Greenslade, F. C., McCormack, J. J., Hirsch, A. F., and Davanzo, J. P. (1973), *Biol. Reprod. 8,* 306.
Grey, R. D., Wolf, D. P., and Hedrick, J. L. (1974), *Dev. Biol. 36,* 44.
Gusseck, D. J., and Hedrick, J. L. (1971), *Dev. Biol. 25,* 337.
Hedrick, J. L., Smith, A. J., Yurewicz, E. C., Oliphant, G., and Wolf, D. P. (1974), *Biol. Reprod. 11,* 534.
Holden, K. G., Yim, M. C. F., Griggs, L. J., and Weisbach, J. A. (1971a), *Biochemistry 10,* 3105.
Holden, K. G., Yim, M. C. F., Griggs, L. J., and Weisbach, J. A. (1971b), *Biochemistry 10,* 3110.
Johnston, J. P., and Ogston, A. G. (1946), *Trans. Faraday Soc. 42,* 789.
Katagiri, C. (1966), *Embryologia 9,* 159.
Katagiri, C. (1968), *SABCO J. 4,* 33.
Katagiri, C. (1973), *Dev. Growth, Differ. 15,* 81.
Lee, P. A. (1967), *J. Exp. Zool. 166,* 99.
Metz, C. B. (1967), in Fertilization, Vol. 1, Metz, C. B., and Monroy, A., Ed., New York, N.Y., Academic Press, p 163.
Monroy, A. (1965), Chemistry and Physiology of Fertilization, New York, N.Y., Holt, Rinehart, and Winston.
Ouchterlony, O. (1948), *Acta Pathol. Microbiol. Scand. 25,* 186.
Oliphant, E. E. (1970), Ph.D. Thesis, University of California, Davis.
Shaver, J. R., Barch, S. H., and Attinasi, L. C. (1973), *Biol. Reprod. 8,* 112.
Shaver, J. R., Barch, S. H., and Umpiere, C. C. (1970), *J. Embryol. Exp. Morphol. 24,* 209.
Shivers, A. L. (1965), *Biol. Bull. 128,* 328.
Shivers, A., and James, J. H. (1970a), *Anat. Rec. 166,* 541.
Shivers, A., and James, J. H. (1970b), *Nature (London) 227,* 183.
Steinke, J. H., and Benson, D. G., Jr. (1970), *J. Morphol. 130,* 57.
Waddell, W. J. (1956), *J. Lab. Clin. Med. 48,* 311.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.
Wolf, D. P. (1974), *Dev. Biol. 38,* 14.
Wolf, D. P., and Hedrick, J. L. (1971a), *Dev. Biol. 25,* 348.
Wolf, D. P., and Hedrick, J. L. (1971b), *Dev. Biol. 25,* 360.
Wyrick, R. E., Nishihara, T., and Hedrick, J. L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2067.

Fucosyl-Glycoprotein and Precursor Pools in HeLa Cells†

Peter D. Yurchenco‡ and Paul H. Atkinson*

ABSTRACT: An enzymatic-radioactive isotope method has been developed for the direct quantitation of L-fucose in amounts as low at 0.5 ± 0.05 nmol. Fucose kinase is used to transfer [^{32}P]phosphate from ATP to [^{3}H]fucose. The labeled enzymatic products are then separated electrophoretically and the amount and specific activity of the fucose are determined from the known specific activity of the phosphate donor. This assay has been used to measure the GDP-L-fucose and macromolecular fucose in HeLa cells after extraction and purification of the sugar. It has been determined there are 0.5 nmol of GDP-L-fucose in 10^7 cells with a nine- to tenfold dilution of specific activity in converting L-[^{3}H] fucose to GDP-L-[^{3}H]fucose. After 2 to 3 days of labeling, the GDP-L-[^{3}H]fucose pool is essentially at equilibrium with the macromolecular pool, and hence it can be concluded that the dilution of label is due to a nine- to tenfold contribution to GDP-L-fucose from an endogenous source, as compared to exogenously supplied fucose. The fucosyl-glycoprotein pool has been shown to be much larger containing 6 to 8 nmol of fucose in 10^7 cells. It has further been shown that GDP-fucose is the only soluble fucose intermediate present in significant amount.

Radioactive fucose is a useful precursor for studying the biosynthesis of glycoproteins (Bekesi and Winzler, 1967; Jabbal and Schachter, 1971; Trujillo and Gan, 1971) including those in the HeLa cell surface (Shen and Ginsburg, 1968; Atkinson and Summers, 1971; Atkinson, 1973). In HeLa cells, radioactive fucose is found almost entirely in glycoprotein (Shen and Ginsburg, 1968; Atkinson, 1975) and the label is not distributed into other sugars (Kaufman and Ginsburg, 1968). However, the rate at which the radioactive fucose is incorporated into glycoprotein is not necessarily a reflection of the true rate of synthesis because the soluble precursor pool, GDP-fucose, can be derived from two sources: namely, exogenous fucose (Bekesi and Winzler, 1967; Kaufman and Ginsburg, 1968; Ishihara et al., 1968; Ishihara and Heath, 1968) and endogenously synthesized GDP-mannose (Foster and Ginsburg, 1961). Radioactive GDP-fucose will thus be diluted to an extent depending on the endogenous contribution, and thus the specific radioactivity may vary with growth conditions (Nowakowski et al., 1972). Therefore, the determination of glycoprotein synthetic rates from the use of radioactive precursors will depend on measuring GDP-fucose. Furthermore, it may be possible to infer precursor-product relationships from mea-

† From the Department of Pathology (P.H.A.) and Department of Developmental Biology and Cancer, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461. *Received December 8, 1974.* This investigation was supported by Research Grants from the National Institutes of Health (CA 13402 and CA 06576) and National Institutes of Health Training Grant No. 5T5 GM 1674 from the National Institute of General Medical Sciences, as well as from the Damon Runyon Memorial Fund (DRG 1204).

‡ Data in this paper are from a thesis which was submitted in partial fulfillment for the Degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.

surement of the specific radioactivity of fucose in various macromolecular pools (e.g., internal material, cell surface glycoprotein, glycoprotein released to the medium) after pulse labeling with radioactive fucose. Measurement of fucose in these various pools from the number of cells deemed feasible for one point in a kinetic series is difficult for two reasons. Firstly, the quantity of fucose-labeled material recoverable from some pools is very small and, secondly, the fucose must be extracted and purified before unambiguous quantitation can be made.

This paper describes a method for the quantitation of L-fucose with a sensitivity of 0.5 ± 0.05 nmol. GDP-fucose is extracted and purified from cells labeled with [^{3}H]fucose and then hydrolyzed to yield fucose which is further purified. The remaining insoluble cell material is hydrolyzed to release fucose from macromolecular material which is then purified from other contaminating sugars. L-Fucose kinase is used to transfer a terminal radioactive phosphate from [γ-^{32}P]ATP to [^{3}H]fucose according to the equation:

$$[^3\text{H}]\text{fucose} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \xrightarrow{\text{fucose kinase}} [^3\text{H}]\text{fucose 1-}[^{32}\text{P}]\text{phosphate} + \text{ADP}$$

Since the specific activity of the [^{32}P]ATP is known, the quantity of fucose converted can be determined. From this, the size and specific radioactivity of [^{3}H]fucose labeled GDP-fucose and glycoprotein-fucose pools in the cells were calculated.

Experimental Procedures

Cells, Radioactive Labeling, and Materials. HeLa S_3 cells were grown as previously described (Atkinson and Summers, 1971) in minimal essential medium supplemented with 3.5% calf serum, 3.5% fetal calf serum (Grand Island Biological Co., New York, N.Y.), and 1% glutamine. Cells were labeled with 1.5 μCi/ml of L-[^{3}H]fucose as the cells grew from a density of 1.5–2.0 × 10^5/ml to 7–10 × 10^5/ml in a period of 2–3 days. Cells, tested periodically for mycoplasma contamination, were found to be free by both a culturing procedure (Levine et al., 1968) and by an enzymatic assay (Levine, 1972).

α-L-Fucose 1-phosphate (Prihar and Behrman, 1973) was a generous gift from Dr. H. S. Prihar, Department of Biochemistry, Ohio State University, Columbus, Ohio. L-[^{14}C]Fucose 1-phosphate and L-[^{14}C]fuconic acid were generous gifts from Dr. H. Schachter, Department of Biochemistry, University of Toronto. Other radioactive compounds included: L-[6-^{3}H]fucose (13.4 Ci/mmol, New England Nuclear, Boston, Mass.) (fucose labeled in position 6 was used unless otherwise noted), L-[1,5,6-^{3}H]fucose (1.025 Ci/mmol, New England Nuclear), L-[1-^{3}H]fucose (>500 mCi/mmol, Amersham Searle, Arlington Hts., Ill.), GDP-L-[U-^{14}C]fucose (170 mCi/mmol, Amersham Searle), and [γ-^{32}P]ATP (initially >10 Ci/mmol, New England Nuclear).

Extraction of GDP-Fucose. Cells (8×10^8) labeled with L-[^{3}H]fucose were harvested by centrifugation, washed with two 40-ml portions of minimal essential medium, and extracted twice with 5–6 vol of 60% ethanol in a boiling water bath for 5 min each (Kaufman and Ginsburg, 1968). The combined extracts were centrifuged at 900g for 5 min and the pellet used for determination of macromolecular fucose. The supernatant was re-centrifuged at 33,000g for 20 min and 1 ml of the supernatant mixed with 0.5 μCi of GDP-L-[^{14}C]fucose ("control"). The remainder ("sample") and control extracts were evaporated to dryness under vacuum at 30°, redissolved in 1 ml of water, and centrifuged at 33,000g for 20 min to remove ethanol-soluble but water-insoluble material (glycoprotein).

Chromatography and High Voltage Paper Electrophoresis. Descending paper (Whatman, 3MM) chromatography (Grollman et al., 1965) was carried out with: (a) 95% ethanol–1.0 *M* ammonium acetate (7:3; solvent I); (b) isobutyric acid–28–30% ammonium hydroxide (5:3; solvent II); (c) pyridine–ethyl acetate–water (1.0:3.6:1.15, upper organic phase; solvent III). High voltage paper electrophoresis (HVPE)[1] was carried out on 4-cm wide strips of Whatman No. 3MM paper in pyridine–acetic acid–water (1:10:89, pH 3.6). Air-dried paper strips from either chromatography or HVPE, when analyzed for radioactivity, were cut into 1-cm or 0.25-in. fractions and eluted with 1.0 ml of water, and aliquots (10–50 μl) were counted. Material pooled for further analysis was passed through a Swinnex 0.22-μ (Millipore Corp.) filter to remove suspended matter. Ascending thin-layer chromatography (TLC) utilized Kieselguhr G (Merck, Germany) plates, spread 300-μ thick and impregnated with 0.15 *M* NaH_2PO_4 developed with ethyl acetate–methanol–1-butanol–water (16:3:3:2) (TLC system I; Talukder, 1971) or commercially spread silica gel G plates, 250-μ thick (Analtech, Pittsburgh, Pa.), impregnated with 0.03 *M* H_3BO_3 and developed with 1-butanol–acetone–water (4:5:1) (TLC system II; Lato et al., 1968). Sugar standards on paper strips were stained with $AgNO_3$ (Trevelyan et al., 1950). Unlabeled nucleotides and nucleotide sugars were located by ultraviolet (uv) adsorption. Inorganic phosphate and pyrophosphate were located by an ammonium molybdate spray reagent (Kakáč, 1963). Unlabeled fucose phosphate was detected by spraying the horizontally hung chromatogram with alkaline phosphatase (Worthington, 0.02 mg/ml, 34 units/mg, in 20 m*M* Tris-HCl, pH 8.2), incubating the paper in a humidified incubator at 37° for 25 min, and then air drying the paper and staining with $AgNO_3$ reagent. Desalting was performed with small (0.5 cm × 8.7 cm) or large (1.5 × 20 cm) columns of Amberlite MB-1 ion-exchange resin equilibrated with distilled water. Unless otherwise stated, Sephadex G-25, fine (0.9 × 142 cm), equilibrated and eluted with 0.05 *M* ammonium acetate and Sephadex G-10 (1.5 × 92 cm) equilibrated and eluted with distilled water were used for gel filtration.

Protein and Radioactivity Determinations. Protein concentration was determined by the method of Lowry et al. (1951) modified so each assay tube contained 0.1 μmol of dithiothreitol, or by the Cl_3CCOOH precipitation method of Bucher (1947). Radioactivities in aqueous samples were determined in a Beckman LS-233 liquid scintillation spectrometer as previously described (Atkinson and Summers, 1971). Radioactivity in samples on paper was determined after addition of 1.0 ml of water to the samples and 6.0 ml of scintillation fluid. The scintillation spectrometer windows were set to discriminate ^{3}H and ^{32}P radioactivity with a spill of 0.5% of ^{32}P into the ^{3}H channel, and to discriminate ^{3}H and ^{14}C radioactivity with a spill of 10–15% of ^{14}C into the ^{3}H channel. With these settings the tritium counting efficiency in water was determined to be 13.6%, the ^{14}C efficiency was 32%, and the ^{32}P efficiency was 70%. Radioactivity measurements have been corrected for quenching rel-

[1] Abbreviations used are: HVPE, high voltage paper electrophoresis; TLC, thin-layer chromatography; Cl_3CCOOH, trichloroacetic acid; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid, disodium salt; F-1-P, fucose 1-phosphate.

Table I: Fucose Kinase Purification.

Purification Step[a]	Protein[b] (mg/ml)	Total Vol[c] (ml)	Sp Act. (Units/mg of Protein)	Total Act. (Units)
Crude	32.6	385	0.09	1130
Ammonium sulfate (0–30%)	23.8	146	1.20	4170
Clarification	12.8	106	4.24	5750
Sephadex G-100	3.2	123	6.03	2370
DEAE-Cellulose	5.9	2	341	4020

[a] Described under Experimental Procedures. [b] Measured by the method of Bucher (1947). [c] Total volumes expressed are corrected on the assumption that all material in a step was processed in the next step. The actual volumes used for further purification were, respectively, 385, 122, 88, 81, and 1.3 ml; the remainder was discarded.

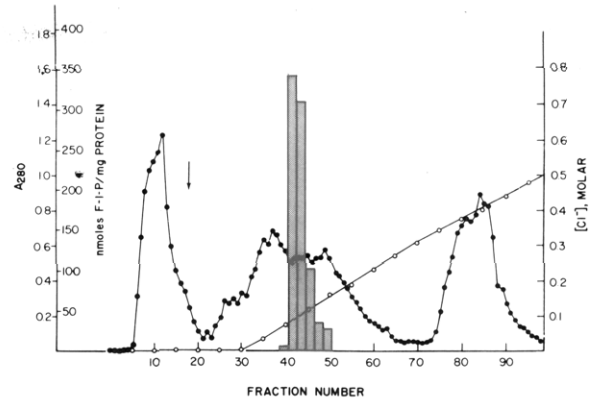

FIGURE 1: Purification of fucose kinase by DEAE-cellulose. Sephadex G-100 fractions were chromatographed on DEAE-cellulose (Experimental Procedure). Absorbance at 280 nm (●) and chloride concentration (○) were determined. Fucose kinase activity is expressed as units per milligram of protein (bar graph). Peak activity for fucose dehydrogenase is indicated with an arrow.

ative to that in water (and can be converted to disintegrations per minute by dividing by the above efficiencies).

Preparation of Fucose Kinase. The purification of fucose kinase from pig liver, based on the methods of Ishihara et al. (1968) and Jabbal and Schachter (1971), was modified as described below. About 150 g of fresh pig liver (Max Insel Cohen Inc.) was homogenized for 3–4 min in 4 vol of 0.25 *M* sucrose containing 1 m*M* dithiothreitol, 0.15 m*M* $PhMeSO_2F$, and 0.1 m*M* EDTA, filtered through cheesecloth, and centrifuged at 14,500*g* for 1 hr. The supernatant was adjusted to 30% saturation with ammonium sulfate and centrifuged at 33,000*g* for 30 min, and the pellet was resuspended in approximately 120 ml of 0.03 *M* sodium phosphate buffer (pH 7.4), 10% glycerol (w/v), 1 m*M* dithiothreitol, 0.15 m*M* $PhMeSO_2F$, and 0.1 m*M* EDTA (standard buffer). This fraction was then centrifuged a second time at 33,000*g* for 1 hr, the pellet discarded, and blue dextran added to the clarified supernatant. A column of Sephadex G-100 (2.7 × 80 cm), preequilibrated several days with standard buffer and loaded with a 50–70-ml aliquot of preparation, was eluted with standard buffer in 10-ml fractions. The bulk of the protein was excluded from the column while the fucose kinase activity, found to be just included, was pooled and up to 350 mg of protein of this material was loaded onto a column of DEAE-cellulose (Whatman DE-52; 2.5 × 20 cm) equilibrated with standard buffer. The column was then washed with 50–60 ml of standard buffer and eluted with NaCl in a linear concentration gradient (500 ml of standard buffer and 500 ml of 0.55 *M* NaCl). Fractions (10 ml) were collected and chloride concentration was determined by Mohr titration (Pierce and Haenisch, 1958). Peak fucose kinase activity eluted at approximately 0.1 *M* chloride and about six to eight fractions on either side of the peak were pooled in pairs, precipitated by 0.48 g/ml of ammonium sulfate, redissolved in 1–2 ml of standard buffer, and stored in liquid nitrogen where they were stable indefinitely.

Enzyme Assay. Single Label Assay of Fucose Kinase. The incubation mixture was as described (Ishihara et al., 1968) except the pH conformed to the enzyme optimum as determined here (pH 7.3) and L-[6-^{3}H]fucose was used as substrate in a total volume of 300 μl. L-[1-^{3}H]Fucose and L-[1,5,6-^{3}H]fucose could not be used since the tritium label is completely lost from position 1 when incubated with enzyme. Tubes, incubated for 20 min at 37°, were then boiled 1 min, cooled on ice, and centrifuged to remove precipitated protein. Supernatant (50 μl) was either chromatographed on paper in solvent I overnight or subjected to paper electrophoresis at 37.5 V/cm for 3.5 hr. One unit of enzyme activity is defined as the amount of enzyme needed to convert 1 nmol of fucose to fucose phosphate in 20 min at 37°.

^{3}H–^{32}P Double Label Assay of Fucose Kinase. The incubation mixture (in a total of 250 μl) consisted of 150–200 μg of protein containing fucose kinase (150–600 units/mg), a 100-μl [^{3}H]fucose sample derived from cells (of unknown specific activity) or from a 10-nmol [^{3}H]fucose standard (of known specific activity; 300,000 cpm/10 nmol), and 100 μl containing 100 nmol of [^{32}P]ATP (approximately 6 × 10^6 cpm), 1 μmol of $MgCl_2$, 2.5 μmol of KF, and 20 μmol of Tris-HCl (pH 7.3). After incubation for 2.5 hr at 37°, tubes were mixed with 0.5 ml of cold 95% ethanol and centrifuged, and 150-μl aliquots of supernatant were electrophoresed on paper strips 148 cm long for 7–9 hr at 36 V/cm.

Fucose Dehydrogenase. The same assay system as for fucose kinase (single label) was used except that the enzyme product, fuconic acid, was found to migrate slower than fucose phosphate in HVPE ($R_{F\text{-}1\text{-}P}$[1] 0.53) and chromatographed slightly slower than fucose in solvent I.

Results

Enzyme Purification and Characteristics. Ammonium sulfate precipitation, clarification, and Sephadex chromatography resulted in several-fold purification and activation of the fucose kinase (Table I). Ion exchange chromatography on DEAE-cellulose provided the largest single step increase in specific enzyme activity (about 50-fold compared with the previous step and about 3500-fold compared to the crude homogenate) as well as some activation (Table I) and also removed fucose dehydrogenase activity which eluted from the column with the wash (Figure 1). Fucose kinase specific activity varied in preparation to preparation from 100 to >600 units/mg of protein.

Unlike the dehydrogenase, fucose kinase was markedly inhibited by 1 m*M* *N*-ethylmaleimide. Enzyme activity was linearly dependent on protein concentration in the range 0–100 μg/ml, in good agreement with previously published results (Ishihara et al., 1968). The pH optimum of the enzyme was 7.3 and the K_m for the purified enzyme with L-fucose as substrate was 1.0×10^{-4} *M* and that for ATP as substrate was 2.9×10^{-3} *M*. The fucose 1-phosphate prod-

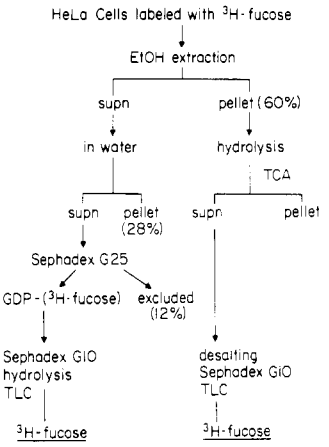

FIGURE 2: Flow chart for isolation and purification of GDP-fucose and glycoprotein-fucose pools. Prior to assay with fucose kinase, [^{3}H]fucose was isolated and purified from HeLa cell soluble and macromolecular pools as described in the text.

uct was authenticated with standard by chromatography in solvents I and II.

Purified fucose kinase was incubated with various labeled sugars as substrate (0.33 m*M*) and the percent conversion to sugar phosphates determined (Table II). The enzyme preparation showed activity (relative to fucose) with a number of sugars, especially glucose and galactose. All sugar phosphates (except that of rhamnose) migrated at a different rate as compared to fucose phosphate (Table II). It was found that by electrophoresing the enzyme products for 6 hr or more it is possible to completely separate these phosphates.

Purification of Fucose from HeLa Cell GDP-Fucose and Glycoprotein-Fucose Pools. Ethanol-extracted soluble and macromolecular material from cells radioactively labeled 2–3 days with [^{3}H]fucose were prepared as outlined under Experimental Procedures (see flow chart, Figure 2). In order to determine both the specific radioactivity and the quantity of GDP-[^{3}H]fucose per given number of cells, the sample aliquot containing about 88% of the total soluble extract and the control containing GDP-[^{14}C]fucose were processed independently through the subsequent steps (Figure 2). GDP-Fucose, on Sephadex G-25, eluted anomalously, and advantageously, after free fucose since it was seen to be substantially separated from earlier eluting material including macromolecular fucose, fucose 1-phosphate, and free sugars. The peak containing GDP-fucose was pooled, concentrated, and further chromatographed on Sephadex G-10 where it was excluded, thus separating it from remaining free sugars. Material excluded on G-10 cochromatographed (97%) with GDP-fucose in solvent II and thus the labeled material was essentially radiochemically pure at this step. This material was pooled and hydrolyzed (1 *N* HCl, 60 min, 100°), deacylating *N*-acetyl sugars, and GDP-fucose was hydrolyzed (100%), releasing free fucose. The hydrolyzed labeled material was eluted from a column of Amberlite MB-1 (removing hexosamines and salts), concentrated, and chromatographed on TLC system I. This step separated fucose from glucose, fructose, galactose, mannose, remaining amino sugars, arabinose, rhamnose, xylose, and most of ribose. Fucose-containing fractions were pooled, desalted on Amberlite, reduced in volume, and chromatographed on TLC system II removing any remaining ribose. This material (Figure 2), representing the GDP-fucose pool, was saved for assay with fucose kinase.

Table II: Specificity of the Fucose Kinase Preparation for Various Sugars.[a]

	Rel Rates of Formation[b]	$R_{F\text{-}1\text{-}P}$ of Sugar Phosphates[c]
L-Fucose	100	1.00
D-Glucose	122	0.95
D-Galactose	130	0.96
D-Mannose	80	0.95
D-Glucosamine	0	0.05
N-Acetyl-D-glucosamine	3	1.38
D-Ribose	19	1.07
D-Xylose	0.6	1.07
L-Rhamnose	0.3	1.00

[a] Fucose kinase, purified on DEAE-cellulose, was incubated with 0.1 μmol of various ^{3}H or ^{14}C-labeled sugars for 30 min. Relative activities were expressed as well as the $R_{F\text{-}1\text{-}P}$ values in the electrophoretic system. Reaction mixtures were chromatographed in solvent I and subjected to HVPE for 3.5 hr with authentic fucose 1-phosphate. Except for glucosamine, products of the various substrates chromatographed and migrated similarly to fucose 1-phosphate. There was no labeled product in the position of authentic glucosamine 6-phosphate with radioactive glucosamine as substrate. [b] The figures express the rate of sugar phosphate formation relative to the rate of fucose 1-phosphate formation. [c] $R_{F\text{-}1\text{-}P}$ is the migration of sugar phosphate in HVPE relative to the migration of fucose 1-phosphate. These differences in migration rates allow satisfactory separation of sugar phosphates from fucose phosphate after electrophoresis for 6 or more hr (Figure 4).

The quantity of GDP-[^{3}H]-fucose radioactivity originally present in the control aliquot, and hence in the given number of cells, was determined from the recovery of ^{14}C radioactivity derived from the initially added GDP-[^{14}C]fucose. An estimate of cell pool was thus not dependent on whether the Control is recovered to the same extent as the Sample. Such determinations were made only after the extracted GDP-[^{3}H]fucose or [^{3}H]fucose had reached radiochemical purity. Recoveries of ^{14}C radioactivity at the final step in purification were typically 10–15%. The large sample aliquot was utilized only for determination of the specific radioactivity of the GDP-[^{3}H]fucose pool. Since ^{32}P was used in this determination, GDP-[^{14}C]fucose could not conveniently be utilized as a tracer in this aliquot and thus the independent processing of the extra control sample was necessary.

In order to determine the specific activity of [^{3}H]fucose in the growth medium it was necessary to quantitate the free fucose in the serum. Calf serum (0.5 ml) plus 0.5 ml of fetal calf serum were mixed with [^{3}H]fucose as marker, and this was purified through the same chromatographic steps as GDP-fucose and saved for assay by fucose kinase.

Sixty percent of the [^{3}H]fucose was released by mild acid hydrolysis (0.1 *N* HCl, 100°, 45 min) from the macromolecular material left after ethanol extraction of the soluble pool (Figure 2). Since hydrolysis of smaller amounts of material resulted in >90% release (data not shown) the remaining 30–40% was trapped in the residue pellet. The viscous material obtained after hydrolysis was centrifuged to remove particulate matter (not required when smaller amounts are hydrolyzed) and the supernatant mixed with an equal volume of 10% cold trichloroacetic acid. The precipitate which formed was removed by centrifugation at 33,000g for 30 min and this supernatant desalted on a large Amberlite column. The eluate (90–100 ml) was reduced to a small volume and chromatographed on a Sephadex G-10

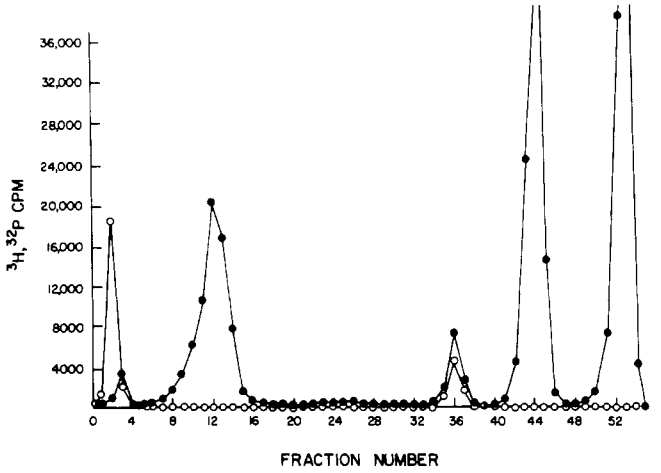

FIGURE 3: Double-label fucose kinase assay: 10 nmol of "standard", HVPE profile. An example of a high voltage paper electrophoresis profile after 10 nmol of L-[6-^{3}H]fucose was incubated with [γ-^{32}P]ATP and fucose kinase preparation for 1.5 hr as described under Experimental Procedures (electrophoresis was at 37.5 V/cm for 3 hr); 1-cm strips were assayed for radioactivity. The anode is to the right. The ^{3}H peaks (O) (as determined in other electrophoregrams of authentic standards) from left to right are (a) fucose and (b) fucose phosphate. The ^{32}P peaks (●) from left to right are: (a) unknown (small peak just ahead of fucose), (b) probably AMP and ADP, since both have been shown to migrate at about the same rate as this peak (data not shown), (c) fucose phosphate, (d) ATP, and (e) inorganic phosphate. Pyrophosphate has been shown to migrate ahead of inorganic phosphate toward the anode. Fuconic acid, although not present, is known to migrate midway between fucose and fucose phosphate with an $R_{F\text{-}1\text{-}P}$ of 0.53. $R_{F\text{-}1\text{-}P}$ values for the other sugar phosphates are shown in Table II. Only fucose phosphate appears as a double labeled peak.

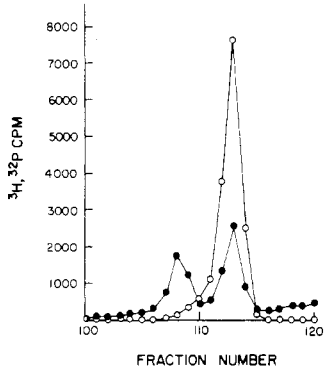

FIGURE 4: Double-label fucose kinase assay of fucose derived from HeLa GDP-fucose. Sample was incubated with [γ-^{32}P]ATP and fucose kinase and the products were separated by electrophoresis at 37 V/cm for 7 hr and 0.25-in. strips were analyzed for radioactivity. The slower moving single label ^{32}P peak is a contaminant with an $R_{F\text{-}1\text{-}P}$ value of 0.95, the same as for hexose phosphates (Table II). The double-labeled peak separated from hexose phosphate is fucose phosphate. This figure shows the HVPE profile only in the region of fucose phosphate: (O) ^{3}H cpm; (●) ^{32}P cpm.

column and the ^{3}H radioactivity chromatographed homogeneously as free sugar. About a 10% aliquot of this material was reduced in volume and chromatographed in TLC solvents I and II. The radioactive material in all cases chromatographed as did authentic fucose. This purified fucose, representing the macromolecular pool (Figure 2), was saved for assay by fucose kinase.

The total macromolecular [^{3}H]fucose was determined by the summation of radioactivity in the ethanol-insoluble material, the insoluble precipitate formed after redissolving the ethanol extract in water, and soluble macromolecular material in the ethanol extract which was excluded from the Sephadex G-25 column in the purification of GDP-fucose.

Assay of Fucose. Ten nanomoles of a standard [^{3}H]fucose solution was phosphorylated with [^{32}]ATP and the products separated by HVPE. The ^{3}H and ^{32}P double-labeled fucose 1-phosphate (F-1-P) was well separated from fucose and phosphates (Figure 3). This procedure defines the specific ^{32}P radioactivity of fucose 1-phosphate which must also equal the specific radioactivity of the [γ-^{32}P]ATP used. The [^{3}H]fucose purified from the cell GDP-fucose, macromolecular fucose material, and serum were similarly phosphorylated with [^{32}P]ATP and the products separated by HVPE. The ^{32}P-^{3}H double-labeled F-1-P product from the cells was substantially separated from a ^{32}P-labeled unknown contaminant ($R_{F\text{-}1\text{-}P}$ 0.95) by high voltage paper electrophoresis (Figure 4). The specific tritium radioactivity of a fucose sample of unknown quantity was then determined from the known specific activity of the [^{32}P]ATP. Since the ratio of fucose:fucose phosphate radioactivity can be determined from the electropherogram (Figure 3), the amount of fucose initially present in the incubation mixture can be determined without complete conversion of substrate to product. The ^{3}H specific activity of a fucose sample was calculated reproducibly from several assay tubes and furthermore this calculated value was found to be independent of the amount of sample incubated with enzyme (data not shown).

The amount of free fucose present in the calf-fetal calf serum mixture was determined by fucose kinase to be an average of 3.5 nmol/ml of serum (Table III). Since the cells were grown in 1 l. of medium in the presence of 70 ml of serum and 1500 μCi of [^{3}H]fucose (13.4 Ci/mmol with 3×10^5 cpm/μCi), there was 357 nmol of L-fucose initially present in the medium, and the actual initial specific activity of [^{3}H]fucose was 1.26×10^6 cpm/nmol. Recent experiments (Yurchenco, 1975) have shown that HeLa cells release more fucose into the growth medium than they consume (net release of about 0.1 nmol/hr per 10^7 cells). Thus, cells growing in the same growth medium for 2 to 3 days accumulate fucose to give a concentration of about 0.5 and 0.6 μM, respectively (9 and 7.5×10^5 cpm/nmol).

In experiment 1 (Table III) in cells labeled for 3 days to a density of 10^6 per ml, the GDP-L-[^{3}H]fucose specific activity was determined to be 7.23×10^4 cpm/nmol and the macromolecular fucose was determined to be 7.76×10^4 cpm/nmol. There were 0.47 nmol of GDP-L-fucose and 6.1 nmol of macromolecular fucose in 10^7 cells. The dilution of specific activity of label in converting L-[^{3}H]fucose in the growth medium to GDP-L-[^{3}H]fucose in the cell was about 1:10. This latter value was calculated from the ratio of the final specific activity of the L-[^{3}H]fucose in the growth medium and the measured GDP-L-[^{3}H]fucose specific radioactivity. In experiment 2, in cells labeled for 2 days to a density of 7.2×10^5 per ml, the GDP-L-[^{3}H]fucose specific activity was determined to be 1.06×10^5 cpm/nmol and the macromolecular fucose was determined to be 0.93×10^5 cpm/nmol. There were 0.46 nmol of GDP-L-fucose and 8.2 nmol of macromolecular fucose. The dilution of specific activity of label in converting L-[^{3}H]fucose to GDP-[^{3}H]fucose was about 1:9.

Soluble Fucose Intermediates. Soluble material, radioactively labeled 2 days with L-[1,5,6-^{3}H]fucose in a separate experiment, was extracted from HeLa cells as described (Experimental Procedures) and chromatographed on paper in solvent I. GDP-L-Fucose was the major component; there

Table III: GDP-Fucose and Macromolecular Fucose Pool Size and Specific Activity in HeLa Cells Labeled with L-[^{3}H]Fucose.

(A) Cells, GDP-Fucose

Expt.	Labeling Time (Days)	Growth Density (Cells/ml)	GDP-L-[^{3}H]Fucose in 10^7 Cells (cpm)[a]	^{3}H per nmol of Fucose (cpm)[b]	GDP-Fucose in 10^7 Cells (nmol)
1	3	10.0×10^5	3.42×10^4	7.23×10^4	0.47
2	2	7.2×10^5	4.86×10^4	1.06×10^5	0.46

(B) Cells, Macromolecular Fucose (Same Cell Cultures as in A)

Expt.	Labeling Time (Days)	Growth Density (Cells/ml)	[^{3}H]Fucosyl-Glycoprotein per 10^7 Cells (cpm)[c]	^{3}H per nmol of Fucose (cpm)	Glycoprotein Fucose in 10^7 Cells (nmol)
1	3	10.0×10^5	4.70×10^5	7.76×10^4	6.1
2	2	7.2×10^5	7.65×10^5	9.30×10^4	8.2

(C) Serum Fucose (Used in Growth Medium)

Expt.	[^{3}H]Fucose Added to 1 ml of Serum (cpm)[d]	^{3}H per nmol of Fucose (cpm)	Fucose per ml of Serum, Uncorrected (nmol)[d]	Fucose per ml of Serum, Corrected (nmol)[d]
A	5.06×10^5	1.51×10^5	3.4	3.3
B	2.25×10^6	5.25×10^5	4.3	3.7

[a] GDP-L-[^{3}H]fucose total counts per minute were determined from the recovery data from ^{3}H and ^{14}C control as described in the text. [b] These values were calculated as described in the text. [c] Macromolecular [^{3}H]fucose total counts per minute were determined by summation of all insoluble precipitate radioactivity as well as soluble macromolecular radioactivity excluded on the Sephadex G-25 column during purification of GDP-fucose. [d] From the specific activity (13.4 μCi/mmol with 3×10^5 cpm/μCi using the Beckman scintillation counter) the amount of fucose contributed by the label was calculated and subtracted from the value calculated for serum.

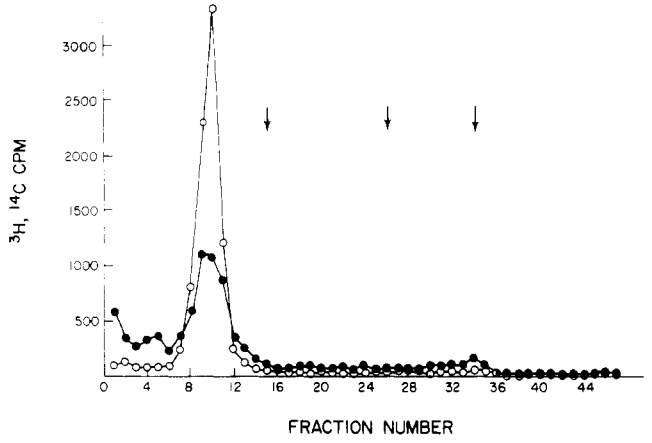

FIGURE 5: Fucose soluble intermediates. HeLa cells were labeled with L-[1,5,6-^{3}H]fucose for 24 hr. Cells were harvested, extracted with ethanol, and centrifuged to remove insoluble material. The extract (●) was mixed with authentic GDP-L-[^{14}C]fucose (○), and chromatographed overnight on paper with solvent I. Strips (1 cm) were assayed for radioactivity. The positions of fucose phosphate, fuconic acid, and fucose were determined from average R_f values of several chromatograms, and their respective positions are marked with arrows from left to right.

were insignificant amounts of L-fucose, L-fucose phosphate, and L-fuconic acid (Figure 5). Erratic background counts are due to the presence, probably, of macromolecular or glycopeptide fucose, as this can be removed by prior Sephadex G-25 chromatography (data not shown).

Discussion

Enzyme Preparation. The fucose kinase preparation as modified in this paper shows high specific activity, absence of dehydrogenase activity, and good stability. With the exception of the pH optimum, the kinetic data obtained were similar to those already published (Ishihara et al., 1968). However, the specificity toward other sugars was significantly different. The activations observed during purification of the enzyme have not been accounted for, although they may be due to the removal of phosphatase activity in each step of purification. It is also of interest that the kinase activity, unlike the dehydrogenase activity, is dependent upon the presence of reduced sulfhydryl groups.

Purification of Fucose. Little fucose-labeled glycolipid material in HeLa is extractable with lipid solvents, either after short (Atkinson, 1975) or long (Shen and Ginsburg, 1967) labeling times. Short-term labeled material, furthermore, when digested with Pronase, released soluble fucose containing material whose behavior in Sephadex G-25 chromatography and high voltage paper electrophoresis was consistent with the behavior of glycopeptides (Atkinson, 1975). Hence, it is assumed that most of the macromolecular fucose is fucosyl-glycoprotein.

The two gel filtration steps removed remaining macromolecules, fucose, and probably all sugars, as well as fucose phosphate and probably all sugar phosphates, from the GDP-fucose (in general, compounds of a molecular weight different from that of nucleotide sugars were probably removed). However, the GDP-fucose was undoubtedly still mixed with other nucleotide sugars. In the hydrolysis step and subsequent thin-layer chromatography, the released fucose was substantially separated from the other sugars also released by hydrolysis from their nucleotides. Hexose phosphates behaved in electrophoresis identically with a contaminant phosphorylated material which was therefore, probably, a hexose insufficiently separated by previous purification steps because of its large excess. However, the chromatographic purifications eliminated most of the sugars from the cell-derived fucose. For example, ribose, in cell hydrolysates, is present in nearly 70-fold excess (Shen and Ginsburg, 1967), yet after purification it is eliminated. Thus, purification to this extent served to reduce sugars other than fucose to manageable proportions in the assay procedure.

The purification procedure was easily modified to isolate [^{3}H]fucose from macromolecular material (i.e., glycopro-

tein) after release of fucose by mild acid hydrolysis and purification through a Sephadex G-10 step and the TLC systems.

Sensitivity of Assay. The main factor which limits the sensitivity of the fucose assay is the amount of nonspecific ^{32}P background on the electropherogram in the location of the fucose phosphate peak. Such background can be determined by inspection (see Figure 4) with little ambiguity and at worst leads to an error of only 0.05 nmol under the present assay conditions. However, for any given quantity of fucose being assayed, this background quantity is a fixed amount and thus the lower limit of sensitivity of the assay will be determined by the percent error deemed acceptable. For example, it would be 0.5 nmol if one accepts a 10% variation. Increasing the percentage of fucose phosphate converted from fucose will increase the sensitivity in a given assay by increasing the ratio of fucose phosphate counts per minute (cpm) to background cpm (e.g., using a much higher specific activity of enzyme or by increasing incubation time).

Several sensitive assays for L-fucose have been described. Finch et al. (1969) measured 25 nmol from hydrolysates of glycoproteins and the sensitivity of this technique is theoretically 10 times greater by use of fluorometric techniques (Finch et al., 1969; Estabrook and Maitra, 1962; Maitra and Estabrook, 1964). Fucose was also quantitated by comparison of sugars equilibrium labeled with radioactive glucose with the directly measurable specific radioactivity of ribose (Shen and Ginsburg, 1967). By reducing with sodium [^{3}H]borohydride and separating the resulting sugar alcohols chromatographically, it is possible to detect approximately 2 nmol of fucose and theoretically much less (Murakami and Winzler, 1967). Combined with the purification techniques for fucose just described, and given that the chromatographic system used to separate fucitol from mannitol and galactitol can separate fucitol from any contaminating alcohols (e.g. glucitol) formed from substances not sufficiently removed from cell extracts, then tritium borohydride reduction could be used to quantitate fucose from soluble pools as well as from glycoprotein hydrolysates with probably greater sensitivity than described here. However, short-term labeling experiments for studies on glycoprotein metabolism are feasible only with [^{3}H]fucose due to the low specific activity (and high cost) of [^{14}C]fucose. Thus, assays with tritium borohydride in short term labeling experiments would be precluded. Gas–liquid chromatography (GLC) has been used to quantitate fucose from sugar mixtures after the formation of Me_4Si or alditol acetate derivatives (for example, see Sweeley and Vance, 1967; Lehnhardt and Winzler, 1968). From one paper (Albersheim et al., 1967) it can be estimated that about 0.5 nmol of fucose injected into the GLC detector can be quantitated with accuracy. However, since it is usual practice to inject only about 3 μl or less out of a practical 20-μl volume of solvent used to dissolve derivative, the actual minimal sensitivity is about 3.5 nmol. The GLC technique might allow one to form the derivatives directly from the partially purified fucose just prior to TLC (as described here); however, the time saved would be offset by the 4–5 days it takes to prepare the derivative. Thus here, neither less time nor greater sensitivity would be achieved by the use of GLC.

Specificity of Assay. The enzyme preparation is not exclusively specific for fucose (Table II), though some sugars, such as *N*-acetylglucosamine, are poor substrates. Although the enzyme preparation exhibits high activity with glucose, galactose, and mannose, and moderate activity with ribose, these sugars are (a) efficiently separated from fucose in the two TLC systems and (b) their phosphates exhibit different migration rates in the HVPE system after phosphorylation which allow satisfactory separation from fucose phosphate if electrophoresis is carried out for more than 6 hr. Only L-rhamnose phosphate, another deoxyhexose derivative, was shown to co-migrate with fucose phosphate in this system; however, L-rhamnose is virtually not phosphorylated by this enzyme preparation and is also well separated by the TLC systems.

Specificity in this fucose kinase assay, therefore, rests on the purification procedure, on the ability of extended HVPE to separate the phosphorylated products, and on the specificity of the enzyme itself.

GDP-L-Fucose Pool Size and Specific Radioactivity. When HeLa cells are incubated in the presence of 0.36 μM L-[^{3}H]fucose (initial concentration) for 2 to 3 days, it has been determined there are 0.5 nmol of GDP-fucose in 10^7 cells. GDP-L-Fucose can be quantitated spectrophotometrically to a concentration of about 1 μM. Using spectrophotometry, the GDP-fucose pool size in HeLa cells was estimated to be about 1 nmol per 10^7 cells in the absence of fucose in the medium (Kaufman and Ginsburg, 1968). At about 0.5 μM extracellular fucose, the ratio of the exogenous:endogenous fucose contribution to GDP-fucose is 1:10. However, at much higher exogenous fucose concentrations in the millimolar range this ratio probably increases as the nucleotide sugar pool increases in size (Kaufman and Ginsburg, 1968).

Macromolecular Fucose Pool and Specific Radioactivity. The pool size of macromolecular fucose was found to be larger than the GDP-L-fucose pool with 6.1–8.2 nmol in 10^7 cells. This is in good agreement with the average value of 6.7 nmol in 10^7 cells as calculated by Shen and Ginsburg (1967) by an entirely different assay procedure.

In experiments from one cell culture, after 3 days of labeling, the GDP-[^{3}H]fucose specific radioactivity was 7.22×10^4 ^{3}H cpm per nmol while the macromolecular fucose specific activity was 7.76×10^4 ^{3}H cpm per nmol. It is perhaps surprising that the latter value should be about 7% greater than the former value, since GDP-fucose is the precursor to macromolecular, or glycoprotein, fucose (Jabbal and Schachter, 1971). In another cell culture, after 2 days labeling, the GDP-fucose specific activity was found to be 1.06×10^5 ^{3}H cpm per nmol while the macromolecular fucose specific activity was found to be 0.93×10^5 ^{3}H cpm per nmol, i.e. the latter value was about 12% less than the former value. Both experiments demonstrate the smaller GDP-fucose precursor pool is approximately at equilibrium with the much larger macromolecular fucose product pool. However, the higher specific activity of macromolecular fucose after 3 days of labeling is too large to be accounted for by a simple error in the fucose assay. (With these numbers, the actual variation introduced by the assay would be less than 1% of the specific radioactivity.) The glycoprotein pool is much larger and hence not subject to rapid equilibrium changes. Changes in cell growth conditions, such as the reaching of high densities (10^6 cells/ml) and pH alterations, might easily cause a small alteration in the ratio of exogenous to endogeneous input into nucleotide sugar. This would result in a change in the GDP-fucose specific activity while the macromolecular pool, because of its great size, would not appreciably change.

Intermediates. The rate limiting step in the synthesis of

fucosyl-glycoproteins from fucose apparently lies in the GDP-fucosyl transferase step since GDP-fucose is the only soluble intermediate present in significant amount. The quantity of glycoprotein acceptor for fucose cannot be the rate limiting step, since inhibition of cellular protein synthesis with cycloheximide shows this pool to be very large and not totally depleted even in ¾ hr (Atkinson, 1975). These results for HeLa cells differ from published results for other cells (Trujillo and Gan, 1971) and presumably, therefore, the cell type and perhaps growth conditions dictate the ratio between soluble fucose intermediates.

Conclusions

With the described assay, it is possible to measure very small quantities of fucose and hydrolysable fucose derivatives in cells. Combined with the use of radioisotopic labeling of cells, a powerful tool is now available to investigate the kinetics, pool sizes, and the relative contributions from endogenous and exogenous sources for fucosyl-glycoproteins and intermediates in animal cells under various growth conditions.

References

Albersheim, P., Nevins, D. J., English, P. D., and Karr, A. (1967), *Carbohydr. Res. 5,* 340–345.

Atkinson, P. H. (1973), *Methods Cell Biol. 7,* 157–188.

Atkinson, P. H. (1975), *J. Biol. Chem. 250,* 2123–2134.

Atkinson, P. H., and Summers, D. F. (1971), *J. Biol. Chem. 246,* 5162–5175.

Bekesi, J. G., and Winzler, R. G. (1967), *J. Biol. Chem. 242,* 3873–3879.

Bucher, T. (1947), *Biochim. Biophys. Acta 1,* 292–314.

Estabrook, R. W., and Maitra, P. K. (1962), *Anal. Biochem. 3,* 369–382.

Finch, P. R., Yuen, R., Schachter, H., and Moscarello, M. A. (1969), *Anal. Biochem. 31,* 296–305.

Foster, D. W., and Ginsburg, V. (1961), *Biochim. Biophys. Acta 54,* 376–378.

Grollman, A. P., Hall, C. W., and Ginsburg, V. (1965), *J. Biol. Chem. 240,* 975–981.

Ishihara, H., and Heath, E. C. (1968), *J. Biol. Chem. 243,* 1110–1115.

Ishihara, H., Massaro, D. J., and Heath, E. C. (1968), *J. Biol. Chem. 243,* 1103–1109.

Jabbal, I., and Schachter, H. (1971), *J. Biol. Chem. 246,* 5154–5161.

Kakáč, B. (1963), in Paper Chromatography, Hais, I. M., and Macek, K., Ed., Prague, Publishing House of the Czechoslovak Academy, p 772.

Kaufman, R. L., and Ginsburg, V. (1968), *Exp. Cell Res. 50,* 127–132.

Lato, M., Brunelli, B., and Cuiffini, G. (1968), *J. Chromatogr. 34,* 26–34.

Lehnhardt, W. F., and Winzler, R. J. (1968), *J. Chromatogr. 34,* 471–479.

Levine, E. M. (1972), *Exp. Cell Res. 74,* 99–109.

Levine, E. M., Thomas, L., McGregor, D., Hayflick, L., and Eagle, H. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 583–589.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

Maitra, P. K., and Estabrook, R. W. (1964), *Anal. Biochem. 7,* 472–484.

Murakami, M., and Winzler, R. J. (1967), *J. Chromatogr. 28,* 344–350.

Nowakowski, M., Atkinson, P. H., and Summers, D. F. (1972), *Biochim. Biophys. Acta 266,* 154–169.

Prihar, H. S., and Behrman, E. J. (1973), *Biochemistry 12,* 997–1002.

Shen, L., and Ginsburg, V. (1967), *Arch. Biochem. Biophys. 122,* 474–480.

Shen, R. L., and Ginsburg, V. (1968), in Biological Properties of the Mammalian Surface Membrane, Manson, L. A., Ed., Philadelphia, Pa., Wistar Institute Press, pp 67–71.

Sweeley, C. C., and Vance, D. E. (1967), in Lipid Chromatographic Analysis, Vol. I, Marinetti, G. V., Ed., New York, N.Y., Marcel Dekker, pp 465–495.

Talukder, M. Q-K. (1971), *J. Chromatogr. 57,* 391–395.

Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature (London) 166,* 444–445.

Trujillo, J. L., and Gan, J. C. (1971), *Biochim. Biophys. Acta 230,* 610–619.

Yurchenco, P. D. (1975), Ph.D. Thesis, Sue Golding Graduate Division of the Albert Einstein College of Medicine, New York, N.Y.