Proc. Natl. Acad. Sci. USA
Vol. 74, No. 10, pp. 4205–4209, October 1977
Biochemistry

Effect of galactose oxidase, with and without prior sialidase treatment, on the viability of erythrocytes in circulation*

(neuraminic acid/neuraminidase/chicken erythrocytes/mammalian erythrocytes)

W. C. BELL, G. N. LEVY, R. WILLIAMS, AND DAVID AMINOFF†

Departments of Internal Medicine (Simpson Memorial Institute) and Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

Communicated by J. L. Oncley, July 11, 1977

ABSTRACT **Previous studies have shown that sialidase-treated mammalian erythrocytes were rapidly eliminated from circulation. In contrast, chicken asialoerythrocytes remained fully viable. This investigation was undertaken to ascertain the reason for this difference in behavior as well as to determine the extent of the similarity of the physiological mechanism for the elimination from circulation of asialoglycoproteins and mammalian asialoerythrocytes. To that end, erythrocytes from dogs, rabbits, and chickens were each subjected to the action of galactose oxidase (D-galactose:oxygen 6-oxidoreductase; EC 1.1.3.9) both before and after sialidase (acylneuraminyl hydrolase; EC 3.2.1.18) treatment. The viability of the autologously transfused erythrocytes in circulation was monitored by $Na_2{}^{51}CrO_4$ labeling. Galactose oxidase had no deleterious effect on the viability of either dog or chicken erythrocytes, nor did it restore the viability of dog or rabbit asialoerythrocytes. On the other hand, desialated chicken erythrocytes, which were fully viable, were rendered nonviable upon treatment with galactose oxidase. It may be concluded therefore that (*a*) the physiological mechanism of elimination of mammalian asialoerythrocytes from circulation is not the same as that for plasma asialoglycoproteins and (*b*) the treatment of chicken asialoerythrocytes with galactose oxidase results in the oxidation at carbons 6 of the galactosyl- or *N*-acetylgalactosaminyl residues, thereby rendering the erythrocytes nonviable.**

Almost all the sialic acid known to be present on the erythrocyte surface is released upon treatment with sialidase (neuraminidase; acylneuraminyl hydrolase; EC 3.2.1.18) (1). Autologous transfusion of the sialidase-treated erythrocytes results in their rapid elimination from circulation in rats (2, 3), rabbits (1, 3–6), dogs (1), goats (1), and humans (7), but not in chickens (1).

The asialoglycoproteins are recognized by the liver by virtue of the galactose residues that are exposed upon removal of the sialic acid (8). While, in general, galactose has been identified as the sugar penultimate to sialic acid in plasma glycoproteins, that role has also been ascribed to *N*-acetyl-D-galactosamine in both erythrocyte glycoproteins and glycolipids, e.g., in the M and N blood group substances (9). Fortunately D-galactose and *N*-acetyl-D-galactosamine are both susceptible to oxidation with galactose oxidase (D-galactose:oxygen 6-oxidoreductase; EC 1.1.3.9) (10, 11) and in both cases it is C6 of the monosaccharide that is oxidized. Sialic acid is most frequently attached either to C3 or C6 of either galactose or *N*-acetylgalactosamine. Oxidation at C6 with galactose oxidase, $-CH_2OH \rightarrow -CHO$, prevents the reattachment of sialic acid.

A significant difference between mammalian and chicken erythrocytes is the presence of a nucleus in the latter but not in the former and, as was postulated in a previous communication (1), the nucleated chicken erythrocyte would have the capability to regenerate the sialic acid on the cell surface by virtue of the presence of CMP-*N*-acylneuraminate synthetase (CTP:*N*-acylneuraminate cytidyltransferase; EC 2.7.7.43) in the nucleus. The synthetase has not been detected in mammalian red cells (12), but has been detected in most nucleated cells and is localized only within the nucleus of the cell (13–15). It might be argued, therefore, that the nucleated chicken erythrocyte would have the capability to make available CMP-*N*-acylneuraminate to the sialyltransferase to resialate the cell surface and thereby remain in circulation. Treatment of chicken asialoerythrocytes with galactose oxidase could be expected to interfere with the resialation process.

This investigation was therefore undertaken with two objectives in mind: (*a*) to ascertain whether the mechanism for the removal of asialoerythrocytes from circulation is similar to that for asialoglycoproteins (8), and (*b*) to ascertain the reason for the observed differences in the viability of chicken and mammalian erythrocytes. A preliminary report of these studies has already appeared (16).

MATERIALS AND METHODS

White leghorn chickens (1–2 kg), mongrel dogs (13–18 kg), and New Zealand White rabbits (3–5 kg) were used. EDTA-Vacutainers were purchased from Becton-Dickenson Corp. Galactose oxidase (68 Worthington units/mg of protein) and horseradish peroxidase (698 Worthington units/mg) were purchased from Worthington Biochemicals and coffee bean α-galactosidase from Boehringer Mannheim. Isotonic bacteriostatic saline was purchased from Invenex; *o*-tolidine from Eastman Organic Chemicals; Ampholine, pH 3–10, from LKB Instruments; Sephadex G-75 superfine from Pharmacia Fine Chemicals; ^{51}Cr, as $Na_2{}^{51}CrO_4$, from E. R. Squibb and Sons; NaB^3H_4 from New England Nuclear Corp.; $NaBH_4$ from Sigma Chemical Co.; NCS tissue solubilizer from Amersham-Searle; PM-10 membranes from Amicon Corp. Phosphate-buffered saline, pH 7.0 and pH 7.4, containing 1 mM $CaCl_2$, and isotonic saline, containing 1 mM $CaCl_2$ and adjusted to pH 7.0 with $NaHCO_3$, were prepared from reagent grade chemicals.

Analytical procedures

Total sialic acid was determined by a modification (17) of the Svennerholm resorcinol procedure (18). Free sialic acid was determined by the thiobarbituric acid assay (19). Radioactivity of samples labeled with ^3H was determined by liquid scintillation. Radioactivity of ^{51}Cr-labeled cells was determined in a gamma well counter.

Paper chromatography was performed by the descending

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* This is the third article in the series "The role of sialic acid in the survival of erythrocytes in circulation." The second article is ref. 30.

† To whom reprint requests should be addressed.

technique using Whatman 3 MM paper and butanol/pyridine/water 6:4:3 (vol/vol). After chromatography, the papers were cut into 1-cm sections and the radioactivity was determined by liquid scintillation in toluene/2,5-diphenyloxazole.

Purification of sialidase

A lyophilized culture filtrate (1 g) of the Ogawa strain of *Vibrio cholerae* was suspended in 90 ml of 1% glycine and dialyzed overnight against 4 liters of 1% glycine. This solution was then used to suspend 5 g of Sephadex G-75 superfine. After the addition of 5 ml of Ampholine, pH 3–10, the slurry was subjected to flat-bed isoelectric focusing (20, 21). After 16 hr the gel was sliced and the resulting fractions were assayed for sialidase activity. The activity was determined with 1% ovine submaxillary mucin as substrate in 0.5 M acetate buffer containing 1 mM $CaCl_2$ (1 unit of activity was defined as that amount of enzyme liberating 1 μmol of sialic acid per min at 37° from the ovine submaxillary mucin). Fractions containing sialidase activity were pooled and the Ampholine was removed by permeation through Sephadex G-75. The active fractions were then pooled and concentrated 10-fold by ultrafiltration through an Amicon PM-10 membrane. This procedure results in a purification of 17.4-fold, with a yield of 66%. Such preparations contain no detectable protease or other glycosidase activity. This is a very rapid method of obtaining sialidase with suitable activity.

Stroma

Production. Blood samples were drawn into EDTA-Vacutainers and the stroma were prepared by the method of Dodge *et al.* (22). The stromal pellet was then suspended in saline and stored at −20° until used.

Optimal Concentration of Galactose Oxidase for Oxidation. Stroma were prepared from dog erythrocytes. The total sialic acid content of the preparation, as determined by the resorcinol technique (17), was used to calculate the equivalent volume of packed erythrocytes. Equal portions of stroma were distributed into tubes and desialated with 0.2 unit of sialidase per ml of packed erythrocytes for 30 min at 37° in isotonic saline containing 1 mM $CaCl_2$ (1). Dilutions of galactose oxidase equivalent to 0, 2, 4, and 6 units/ml of packed erythrocytes were added. Each tube contained 12 units of horseradish peroxidase and 0.685 μmol of *o*-tolidine. The tubes were incubated for 30 min at 37° and the reactions stopped by the addition of a mixture of 50 μl of 0.5 M EDTA (pH 10.5) and 100 μl of 2% Triton X-100. The samples were then centrifuged at 500 × *g* for 10 min, and the absorbance of the supernatant solutions was determined at 425 nm to measure the extent of the oxidation.

Erythrocytes

Preparation. Blood was obtained from the medial ear artery of rabbits, cephalic vein of dogs, and wing vein of chickens. The erythrocytes were then sedimented at 500 × *g* for 4 min. Serum and buffy coat were removed and the erythrocytes were resuspended in 4 volumes of sterile isotonic saline. The cells were washed three times with isotonic saline and once with isotonic saline containing 1 mM $CaCl_2$. Packed cell volume of the washed erythrocytes was determined in duplicate in hematocrit tubes.

Enzyme Treatment and ^{51}Cr Labeling. Sialidase (0.2 unit) and $Na_2{}^{51}CrO_4$ (1.5 μCi/kg) were added to each ml of the packed erythrocytes. The cell suspensions were then diluted with $CaCl_2$/saline to yield a final microhematocrit value of 50%. Suspensions were incubated at 37° for 30 min with gentle agitation. To some of the suspensions were added 5 units of galactose oxidase per ml of packed erythrocytes and the cell suspensions were incubated an additional 30 min at 37°.

The cells were then sedimented at 500 × *g* for 5 min and washed three times with sterile isotonic bacteriostatic saline. The supernatant and washes were retained. The cell pellet was then suspended in sterile isotonic bacteriostatic saline to the initial hematocrit value of the sample. The radioactivity of samples (0.5 ml) of the cells, supernatant solution, and washes was determined. The cell suspensions contained 80–95% of the radioactivity used. The supernate and washes were also assayed for free sialic acid (19).

Injection and Determination of Radioactivity. The labeled erythrocytes were autologously transfused and blood samples were obtained from the transfused animals 30 min after transfusion and every 24 hr thereafter until the radioactivity of the samples reached 50% (the half-life) of that exhibited by the 24-hr sample. The counts, using 0.5-ml samples, were corrected for ^{51}Cr decay by the inclusion of the 24-hr sample at each counting interval. The radioactivity of subsequent samples was also corrected for any change in hematocrit reading, which varied from day to day.

Labeling with NaB^3H_4 after Treatment with Galactose Oxidase. The procedure used was essentially that described by Gahmberg and Hakomori (23). The buffy coat was removed and the erythrocytes were washed three times with phosphate-buffered saline, pH 7.4. After the final wash, the cells were suspended in phosphate-buffered saline, pH 7.0, containing 1 mM $CaCl_2$, and incubated at 37° for 30 min with sialidase, 0.2 unit/ml of packed erythrocytes (1). Control cells were treated in a similar manner, except that the sialidase was omitted. To cells to be treated with galactose oxidase, 5 units were then added per ml of packed erythrocytes (24) and the cell suspensions were incubated for an additional 30 min. The cells were sedimented at 500 × *g* for 4 min, washed three times with phosphate-buffered saline, pH 7.4, and suspended in an equal volume of phosphate-buffered saline, pH 7.4, containing 0.25 mCi of NaB^3H_4 per 0.8 ml of packed erythrocytes. The cell suspensions were incubated at room temperature for 30 min and then diluted by the addition of 1 mg of KBH_4 in 5 ml of phosphate-buffered saline, pH 7.4, to complete reduction. The cells were finally washed with phosphate-buffered saline, pH 7.4, until the supernatant solutions were essentially free of radioactivity.

The labeled cells were lysed by the addition of phosphate-buffered saline, pH 7.4, (diluted 1:9 vol/vol) and the stromal pellet was suspended in 0.5 ml of phosphate-buffered saline, pH 7.4. Samples were taken for determination of radioactivity. Tissue solubilizer (0.5 ml) was added to each sample and the mixtures were incubated overnight at 50°. To each sample, 9 ml of counting fluid (8.4 g of 2,5-diphenyloxazole, 0.21 g of 1,4-bis[2(5-phenyloxazolyl)]benzene in 2.1 liters of toluene and 1.8 liters of Triton X-100) was added and the radioactivity of the samples was determined.

CMP-*N*-acylneuraminate synthetase

The enzyme was assayed as described by Kean (12, 25). Chicken and rat erythrocytes were lysed and assayed for the enzyme. A sample of rat liver was also assayed alone and together with chicken erythrocyte lysate to provide a positive control.

RESULTS

Optimal Enzyme Concentrations for Erythrocyte Treatment. In a previous report (1) we had demonstrated that

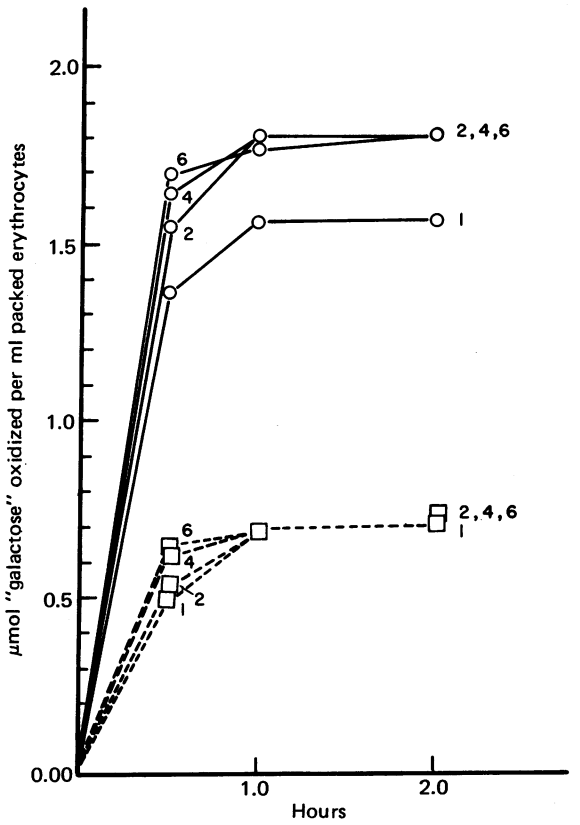

FIG. 1. Rate and extent of oxidation of asialoerythrocyte stroma from (□) dogs and (○) rabbits with various amounts of galactose oxidase (1, 2, 4, and 6 units). See *text* for experimental details.

maximal amounts of sialic acid were released when erythrocytes were treated with sialidase under conditions described above. This procedure was adopted to prevent the deleterious effects of prolonged incubations leading to increased fragility of the treated cells. The same time period was also chosen for optimal galactose oxidase treatment.

Stroma were prepared from washed dog erythrocytes (chicken erythrocytes were excluded because of difficulties in obtaining stromal preparations without contamination with nuclear material), and resorcinol assays for total sialic acid enabled us to calculate the volume of stroma equivalent to 1 ml of packed erythrocytes. These stroma were then treated with sialidase followed by 2, 4, and 6 units of galactose oxidase for 30, 60, and 120 min. The μmol of galactose and/or *N*-acetylgalactosamine oxidized per ml of packed erythrocytes is shown in Fig. 1. The maximal value for oxidizible galactose and/or *N*-acetylgalactosamine in dog erythrocytes was found to be approximately the same as the value found for sialic acid present on the cell surface. On the basis of these preliminary experiments, we decided to use 5 units of galactose oxidase for a 30-min incubation at 37° in all subsequent experiments using galactose oxidase. Under these conditions a minimal amount of hemolysis occurred (less than 0.1%) of the cells to be reinjected. This amount of galactose oxidase was also shown to be optimal for oxidation of human erythrocytes (24).

Survival of Enzyme-Treated Erythrocytes. About 70% of the dog erythrocytes treated with galactose oxidase alone survived in the circulation for 24 hr; their half-life was 25 days. These findings were comparable to those obtained with untreated control erythrocytes (Table 1). On the other hand, treatment with sialidase yielded a cell population of which only

Table 1. Effect of sialidase and galactose oxidase on erythrocytes of various species

Animal	Treatment	% survival after 24 hr	Half-life of survivors, days
Dog	Control	83	25
	Sialidase	12	2
	Galactose oxidase	70	25
	Sialidase + galactose oxidase	18	3
Rabbit	Control	86	12
	Sialidase	34	3
	Galactose oxidase	15	4
	Sialidase + galactose oxidase	22	3.5
Chicken	Control	89	8.5
	Sialidase	100	7.5
	Galactose oxidase	100	8.5
	Sialidase + galactose oxidase	2	2

12% survived the first 24 hr, and their half-life was estimated to be 2 days. Treatment of dog erythrocytes with both sialidase and galactose oxidase gave cells of which only 16–20% survived the first 24 hr, and the survivors exhibited a half-life of 3 days (Table 1).

In contrast to the dog erythrocytes, only 15–30% of rabbit erythrocytes survived for 24 hr after treatment with sialidase, galactose oxidase, and sialidase plus galactose oxidase, with roughly the same half-life, 3, 4, and 4 days, respectively (Table 1).

Sialidase-treated chicken erythrocytes gave 24-hr survivals of 100% compared to 89% in the untreated control cells. Galactose oxidase-treated cells exhibited a similar 24-hr survival (100%). However, when galactose oxidase and sialidase were used in concert, virtually all the erythrocytes were removed from the circulation in 24 hr (98%) and the half-life of the survivors was 2 days (Table 1).

Erythrocyte Labeling with NaB^3H_4 after Treatment with Galactose Oxidase. Gahmberg and Hakomori (23, 24) and Steck and Dawson (26) have used treatment of erythrocytes with galactose oxidase followed by ^3H-labeled borohydride to label the surface components of human erythrocytes. Carraway *et al.* (27) have used a similar procedure to investigate the species variability in the surface glycoproteins of bovine and equine erythrocytes. We have adapted these techniques to establish the variability of response to galactose oxidase among dog, chicken, and rabbit erythrocytes.

The labeling pattern obtained from dogs (Table 2) demonstrated that without prior desialation, dog erythrocytes treated with galactose oxidase did not incorporate significantly higher levels of tritium than did the untreated control cells. The removal of sialyl residues by sialidase and subsequent galactose oxidase treatment increased the level of tritium incorporation 8- to 16-fold.

Rabbit erythrocytes, on the other hand, exhibited a significantly different response to labeling than that observed in dog cells. Rabbit cells treated with galactose oxidase alone were labeled almost as efficiently as those treated with sialidase and galactose oxidase (Table 2).

Chicken erythrocytes showed little incorporation of tritium regardless of prior enzymatic treatment. Some incorporation was observed after sialidase and galactose oxidase treatment, but the amount incorporated was considerably less than that

Table 2. Incorporation of tritium into erythrocytes after enzyme treatment

Animal	Treatment	dpm × 10^{-4}/ ml packed cells	dpm × 10^{-3}/ million cells
Dog	Control	2.2, 2.7	1.6, 1.9
	Sialidase	4.6, 3.2	3.3, 2.3
	Galactose oxidase	2.3, 2.3	1.6, 1.6
	Sialidase + galactose oxidase	32.1, 24.5	22.9, 17.5
Chicken	Control	1.5	1.9
	Sialidase	3.1	3.9
	Galactose oxidase	2.0	2.5
	Sialidase + galactose oxidase	4.8	6.0
Rabbit	Control	2.1	1.5
	Sialidase	2.0	1.4
	Galactose oxidase	26.3	18.9
	Sialidase + galactose oxidase	25.9	18.6

of the other species. This result was not significantly altered by correcting for the larger size of chicken erythrocytes (Table 2).

Identification of Labeled Sugar. Samples of erythrocytes of the three species studied were subjected to treatment with sialidase, galactose oxidase, or sialidase followed by galatose oxidase. The erythrocytes were then labeled with NaB^3H_4 and incubated with α-galactosidase. The supernatants of these incubations were analyzed by paper chromatography followed by liquid scintillation counting. Some [^{3}H]galactose was obtained from dog erythrocytes treated with sialidase and galactose oxidase. The major labeled compound obtained from rabbit erythrocytes treated either with galactose oxidase alone or sialidase followed by galactose oxidase was shown to be galactose.

CMP-*N*-Acylneuraminate Synthetase and Chicken Erythrocytes. No synthetase could be demonstrated in chicken or rat erythrocytes, although the presence of the enzyme was readily demonstrated in rat liver. Admixture of rat liver with chicken erythrocytes was still positive, thereby ruling out the possibility of an inhibitor of the synthetase in the chicken erythrocytes.

DISCUSSION

Ashwell and Morell (8) have demonstrated that elimination of asialoglycoproteins from the circulation can be effectively reversed by their treatment with β-galactosidase or by oxidation with galactose oxidase. This identified the exposed galactosyl residue on the oligosaccharide chain of the asialoglycoprotein as the recognition site for their removal from circulation by the liver.

Oxidation of the asialoerythrocytes with galactose oxidase did not restore their viability. Thus, it would appear that the mechanism for removal of asialoerythrocytes is not the same as that for removal of asialoglycoproteins. The demonstration by Jancik and Schauer (4) that β-galactosidase does not restore the viability of asialoerythrocytes in rabbits would be further evidence that the two systems are analogous but not identical.

The results with the chicken erythrocytes may indicate that while sialidase treatment exposes galactosyl and/or *N*-acetylgalactosaminyl residues, these residues are quickly masked again *in vivo* to maintain the initial viability of the erythrocytes. Galactose oxidase treatment alone has no effect, but galactose oxidase subsequent to sialidase treatment is effective in destroying the viability of the erythrocytes. A plausible explanation would be that the oxidation at C6 of galactose or *N*-acetylgalactosamine prevents regeneration of the sialic acid on the cell surface. However, our inability to find CMP-*N*-acylneuraminate synthetase activity, presumably required for such resialation, argues against this explanation.

Regoeczi *et al.* (28) were the first to note that asialo-α_1-acid glycoprotein was not removed from the circulation of chickens in the same manner as it was from that of mammals. Lunney and Ashwell (29) further investigated this problem and demonstrated that in chickens the liver cells bind a terminal *N*-acetylglucosaminyl residue rather than a terminal galactosyl residue, as is the case in mammalian liver.

In summary, it may be stated that the present observations support the following conclusions: (*a*) the physiological mechanisms in mammals for the elimination from circulation of asialoerythrocytes and asialoglycoproteins, while analogous in many respects, are not identical; (*b*) the viability of chicken asialoerythrocytes cannot be attributed to the presence of CMP-*N*-acylneuraminate synthetase in the nucleus with its capability to resialate the erythrocyte surface; and (*c*) rabbit erythrocytes have terminal α-galactosyl residues that are susceptible to oxidation with galactose oxidase, and this treatment renders them nonviable. In contrast, cell surface carbohydrate components of dog and chicken erythrocytes are not susceptible to oxidation with galactose oxidase.

This project was supported in part by Grant HL 17881 from the National Institutes of Health, and in part by Contract N 0014-76-C-0269 from the Office of Naval Research.

1. Aminoff, D., Bell, W. C., Fulton, I. & Ingerbrigtsen, N. (1977) *Am J. Hematol.* **1**, 419–432.
2. Gregoriadis, G., Putman, D., Louis, L. & Neerunjun, D. (1974) *Biochem. J.* **140**, 323–330.
3. Durocher, J. R., Payne, R. C. & Conrad, M. E. (1975) *Blood* **45**, 11–20.
4. Jancik, J. & Schauer, R. (1974) *Hoppe Seyler's Z. Physiol. Chem.* **355**, 395–400.
5. Gattegno, L., Bladier, D. & Cornillot, P. (1974) *Carbohydr. Res.* **34**, 361–369.
6. Gattegno, L., Bladier, D. & Cornillot, P. (1975) *Hoppe Seyler's Z. Physiol. Chem.* **356**, 391–397.
7. Jancik, J. & Schauer, R. (1975) *Hoppe Seyler's Z. Physiol. Chem.* **356**, 1329–1331.
8. Ashwell, G. & Morell, A. G. (1974) *Adv. Enzymol.* **41**, 99–128.
9. Zahler, P. (1968) *Vox Sang.* **15**, 81–101.
10. Avigad, G., Amaral, G., Asensio, C. & Horecker, B. L. (1962) *J. Biol. Chem.* **237**, 2736–2743.
11. Sempere, J. M., Gancedo, C. & Asensio, C. (1965) *Anal. Biochem.* **12**, 509–515.
12. Kean, E. L. & Roseman, S. (1966) *J. Biol. Chem.* **241**, 5643–5650.
13. Kean, E. L. (1970) *J. Biol. Chem.* **245**, 2301–2308.
14. Van Dijk, W., Ferwerda, W. & Van den Eijndnen, D. H. (1973) *Biochim. Biophys. Acta* **315**, 162–175.
15. Van den Eijndnen, D. H. (1973) *J. Neurochem.* **21**, 949–958.
16. Bell, W. C., Williams, R. & Aminoff, D. (1976) *Fed. Proc.* **35**, 1432.
17. Cassidy, J. T., Jourdian, G. W. & Roseman, S. (1965) *J. Biol. Chem.* **240**, 3501–3506.
18. Svennerholm, L. (1957) *Biochim. Biophys. Acta* **24**, 604–611.
19. Aminoff, D. (1961) *Biochem. J.* **81**, 384–392.
20. Radola, B. J. (1973) *Ann. N.Y. Acad. Sci.* **209**, 127–143.
21. Radola, B. J. (1974) *Biochim. Biophys. Acta* **386**, 181–195.

22. Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100,** 119–130.
23. Gahmberg, C. G. & Hakomori, S. (1973) *J. Biol. Chem.* **248,** 4311–4317.
24. Gahmberg, C. G. (1976) *J. Biol. Chem.* **251,** 510–515.
25. Kean, E. L. (1972) in *Methods in Enzymology,* ed. Ginsburg, V. (Academic Press, New York), Vol. 28B, pp. 413–421.
26. Steck, T. L. & Dawson, G. (1974) *J. Biol. Chem.* **249,** 2135–2142.
27. Carraway, K. L., Colton, D. G., Shin, B. C. & Triplett, R. B. (1975) *Biochim. Biophys. Acta* **382,** 181–192.
28. Regoeczi, E., Hatton, M. W. C. & Charlwood, P. A. (1975) *Nature* **254,** 699–701.
29. Lunney, J. & Ashwell, G. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 341–343.
30. Aminoff, D., VorderBruegge, W. F., Bell, W. C., Sarpolis, K. & Williams, R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1521–1524.