Bhaduri, A., Christensen, A., and Kalckar, H. M. (1965), *Biochem. Biophys. Res. Commun. 21*, 631.
Darrow, R. A., and Rodstrom, R. (1968), *Biochemistry 7*, 1645.
Davis, L., and Glaser, L. (1971), *Biochem. Biophys. Res. Commun. 43*, 1429.
Kalckar, H. M., Bertland, A. U., II, and Bugge, B. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 1113.
Kobayashi, Y., and Maudsley, D. V. (1970), The Current Status of Liquid Scintillation Counting, New York, N. Y., Grune & Stratton, p 76.
Layne, E. (1957), *Methods Enzymol. 3*, 447.
Maxwell, E. S., and Szulmajster, H. De R. (1960), *J. Biol. Chem. 235*, 308.
Maxwell, E. S., Szulmajster, H. De R., and Kalckar, H. M. (1958), *Arch. Biochem. Biophys. 78*, 407.
Nelsestuen, G., and Kirkwood, S. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 518.
Seyama, Y., and Kalckar, H. M. (1972), *Biochemistry 11*, 40.
Smith, I. (1958), Chromatographic Techniques, London, Heinemann, p 166.
Turner, G. K. (1964), *Science 146*, 183.
Wilson, D. B., and Hogness, D. S. (1964), *J. Biol. Chem. 239*, 2469.

Interaction between Uridine Diphosphate Galactose and Uridine Diphosphate Galactose 4-Epimerase from *Escherichia coli**

Y. Seyama† and H. M. Kalckar

ABSTRACT: Incubation of purified *Escherichia coli* UDPGal-4-epimerase in high concentrations with excess of its specific substrate, UDP-galactose gives rise to a marked increase of the blue fluorescence emission of the bound NADH formed. This type of reduction is reminiscent of the concerted reduction brought about by 5′UMP and free galactose. Both reactions can be characterized as reductive inhibitions, since the fluorescent enzymes show only a small fraction of the catalytical activity of the native epimerase. However, labeling of the 1-H of the galactose moiety of UDP-galactose with ^3H does not elicit a transfer of ^3H to the bound DPN^+ of epimerase as seen in the concerted reduction by free galatose labeled in the same position. Also, intermediates formed from UDP-hexoses are bound much stronger to reduced epimerase than UDP-hexoses. The intermediates or their derivatives can be released from epimerase by heating at 60° for 5 min. They are nonreducible by sodium borohydride.

It has been shown that addition of an excess of UDP-glucose[1] to *Escherichia coli* epimerase is able to generate DPNH epimerase in significant amount (Wilson and Hogness, 1964). Recently, we have shown that UDP-galactose added in excess to *E. coli* epimerase gives rise to a striking increase in fluorescence emission at 450 mμ (Kalckar *et al.*, 1970).

This reaction is very reminiscent of the reduction of *E. coli* epimerase by free D-galactose (or L-arabinose) and 5′UMP (Seyama and Kalckar, 1972). Epimerase isolated from yeast can also be reduced by these two sugars in the presence of 5′UMP (Bhaduri *et al.*, 1965; Kalckar *et al.*, 1970; Bertland *et al.*, 1971), but it does not form a fluorescent enzyme upon addition of UDP-galactose or UDP-glucose (Bhaduri *et al.*, 1965).

In the present paper we have first ascertained that the generation of fluorescent *E. coli* epimerase by UDP-galactose is due to the interaction with the nucleotide itself and not with hydrolysis products such as galactose or glucose and 5′UMP.

It was shown in the previous paper (Seyama and Kalckar, 1972) that the generation of fluorescent epimerase by D-[1-^3H]-galactose gives rise to a tritiated epimerase (enzyme-bound [^3H]DPN). It therefore was of interest whether a related reaction might also take place upon addition of UDP-galactose to epimerase, the nucleotide being labeled in the corresponding position of the galactose moiety.

Materials and Methods

Enzymes. UDP-Galactose 4-epimerase from *E. coli* K-12 strain, 3092cy$^-$ (Kalckar *et al.*, 1970), was purified according to the procedure of Wilson and Hogness (1964) with slight modification. The hydroxylapatite chromatography step gave very low recovery of activity, and it was possible to obtain the activity with somewhat improved recovery only when we used an elution gradient of 0.015–0.060 M potassium phosphate buffer (pH 6.5). The activity was measured by the method of Darrow and Rodstrom (1968). The purest enzyme preparation showed a catalytic activity of 5×10^3 to 6×10^3 μmoles per mg of protein per hr at 25°. Electrofocussing (courtesy of Dr. D. Stathakos of our department) revealed the presence of 20–30% of other protein, presumably inpurities. Large-scale preparation from 500-l. fermentation has been

* From the Biochemical Research Laboratory, Massachusetts General Hospital, and from the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts. *Received June 16, 1971.* This investigation has been supported by grants from the National Institutes of Health AM 05507 and AM 05639, and from the National Science Foundation GB-8702.

† Present address: Biochemistry Department, University of Tokyo.

[1] Abbreviations used are: UDP-glucose 4-epimerase (EC 5.1.3.2.), UDP-galactose 4-epimerase; uridine 5′-(α-D-galactopyranosyl pyrophosphate), UDP-galactose and UDPGal; uridine 5′-(α-D-glucopyranosyl pyrophosphate), UDP-glucose and UDPGlc; 2-mercaptoethanol, 2-ME; 1-H, hydrogen in 1 position of sugar.

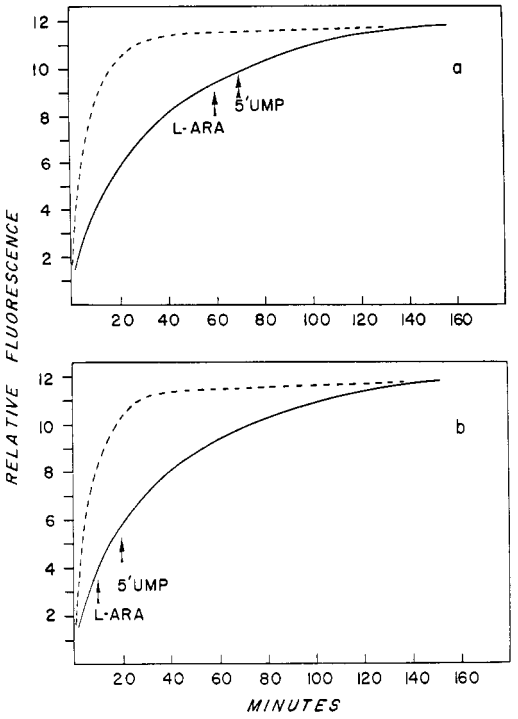

FIGURE 1: Effect of UDP-galactose on epimerase fluorescence. (a) Effect of L-arabinose and 5'UMP added at later stage of fluorescence increase; (b) effect of L-arabinose and 5'UMP added at earlier stage of fluorescence increase. UDP-galactose 4-epimerase from *E. coli* was incubated with 10 mM UDP galactose in 0.1 M Tris-HCl buffer (pH 7.5) including 10^{-3} M 2-ME and 10^{-4} M EDTA at 25° (—), at indicated points, L-arabinose (10 mM) and 5'UMP (0.1 mM) were added, respectively. Another sample of epimerase was incubated with L-arabinose (10 mM) and 5'UMP (0.1 mM) under the same condition but in the absence of UDPGal (- - - -). Fluorescence was measured at 450 mμ with 350-mμ excitation.

made at the New England Enzyme Center. UDP-glucose dehydrogenase was obtained from Sigma Chemical Co.

Radiochemicals. UDP-[^{14}C]galactose and UDP-[^{3}H]galactose (D-[1-^{3}H]galactose) were obtained from New England Nuclear. Radioactivities were measured in a liquid scintillation counter, Packard Tri-Carb Model 3320, using premixed scintillation counting solution, Aquasol, prepared by New England Nuclear. Tritium and ^{14}C in the same sample were determined by the isotope exclusion method (Kobayashi and Maudsley, 1970). Radioactivities on thin-layer plates were measured in the liquid scintillation counter after scraping successive 5-mm wide bands of a developed plate with a razor blade.

Chemicals. UDP-galactose was obtained from Calbiochem. Other reagents were obtained from commercial sources.

Thin-Layer Chromatography. Thin-layer chromatography was done on a precoated plate of Ecteola- and PEI-cellulose obtained from Brinkmann Instruments, Inc. Solvent system of 95% ethanol and 0.1 M ammonium biborate (3:2, v/v) was used with Ecteola-cellulose (Dietrich *et al.*, 1964), and 0.3 M LiCl was used with PEI-cellulose. Unlabeled standards, UDP-galactose, UDP-glucose, DPN$^+$, and DPNH, were detected with an ultraviolet lamp.

Sephadex Column Chromatography. A Sephadex column (1.1 × 15 cm) was prepared with 1 mM Tris-HCl buffer (pH 8.5) and eluted with the same buffer. Fractions of 1 ml were collected.

Other Methods. Fluorescence was measured with a Turner Model 210 Spectro absolute spectrofluorometer (Turner, 1964). Protein was determined by absorption at 280 and 260 mμ (Layne, 1957) using Zeiss PMQII spectrophotometer.

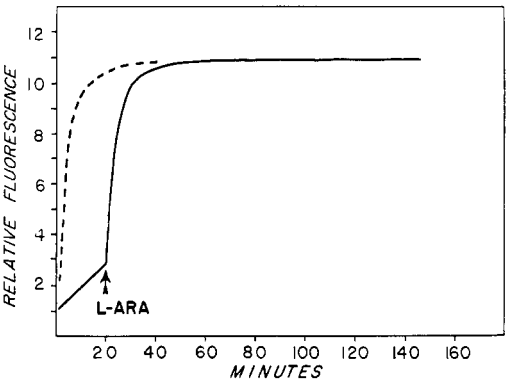

FIGURE 2: Formation of fluorescent epimerase in the presence of two sugars. UDP-galactose 4-epimerase from *E. coli* was incubated with 10 mM D-glucose and 0.1 mM 5'UMP in 0.1 M Tris-HCl buffer (pH 7.5) including 10^{-3} M 2-ME and 10^{-4} M EDTA at 25° (—). At indicated point, 10 mM L-arabinose was added. Another sample of epimerase was incubated with 10 mM L-arabinose and 0.1 mM 5'UMP under the same condition (- - - -).

Results

Effect of UDP-Galactose on Epimerase Fluorescence. If *E. coli* epimerase in concentrations ranging from 0.1 to 1 mg per ml is incubated with large excess of UDP-galactose (ratio UDP-galactose to epimerase ranging from 200 to 2000) practically all of the enzyme will be reduced to a blue fluorescent epimerase. Even smaller excesses (ratios of 10 to 50) will bring about a marked increase in the blue fluorescence of the enzyme, as will appear from the following experiment. Epimerase (1 nmole) was incubated with 20 μmoles of UDP-galactose in 2 ml of 0.1 M Tris-HCl buffer (pH 7.5), 10^{-3} M 2-ME, and 10^{-4} M EDTA. The fluorescence emission at 450 mμ increased 10-fold within 2 hr (Figure 1a,b). This increase of fluorescence was not affected by the addition of L-arabinose or 5'UMP, either in the earlier stage (Figure 1b) or in the later stage (Figure 1a). In contrast, if epimerase was incubated with 10^{-2} M D-glucose and 10^{-4} M 5'UMP, and 10^{-2} M L-arabinose was added during incubation, the rate of increase of fluorescence changed to that of L-arabinose (Figure 2). The epimerase was reacting with the more effective sugar, *e.g.*, L-arabinose, when more than two sugar were present.

The blue fluorescent epimerase formed from reduction by UDPGal has lost more than 90% of its catalytic activity. It can

TABLE I: Reductive Inactivation and Reactivation of *E. coli* Epimerase.

	Fluorescence (Arbitrary Units)	Catalytic Activity (μmoles/mg per hr)
Epimerase, native	1	4000
Epimerase reduced by UDPGal	8	300
Epimerase reoxidation by TDP-4-keto-6-deoxy-Glc	~1	2400

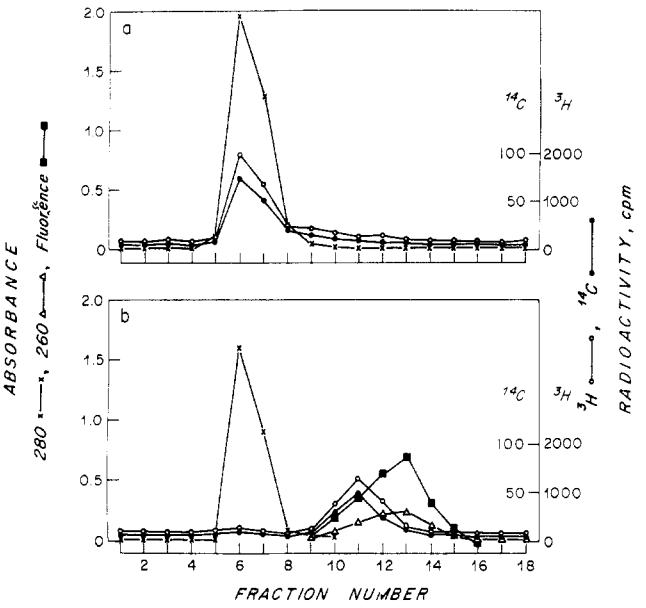

FIGURE 3: Sephadex column chromatography of epimerase incubated with UDP-galactose. (a) Rechromatography of the epimerase peak. Free UDP-hexoses were already removed by the first column. (b) After heating of the epimerase at 60° for 5 min. Sephadex G-50 column (1.1 × 15 cm) was equilibrated with 1 mM Tris-HCl buffer (pH 8.5) and eluted with the same buffer. Fractions of 1 ml were collected, and 5 μl was counted in a liquid scintillation counter.

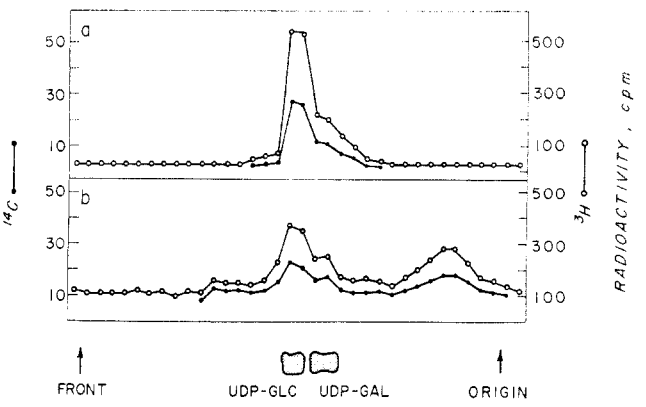

FIGURE 4: Ecteola-cellulose thin-layer chromatograms of sugar nucleotides. (a) Free sugar nucleotides separated from incubation mixture of UDP-galactose 4-epimerase and UDP-galactose by the first Sephadex G-50 column chromatography. (b) Sugar nucleotide fraction separated from epimerase by heating at 60° for 5 min. Radioactivities were measured in liquid scintillation counter after scraping successive 5-mm wide bands of a developed plate. Plate: Ecteola-cellulose (20 × 20 cm), 0.1 mm thick. Solvent: 95% ethanol–0.1 M ammonium biborate (3:2, v/v). Standards, UDP-glucose and UDP-galactose, were detected with an ultraviolet lamp.

be effectively activated by a few micrograms of TDP-4-keto-6-deoxyglucose (see Table I).

Incubation of UDP-Galactose 4-Epimerase with Radioactively Labeled Substrate. UDP-galactose 4-epimerase (6 mg; 50 nmoles was incubated with UDP-galactose (70 nmoles) in 1.8 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 10^{-3} M 2-ME and 10^{-4} M EDTA. The UDP-galactose consisted of a mixture of UDP-[1-^{3}H]galactose (5.4×10^7 cpm) and UDP-[^{14}C]galactose (2.3×10^6 cpm), with a ^{3}H:^{14}C ratio of 23.5:1. The fluorescence increased 2.87-fold during 22 hr at 25°, then 2 μmoles of unlabeled UDP-galactose was added. The fluorescence increased to a final level of 5.22-fold within 3 hr.

Separation of Enzyme-Bound Sugar Nucleotides. To remove excess free UDP-galactose and UDP-glucose, the incubation mixture was passed through a Sephadex G-50 column equilibrated with 1 mM Tris-HCl buffer (pH 8.5). The protein peak contained both ^{3}H and ^{14}C at the ^{3}H:^{14}C ratio of 22:1; 5.5% of the total radioactivity was associated with the enzyme, 94.5% appeared as free UDP-galactose and UDP-glucose. The protein was rechromatographed on Sephadex G-50 column with the same condition as above. The result is shown on Figure 3a. Epimerase after rechromatography in 1 mM Tris-HCl buffer (pH 8.5) was heated at 60° for 5 min and then applied on Sephadex G-50 column. Radioactive materials were split off from the enzyme by this treatment and appeared after the protein peak, but before the pyridine nucleotide (Figure 3b). The tubes containing radioactivities were collected. A portion of this fraction was examined on Ecteola-cellulose thin-layer plate (Figure 4b) together with free UDP-hexoses from the first Sephadex G-50 column (Figure 4a). As free radioactive nucleotides, only UDP-galactose and UDP-glucose were detected (Figure 4a). In contrast, the fraction split off from the enzyme by heating contained one additional peak near origin (Figure 4b). The same fraction was also examined on PEI-cellulose thin-layer plate (Figure 5), where two additional peaks other than UDP-galactose and UDP-glucose could be observed. In the latter system, UDP-galactose and UDP-glucose move together.

In either system, each peak contained both ^{3}H and ^{14}C at the ^{3}H:^{14}C ratio of 22:1.

Sodium Borohydride Reduction of Sugar Nucleotides Split off from the Epimerase by Heating. The sugar nucleotides split off from the epimerase by heating were treated with sodium borohydride to examine the reducibility. The fractions containing radioactivities (4.43 ml) (Figure 3b) were concentrated to 1 ml under nitrogen stream and 25 μl of 20 mM $NaBH_4$ was added. After 1 hr at room temperature, the reaction mixture was passed through Sephadex G-10 column, equilibrated with Tris-HCl buffer (pH 8.5) to remove excess $NaBH_4$. The fractions containing radioactivities were collected and concentrated under nitrogen stream, then examined on PEI-cellulose thin-layer plate. It appeared that no reduction by $NaBH_4$ had taken place. The R_F values before and after $NaBH_4$ treatment were identical.

Discussion

We have shown that UDP-galactose in large excess is able to bring about a marked increase in the fluorescence emission of UDP-galactose 4-epimerase. The rate of increase of fluorescence brought about by addition of UDP-Gal is not influenced by the further addition of L-arabinose and 5′UMP (Figure 1a,b). This is in contrast to the effect of addition of L-arabinose on the rate of fluorescence increase initiated by glucose and 5′UMP (Figure 2). Apparently the epimerase-bound DPN^+ is protected by UDPGal, UDPGlc, or their intermediates so as to prevent admission of L-arabinose and 5′UMP. It may apply too in the case of reduction by sodium borohydride and 5′UMP.

Tritiation experiments using UDP-galactose labeled with ^{3}H (D-[1-^{3}H]galactose) and also universally labeled with ^{14}C showed still another completely different pattern. (i) No ^{3}H remained on the enzyme after heating at 60° for 5 min. (ii) No ^{3}H was incorporated into DPNH or DPN^+. (iii) No trace of

^{14}C-labeled intermediates were detected with the free UDP-galactose and UDP-glucose. (iv) Intermediates or derivatives of intermediates bound to the enzyme were released upon heating of the reduced epimerase at 60°. (v) The ^{3}H:^{14}C ratio remained unchanged in the UDP hexoses as well as in the materials released from the enzyme.

Apparently the 1-H in the galactose moiety of the uridine nucleotide in contrast to that of the free galactose does not interact in the oxidation–reduction of the UDP-galactose 4-epimerase.

Hence, epimerase can participate in various types of oxidation–reductions, that is: (a) the stoichiometrical reduction by a concerted reaction between the 1-H of specific sugars (L-arabinose, D-galactose) and the bound DPN; (b) the stoichiometrical reduction of the bound DPN by an excess of the substrate UDPGal; (c) the catalytic UDPGal–UDPGlc 4 epimerization in which the bound DPN^{+} also seems to be involved.

Assuming that point c is also involved in an oxidation-reduction, the hydrogen in the 1 position cannot be involved in the reaction.

The substrate induced reductive inactivation of epimerase is also of interest. Under the conditions used for determining catalytical activity quantitatively, the substrate UDPGal is usually added in an excess of 10^{4}- to 10^{5}-fold and yet no detectable inactivation takes place over the first 10–15 min even in the absence of UDPG-dehydrogenase. Apparently, the UDPGal-induced inactivation of *E. coli* epimerase requires large amounts of the enzyme. Whether epimerase itself catalyzes the reductive inactivation or an accompanying enzyme ("epimerase reductase") is responsible for the reduction, is a moot question. The reduction is probably a different one from the one involving the 1-H of galactose (Seyama and Kalckar, 1972). Yeast epimerase, for instance, is not reduced by UDPGal, but only by free-galactose and UMP.

The reactivation of epimerase reduced by UDPGal, by incubation with a small amount of TDP-4-keto-6-deoxyglucose (see Table I) poses the question previously raised by Nelsestuen and Kirkwood (1970) whether the 4 position of the glucose or the galactose of the UDP-hexose is the donor of the hydrogen in the epimerase reaction. The experiments by Nelsestuen and Kirkwood on the interaction of borohydride-reduced *E. coli* epimerase with TDP-4-keto-6-deoxyglucose point in this direction. Moreover, Gabriel, and his group (Adair *et al.*, 1971) have recently found that TDPG reacts with epimerase, giving rise to the formation of TDPGal. They also studied the kinetics of the epimerase reaction using TDPG tritiated either in the 3 or in the 4 position; these studies indicated that the removal of tritium from carbon 4 to be rate limiting (Adair *et al.*, 1971).

The results of all these experiments have so far not been able to provide a decisive verdict in favor of the various hypotheses assigning either 2-H (Kalckar *et al.*, 1969), the 3-H (Davis and Glaser, 1971) or the 4-H (Maxwell *et al.*, 1958; Nelsestuen and Kirkwood, 1970) as the hydrogen reacting with the bound DPN of the epimerase in the catalytical reaction.

In a recent study of the interaction between epimerase and 4-T TDPG, a transfer of tritium to epimerase was observed to take place and without significant dilution of the isotope.[2]

The nature of the two double-labeled peaks which migrate differently from UDPGal and UDPGlc is not known, but they were nonreducible with sodium borohydride. It is not known at present whether the true intermediates in the epimerase reaction can be released from the epimerase upon proper denaturation or whether the intermediates were further converted to derivatives.

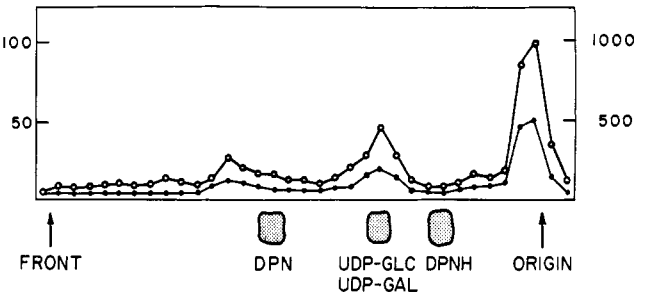

FIGURE 5: PEI-cellulose thin-layer chromatogram of sugar nucleotides. A sample tested on Figure 4b was also examined on PEI-cellulose plate. (●) ^{14}C cpm; (○) ^{3}H cpm. Plate: PEI-cellulose plate (20 × 20 cm), 0.1 mm thick. Solvent: 0.3 M LiCl.

Acknowledgments

We thank Drs. Winfried Boos and Dimitri Stathakos for numerous helpful discussion and Dr. Ottmar Gabriel for a generous sample of TDP-4-keto-6-deoxyglucose. We acknowledge thankfully the technical assistance of Miss Donna Ullrey.

Added in Proof

Recently Maitra and Ankel (1971) have provided more direct evidence for the UDP-4-ketoglucose intermediate in the 4-epimerase reaction.

References

Adair, W. L., Gaugler, R. W., and Gabriel, O. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, Abstr. 376.

Bertland, A. U., II, Bugge, B., and Kalckar, H. M. (1966), *Arch. Biochem. Biophys. 116*, 280.

Bertland, A. U., II, Seyama, Y., and Kalckar, H. M. (1971), *Biochemistry 10*, 1545.

Bhaduri, A., Christensen, A., and Kalckar, H. M. (1965), *Biochem. Biophys. Res. Commun. 21*, 631.

Creveling, C. R., Bhaduri, A., Christensen, A., and Kalckar, H. M. (1965), *Biochem. Biophys. Res. Commun. 21*, 624.

Darrow, R. A., and Rodstrom, R. (1968), *Biochemistry 7*, 1645.

Davis, L., and Glaser, L. (1971), *Biochem. Biophys. Res. Commun. 43*, 1429.

Dietrich, C. P., Dietrich, S. M. C., and Pontis, H. G. (1964), *J. Chromatogr. Sci. 15*, 277.

Kalckar, H. M., Bertland, A. U., II, and Bugge, B. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 1113.

Kalckar, H. M., Bertland, A. U., II, Johansen, J. T., and Ottesen, M. (1969), *in* The Role of Nucleotides for the Function and Conformation of Enzymes, Kalckar, H. M., Klenow, H., Munch-Petersen, A., Ottesen, M., and Thaysen, J. H., Ed., Copenhagen, Munksgaard, p 247.

Kobayashi, Y., and Maudsley, D. V. (1970), The Current Status of Liquid Scintillation Counting, New York, N. Y., Grune and Stratton, p 76.

Layne, E. (1957), *Methods Enzymol. 3*, 447.

Maitra, U. S., and Ankel, H. (1972), *Proc. Nat. Acad. Sci. U. S. 68*, 2660.

[2] Unpublished data by Gabriel and Kalckar.

Maxwell, E. S., Szulmajster, H. de R., and Kalckar, H. M. (1958), *Arch. Biochem. Biophys. 78*, 407.
Nelsestuen, G. L., and Kirkwood, S. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 518.
Seyama, Y., and Kalckar, H. M. (1972), *Biochemistry 11*, 36.
Turner, G. K. (1964), *Science 146*, 183.
Wilson, D. B., and Hogness, D. S. (1964), *J. Biol. Chem. 239*, 2469.

Physicochemical Properties of Some Glycopeptides Released from Human Erythrocyte Membranes by Trypsin*

L. J. Jackson and G. V. F. Seaman†

ABSTRACT: Glycopeptides possessing very weak M and N hemagglutinating inhibitory activity are released both from intact erythrocytes and stroma by the action of trypsin. The variation in chromatographic and electrophoretic behavior between these glycopeptides indicates differences in their molecular weights and net electrical charges. These sialoglycopeptides contain galactosamine and glucosamine and a variety of neutral sugars. The amino acid composition varies significantly from one tryptic glycopeptide to another. One glycopeptide was found to be rich in glutamate and poor in serine, threonine, and aspartate with the NH_2-terminal amino acid being alanine. Two other glycopeptides contained large quantities of serine and threonine, one of these had serine as the NH_2-terminal amino acid while no NH_2-terminal amino acid could be demonstrated in the other glycopeptide.

The M, N blood-group active materials are associated with erythrocyte peripheral zone glycoproteins. All M antigenic determinants contain intrinsic N substance, the specificity of these substances (M, N) apparently being conferred by their terminal neuraminyl groups (Prokop and Uhlenbruck, 1969). No conclusive difference in chemical composition has been found between the M and N blood-group active sialoglycoproteins (Kathan and Adamany, 1967).

Treatment of intact human erythrocytes with trypsin releases glycopeptides which possess low M or N blood-group activity (Seaman and Heard, 1960; Cook *et al.*, 1960; Thomas and Winzler, 1969). It has also been shown that such treatment leads to the loss of M but not N cellular blood-group specificity (Ohkuma *et al.*, 1968). Some of the glycopeptides released into the suspending medium by trypsin treatment have been partially characterized (Winzler *et al.*, 1967).

The present study was undertaken in order to isolate several of the glycopeptides released from either intact human red cells or posthemolytic residues (ghosts) by the action of trypsin and to examine their structure and hemagglutinating inhibitory properties, if any, in relation to the antigenic type of the erythrocytes from which they were derived.

Experimental Sections

Materials

All solutions were made up in water distilled twice in Pyrex glassware. Reagents were of analytical grade unless specified to be otherwise. Standard saline consisted of 0.145 M aqueous sodium chloride solution with the pH adjusted to 7.2 ± 0.2 using 0.5 M aqueous sodium bicarbonate solution. Trypsin (twice crystallized and lyophilized) from bovine pancreas was obtained from the Worthington Biochemical Corp. Rabbit M and N antisera were supplied by Ortho Diagnostics. Outdated O Rhesus-positive bloods were obtained from the Pacific Northwest Red Cross Blood Center, Portland, Ore. Dowex 50W-X2 (200–400 mesh) strongly acidic cation exchanger was obtained from J. T. Baker Chemical Co. Electrophoresis grade acrylamide was purchased from Bio-Rad Laboratories. All grades of Sephadex were supplied by Pharmacia Fine Chemicals, Inc.

Methods

Blood samples were pooled according to their M, N, or MN antigenic specificity after typing with rabbit M and N antisera. Red blood cells from the pooled blood samples were washed four times with standard saline. The washed erythrocytes were then either treated directly with 0.5 mg/ml of trypsin at 38° for 60 min or washed once in 310 mOsmoles/kg of sodium phosphate buffer at pH 5.8 and red cell ghosts prepared as follows. The washed erythrocytes were hemolyzed in 20 mOsmoles/kg of phosphate buffer (Dodge *et al.*, 1963) and the posthemolytic residues dialyzed against doubly distilled water at ≃5°. Cell ghosts were then made up to about 50% v/v suspension in distilled water and trypsin added to give a concentration of 0.5 mg/ml. The system was incubated at 38° for 60 min. The release of the glycopeptides from both intact red blood cells and cell ghosts was followed by assay of the sialic acid liberated into the suspending medium and also by monitoring the gradual decrease, with time, in the electrophoretic mobility of treated erythrocytes and cell ghosts.

Total neutral hexose in the sialoglycopeptides was determined by the phenol–sulfuric acid colorimetric method (Dubois *et al.*, 1956) and the individual sugars by the gas–liquid chromatographic procedure of Lehnhardt and Winzler (1968). Total hexosamines and the individual amounts of glucosamine

* From the Division of Neurology, University of Oregon Medical School, Portland, Oregon 97201. *Received June 24, 1971.* This research was supported by grants from the U. S. Public Health Service (CA 10422 and HE 12787).
† To whom to address correspondence.