan, W. E., D. P. Proctor and J. S. Harrison 1950 Detection of sugars on paper chromatograms. Nature, *166:* 444–445.

Winzler, R. J. 1955 Determination of serum glycoproteins. Methods of Biochemical Analysis, D. Glick, ed. Vol. II, Interscience Publ., New York, pp. 279–312.