1982

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Citation/Publisher Attribution
Urbanowski, J. C., Cohenford, M. A., & Dain, J. A. (1982). Nonenzymatic Galactosylation of Human Serum Albumin. *In Vitro* Preparatio. *Journal of Biological Chemistry, 257*(1), 111-115. Retrieved from http://www.jbc.org/content/257/1/111.
Available at: http://www.jbc.org/content/257/1/111

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 1, Issue of January 10, pp. 111-115, 1982
Printed in U.S.A.

Nonenzymatic Galactosylation of Human Serum Albumin

IN VITRO PREPARATION*

(Received for publication, August 25, 1981)

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Incubation of purified human serum albumin with D-[1-^{14}C]galactose (5 mM) or D-[1-^{14}C]glucose (5 mM) *in vitro* for 7 days under physiological conditions resulted in the time-dependent accumulation of radioactivity into trichloroacetic acid-precipitable material. Comparative studies indicated that the rate of sugar incorporation into albumin increased with increasing pH and temperature of incubation and followed a first order dependence with regard to monosaccharide and albumin concentrations. The extent of nonenzymatic galactosylation of human albumin was approximately 300% greater than the extent of nonenzymatic glucosylation under equivalent experimental conditions. Prolonged dialysis of the modified albumins against a large excess of the unlabeled monosaccharides failed to alter the amount of protein-bound radiolabeled carbohydrate, suggesting that the linkage between sugar and albumin is covalent in nature. The post-translational modification of proteins by nonenzymatic galactosylation may be of physiological significance in individuals with reduced galactokinase or galactose-1-phosphate uridyl transferase activities.

The increasing amount of reports with regard to the nonenzymatic incorporation of glucose into proteins, including lens crystallins (1-4), insulin (5), basic myelin protein (6), erythrocyte membrane proteins (7, 8), collagen (9-11), albumin (12-15), immunoglobulins (13, 14), and hemoglobin (16-20), has encouraged speculation upon the significance of this phenomenon as concerns the pathophysiology of certain disorders of carbohydrate metabolism (3, 5, 16, 21-24). Chronic hyperglycemia, found in many diabetic individuals, enhances this type of post-translational protein modification. For example, quantities of hemoglobin A_{IC}, a nonenzymatically glucosylated form of hemoglobin A, parallel blood glucose concentration (25-27). In addition, the nonenzymatic glycosylation of lens crystallins is implicated in the etiology of both diabetic and galactosemic cataracts (1, 2, 28, 29).

It has been suggested that the clinical sequelae of diabetes may accrue from alterations in protein function occurring as a result of glucose-dependent chemical modification (3). At the present time, the relationship between elevated tissue galactose concentrations and observed pathological complications in galactosemic patients remains obscure. However, we suggest that the hypothesis offered for diabetes may also be applied regarding galactosemia.

* This work was supported in part by United States Public Health Service Research Grant NS05104. 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. Section 1734 solely to indicate this fact.

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We present here a preliminary report of the nonenzymatic galactosylation of human serum albumin *in vitro*, and in addition, we demonstrate that human albumin incorporates galactose both faster and more extensively than glucose under various experimental conditions. A partial description has already appeared (30).

EXPERIMENTAL PROCEDURES

Materials—Human serum was obtained fresh from the University of Rhode Island Health Center (Kingston, RI). Human serum albumin was purified from fresh human serum by affinity chromatography on Affi-Gel blue (Bio-Rad) (31), followed by gel filtration on Bio-Gel P-150 (Bio-Rad) prior to recrystallization (32). D-Galactose and D-glucose were purchased from Sigma. D-[1-^{14}C]Galactose (6.2 mCi/mmol) was obtained from New England Nuclear, D-[1-^{14}C]Glucose (5.8 mCi/mmol) was purchased from International Chemical and Nuclear. Both chemical and radiochemical purity of the labeled sugars was assured at greater than 99% by the manufacturers; subsequent chromatographic and radiographic analysis in our laboratory confirmed the integrity of the tracers.

Incubations—All solutions and materials were sterilized by autoclaving or by ultrafiltration prior to incubation at 37 °C in a shaking water bath. Sodium azide (0.02%) was added to prevent bacterial growth. Unless indicated otherwise, human albumin solutions (10 mg/ml) were prepared in Dulbecco's phosphate-buffered saline, pH 7.4 (33), containing either galactose or glucose at a concentration of 5 mM. Trace amounts of the radiolabeled sugars were added to the mixtures to obtain desired specific activity.

Assays—Protein-bound radioactivity was measured by removing 0.04-ml aliquots of incubation mixtures to 0.05 ml of a bovine serum albumin solution (10 mg/ml) and precipitating with 1.0 ml of cold 10% trichloroacetic acid. The mixtures were then centrifuged in an Eppendorf model 5412 Microfuge, and the pellets were washed twice with 1.0 ml of cold 10% trichloroacetic acid. Precipitates were dissolved in 0.5 ml of Protosol (New England Nuclear) and counted for radioactivity in Aquasol-2 (New England Nuclear). Protein was assessed by the method of Lowry *et al.* (34) with human serum albumin serving as the standard.

RESULTS

Fig. 1 compares the incorporation of radionuclide into acid-precipitable material upon reaction of purified human serum albumin with either D-[1-^{14}C]galactose or D-[1-^{14}C]glucose. Protein accumulation of radioactivity followed a rectangular hyperbola with increasing incubation for 7 days, at which time approximately 1.6 mol of galactose or 0.5 mol of glucose was incorporated per mol of albumin. Despite the equivalence of reaction conditions, both the initial velocity and the degree of association of radionuclide with acid-precipitable material were significantly greater when galactose was employed relative to when glucose was substituted in the incubations. It should be noted that chromatography of a hydrolysate of albumin, obtained at 7 days from the incubation with galactose, described in Fig. 1, resulted in the recovery of radiolabel in both galactose and talose in a ratio of 3:1. The observation is not unexpected since the proposed reaction mechanism

involving an Amadori rearrangement to the ketoamine adduct would destroy the chiral center at C-2 of galactose. Consequently, both galactose and talose should be obtained upon protein hydrolysis. This experiment strongly suggests that galactose, and not an alleged radiolysis contaminant (35), is indeed the modifying agent of albumin, at least with concern

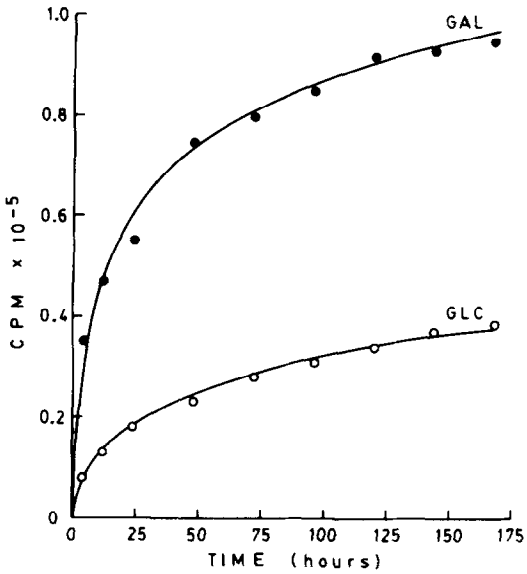

FIG. 1. ***In vitro* incorporation of D-[1-^{14}C]galactose or D-[1-^{14}C]glucose into human serum albumin.** Albumin solutions (10 mg/ml) were prepared in 1 ml of Dulbecco's phosphate-buffered saline (33) containing 1 μCi of either D-[1-^{14}C]galactose or D-[1-^{14}C]glucose and 5 mM of either D-galactose or D-glucose, respectively. Incubations proceeded aseptically at 37 °C in a shaking water bath. Aliquots were removed at indicated times, and radioactivity precipitable by trichloroacetic acid was determined as described under "Experimental Procedures."

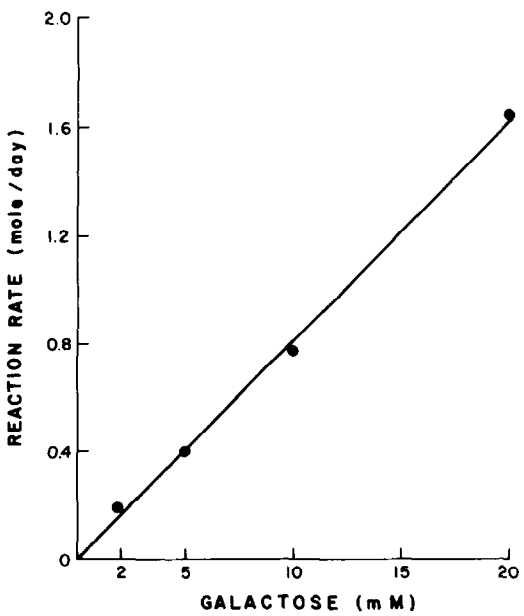

FIG. 2. ***In vitro* incorporation of D-[1-^{14}C]galactose into human serum albumin incubated with increasing concentrations of galactose.** Albumin solutions (10 mg/ml) were prepared in 1 ml of Dulbecco's phosphate-buffered saline (33) containing various concentrations of D-galactose (2–20 mM) and D-[1-^{14}C]galactose to a constant specific activity of 4.44 × 10^5 cpm/μmol. Incubation proceeded at 37 °C in a shaking water bath for 24 h. Protein-bound radioactivity was determined as described under "Experimental Procedures."

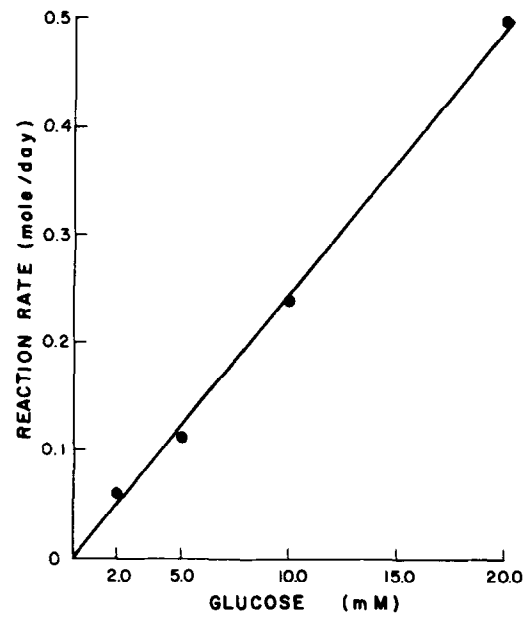

FIG. 3. ***In vitro* incorporation of D-[1-^{14}C]glucose into human serum albumin incubated with increasing concentrations of glucose.** Albumin solutions (10 mg/ml) were prepared in 1 ml of Dulbecco's phosphate-buffered saline (33) containing various concentrations of D-glucose (2–20 mM) and D-[1-^{14}C]glucose to a constant specific activity of 4.44 × 10^5 cpm/μmol. Incubation proceeded at 37 °C in a shaking water bath for 24 h. Protein-bound radioactivity was determined as described under "Experimental Procedures."

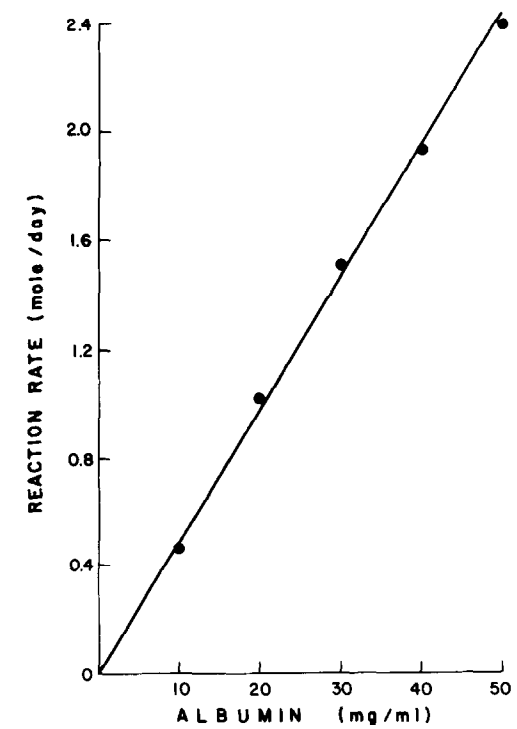

FIG. 4. ***In vitro* incorporation of D-[1-^{14}C]galactose into human serum albumin incubated with increasing concentrations of protein.** Albumin solutions (10–50 mg/ml) were prepared in 1 ml of Dulbecco's phosphate-buffered saline (33) containing 1 μCi of D-[1-^{14}C]galactose and 5 mM D-galactose. Incubation proceeded at 37 °C in a shaking water bath for 24 h. Protein-bound radioactivity was determined as described under "Experimental Procedures."

to the research here described. In addition, the ratio of galactose to talose, 3:1, compares favorably with the ratio of glucose to mannose, 3:1, obtained upon hydrolysis of hemoglobin A_{Ic} (21).

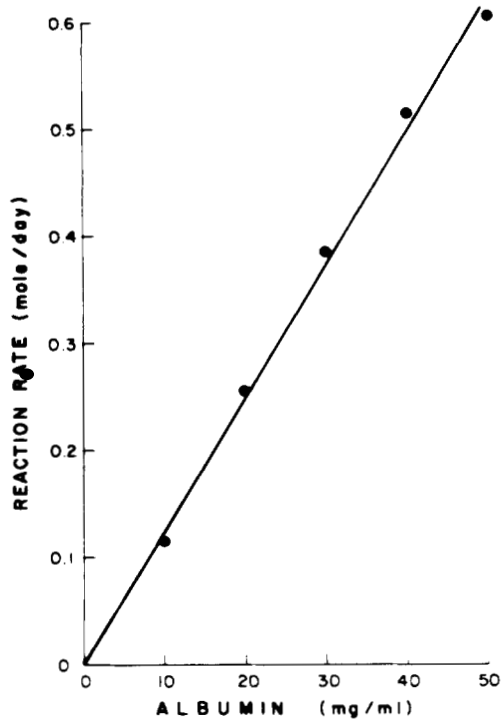

FIG. 5. ***In vitro* incorporation of D-[1-^{14}C]glucose into human serum albumin incubated with increasing concentrations of protein.** Albumin solutions (10–50 mg/ml) were prepared in 1 ml of Dulbecco's phosphate-buffered saline (33) containing 1 μCi of D-[1-^{14}C]glucose and 5 mM D-glucose. Incubation proceeded at 37 °C in a shaking water bath for 24 h. Protein-bound radioactivity was determined as described under "Experimental Procedures."

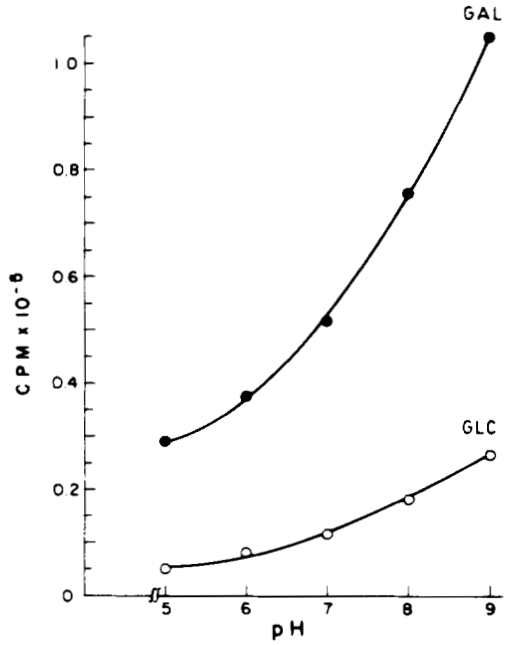

FIG. 6. **Nonenzymatic glycosylation of human serum albumin *in vitro* as a function of pH.** Albumin solutions (10 mg/ml) were prepared in 1 ml of Dulbecco's phosphate-buffered saline (33) modified to contain 15 times the usual concentration of phosphate so as to stabilize the reactions at the pH values indicated. Incubations contained 5 mM of either D-galactose or D-glucose and 1 μCi of either D-[1-^{14}C]galactose or D-[1-^{14}C]glucose, respectively, and were allowed to proceed at 37 °C in a shaking water bath for 24 h. Protein-bound radioactivity was determined as described under "Experimental Procedures." Data are expressed as trichloroacetic acid-precipitable counts per min in total incubation.

We decided next to compare the nonenzymatic galactosylation and the nonenzymatic glucosylation of human albumin *in vitro* with regard to both the extent and the rate of protein incorporation of monosaccharide as a function of various reaction conditions. Figs. 2 through 7 inclusive depict data concerning several of the kinetic and thermodynamic properties of albumin glycosylation *in vitro*. For example, the rate of incorporation of both galactose (Fig. 2) and glucose (Fig. 3) increased linearly with increasing concentration of the sugar; however, despite the equivalence of reaction conditions, nonenzymatic galactosylation occurred significantly faster than nonenzymatic glucosylation. Similarly, when protein glycosylation occurred in the presence of augmented concentrations of albumin, the linear increase in the rate of nonenzymatic galactosylation (Fig. 4) was again substantially greater than any comparative increase in the rate of glucose incorporation (Fig. 5) even though identical reaction conditions were employed. The experimental results described in Figs. 2 to 5 are not surprising, since the reaction mechanism would suggest that the initial rate of albumin glycosylation *in vitro* is first order with regard to both protein and sugar concentrations separately and second order overall.

Additionally, we attempted to make a comparison of the rates of nonenzymatic galactosylation and nonenzymatic glucosylation of human serum albumin *in vitro* under various conditions of pH and temperature. As shown in Figs. 6 and 7, protein incorporation of monosaccharide increased with both increasing pH and increasing temperature of incubation, re-

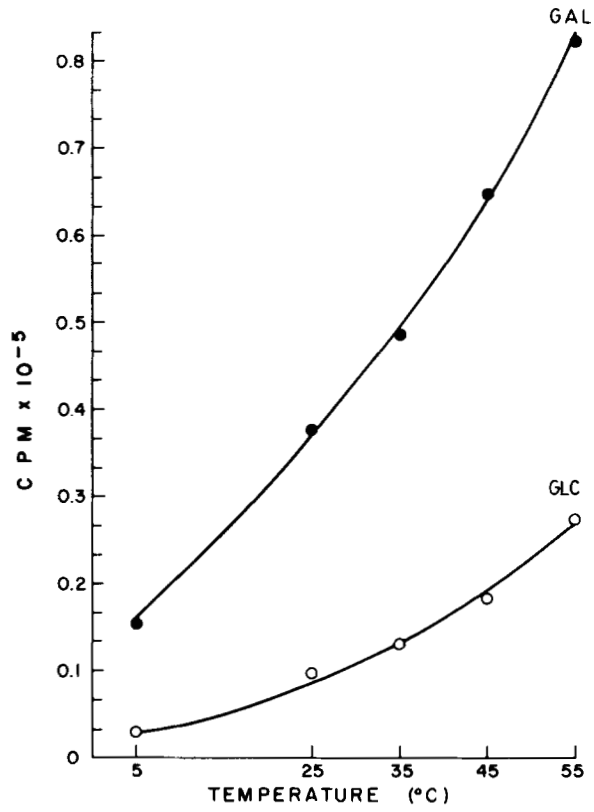

FIG. 7. **Nonenzymatic glycosylation of human serum albumin *in vitro* as a function of temperature.** Albumin solutions (10 mg/ml) were prepared in 1 ml of Dulbecco's phosphate-buffered saline (33) and contained 5 mM of either D-galactose or D-glucose and 1 μCi of either D-[1-^{14}C]galactose or D-[1-^{14}C]glucose, respectively. Incubations proceeded at the indicated temperatures for 24 h, after which protein-bound radioactivity was determined as described under "Experimental Procedures." Data are expressed as trichloroacetic acid-precipitable counts per min in total incubation.

spectively. Once again, the modification of human albumin by galactose exceeded the protein alteration occurring when glucose was substituted in the reaction mixtures.

Finally, it must be emphasized that in all of the experimental circumstances described in Figs. 2 to 7 inclusive, the rate of nonenzymatic galactosylation of human albumin *in vitro* was consistently 4-fold greater than the rate of nonenzymatic glucosylation despite the apparent identity of reaction environments.

DISCUSSION

Evidence for the nonenzymatic incorporation of glucose into proteins, both *in vivo* and *in vitro*, is overwhelming (1–20), and it has been postulated that the enhancement of this phenomenon in diabetes may contribute to the pathophysiology of the disorder (*e.g.* atherosclerotic and microangiopathic vascular disease and neuropathy) (3, 5, 16, 21–24). Working from this hypothesis we suggested that the elevated tissue galactose concentrations and the accompanying pathological complications in galactosemia may be partially related through the nonenzymatic galactosylation of proteins *in vivo*. Accordingly, we decided to examine the nonenzymatic incorporation of galactose into serum proteins *in vitro*, and the results of this study have been presented here.

Additional experiments enabled us to demonstrate that the rate of protein glycosylation *in vitro* followed a first order dependence with regard to monosaccharide (Figs. 2 and 3) and albumin (Figs. 4 and 5) concentrations and increased with increasing pH (Fig. 6) and temperature (Fig. 7) of reaction.

The major finding of this report, however, consists of the observation that galactose nonenzymatically glycosylates human serum albumin *in vitro*, both faster and more extensively than glucose, under apparently equivalent incubation conditions (Figs. 1–7). Reasons for this behavior are not readily evident. Nevertheless, we can speculate that the greater general instability of galactose relative to glucose might render the former more vulnerable to nucleophilic attack by appropriately reactive groups on the protein. In fact, Kelly (36) has documented the approximately 2-fold greater instability of the preferred conformation of galactose, compared to the preferred conformation of glucose, as resulting from an axial orientation of the hydroxyl group on the fourth carbon of galactose. We may suppose that the ensuing steric hindrance could cause the generation of a higher percentage of the chemically more reactive straight-chain form of the sugar (37). Our conjecture is an adequate interpretation for the observed differences in the initial rates of nonenzymatic galactosylation and glucosylation of human serum albumin. However, the disparity in the overall extent of protein incorporation of galactose and glucose remains to be explained. In spite of this, we believe it is possible that, just as certain sugars are more chemically reactive relative to one another, certain groups on the protein may possess various levels of reactivity, especially where specific monosaccharides are concerned. In other words, it is plausible that there may exist some measure of distinctness between protein galactosylation sites and protein glucosylation sites, which might account for the observed disparity regarding incorporation of certain sugars. It should be mentioned that Dolhofer and Wieland (5) also noticed a difference in both the initial rates and the final extent of nonenzymatic incorporation of mannose and glucose into insulin, with the former combining both faster and to a greater degree. Again, according to Kelly (36), the preferred conformation of mannose is significantly more unstable in comparison to that of glucose, due to the presence of an axial hydroxyl group on the second carbon atom of the mannopyranose ring.

It is conceivable that the galactose-dependent chemical modification of proteins *in vivo* might alter both their rate and their path of metabolism, in addition to their proper functioning in the body. For example, the nonenzymatic galactosylation of albumin may affect its ability to transport metabolites such as bilirubin. Indeed, further investigation concerning this phenomenon may provide insight regarding the pathophysiology of certain disorders of carbohydrate metabolism.

REFERENCES

1. Stevens, V. J., Rouzer, C. A., Monnier, V. M., and Cerami, A. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 2918–2922
2. Monnier, V. M., Stevens, V. J., and Cerami, A. (1979) *J. Exp. Med.* **150,** 1098–1107
3. Cerami, A., Stevens, V. J., and Monnier, V. M. (1979) *Metab. Clin. Exp.* **28,** 431–437
4. Chiou, S.-H., Chylack, L. T., Jr., Bunn, H. F., and Kinoshita, J. H. (1980) *Biochem. Biophys. Res. Commun.* **95,** 894–901
5. Dolhofer, R., and Wieland, O. H. (1979) *FEBS Lett.* **100,** 133–136
6. Fluckiger, R., and Winterhalter, K. H. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., ed) p. 208, Academic Press, New York
7. Bailey, A. J., Robins, S. P., and Tanner, M. J. A. (1976) *Biochim. Biophys. Acta* **434,** 51–57
8. Miller, J. A., Gravallese, E., and Bunn, H. F. (1980) *J. Clin. Invest.* **65,** 896–901
9. Rosenberg, H., Modrak, J. B., Hassing, J. M., Al-Turk, W. A., and Stohs, S. J. (1979) *Biochem. Biophys. Res. Commun.* **91,** 498–501
10. Tanzer, M. L., Fairweather, R., and Gallop, P. M. (1972) *Arch. Biochem. Biophys.* **151,** 137–141
11. Robins, S. P., and Bailey, A. J. (1972) *Biochem. Biophys. Res. Commun.* **48,** 76–84
12. Day, J. F., Thorpe, S. R., and Baynes, J. W. (1979) *Fed. Proc.* **38,** 418
13. Day, J. F., Thorpe, S. R., and Baynes, J. W. (1979) *J. Biol. Chem.* **254,** 595–597
14. Day, J. F., Thornberg, R. W., Thorpe, S. R., and Baynes, J. W. (1979) *J. Biol. Chem.* **254,** 9394–9400
15. Guthrow, C. E., Morris, M. A., Day, J. F., Thorpe, S. R., and Baynes, J. W. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4258–4261
16. Dolhofer, RR, and Wieland, O. H. (1979) *FEBS Lett.* **103,** 282–286
17. Bunn, H. F., Gabbay, K. H., and Gallop, P. M. (1978) *Science* **200,** 21–27
18. McDonald, M. J., Shapiro, R., Bleichman, M., Solway, J., and Bunn, H. F. (1978) *J. Biol. Chem.* **253,** 2327–2332
19. Allen, D. W., Schroeder, W. B., and Balog, J. (1958) *J. Am. Chem. Soc.* **80,** 1628–1634
20. Koenig, R. J., Blobstein, S. H., and Cerami, A. (1977) *J. Biol. Chem.* **252,** 2992–2997
21. Bunn, H. F., Haney, D. N., Gabbay, K. H., and Gallop, P. M. (1975) *Biochem. Biophys. Res. Commun.* **67,** 103–109
22. Renold, A. E., Mintz, D. H., Muller, W. A., and Cahill, G. F., Jr. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S., eds) pp. 80–109, McGraw-Hill Book Co., New York
23. Schnider, S. L., and Kohn, R. R. (1980) *J. Clin. Invest.* **66,** 1179–1181
24. Pollak, A., Lubek, G., Schober, E., Simbruner, G., and Waldhauser, F. (1980) *IRCS Med. Sci.-Biochem.* **8,** 474–475
25. McFarland, K. F., Catalano, E. W., Day, J. F., Thorpe, S. R., and Baynes, J. W. (1979) *Diabetes* **28,** 1011–1014
26. Klujber, L., Molnar, D., Kardos, M., Jasyai, V., Soltesz, G., and Mestyan, J. (1979) *Eur. J. Pediatr.* **132,** 289–297
27. Fadel, H. E., Reynolds, A., Stallings, M., and Abraham, E. C. (1981) *Am. J. Obstet. Gynecol.* **139,** 397–402
28. Skalka, H. W., Prchal, J. F., and Conrad, M. E. (1979) *Metab. Pediat. Ophthal.* **3,** 97–101
29. Kinoshita, J. H., Futterman, S., Satoh, K., and Merola, L. O. (1963) *Biochim. Biophys. Acta* **74,** 340–350
30. Urbanowski, J. C., McKinney, R. A., and Dain, J. A. (1981) *Fed.*

Proc. **40,** 782
31. Travis, J., and Pannell, R. (1973) *Clin. Chim. Acta* **49,** 49–52
32. Cohn, E. J., Hughes, W. L., and Weare, J. H. (1947) *J. Am. Chem. Soc.* **69,** 1753–1761
33. Dulbecco, R., and Vogt, M. J. (1954) *J. Exp. Med.* **98,** 167–173
34. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
35. Trueb, B., Holenstein, C. G., Fischer, R. W., and Winterhalter, K. H. (1980) *J. Biol. Chem.* **255,** 6717–6720
36. Kelly, R. B. (1957) *Can. J. Chem.* **35,** 149–158
37. Hayward, L. D., and Angyal, S. J. (1977) *Carbohydr. Res.* **53,** 13–20

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J C Urbanowski, M A Cohenford and J A Dain

J. Biol. Chem. 1982, 257:111-115.
