Role of β_2-Microglobulin in the Intracellular Processing of HLA Antigens†

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ABSTRACT: The biosynthesis of HLA-A, -B, and -C antigens was examined in the two lymphoblastoid cell lines DAUDI and RAJI. In RAJI cells the HLA-A, -B, and -C antigen heavy chains become core-glycosylated in the endoplasmic reticulum as evidenced by their sensitivity to endo-H digestion and tunicamycin treatment. β_2-Microglobulin is present in excess in the endoplasmic reticulum of the RAJI cells and associates with the heavy chain at the time of synthesis of the heavy chain. Pulse–chase experiments demonstrated that the RAJI HLA-A, -B, and -C antigen heavy chains become terminally glycosylated since their changed characteristics included resistance to endo-H digestion, sensitivity to neuraminidase treatment, and incorporation of fucose. DAUDI HLA-A, -B, and -C antigen heavy chains are synthesized normally and become core-glycosylated but not terminally glycosylated. Other glycosylated cell surface proteins, like the HLA-DR antigens, display normal glycosylation in DAUDI cells. Therefore it is unlikely that the absence of terminally glycosylated HLA-A, -B, and -C antigen heavy chains is the result of a general defect in the biosynthetic machinery of DAUDI cells. However, DAUDI cells lack the ability to synthesize β_2-microglobulin, the common subunit of all HLA-A, -B, and -C antigens. Therefore, it seems reasonable to conclude that β_2-microglobulin is of importance for the intracellular transport of newly synthesized HLA-A, -B, and -C antigens.

The classical transplantation antigens called HLA-A, -B, and -C antigens in man are composed of one heavy chain, which displays extensive genetic polymorphism, and one invariant chain, β_2-microglobulin (Rask et al., 1976; Snary et al., 1977). The heavy chain spans the plasma membrane (Walsh & Crumpton, 1977), is glycosylated, and exhibits amino acid sequence homology with immunoglobulin chains (Trägårdh et al., 1979b,c; Orr et al., 1979). β_2m[1] is not glycosylated (Berggård & Bearn, 1968) and does not measurably interact with the lipid bilayer of the plasma membrane. Instead, β_2m is bound to the extracellular portion of the heavy HLA[2] antigen chain (Möller, 1974). Also, β_2m is homologous in amino acid sequence to the immunoglobulin chains (Peterson et al., 1972).

The detailed biochemistry of the HLA antigens makes them excellent model proteins for examining eukaryotic membrane protein biosynthesis (Dobberstein et al., 1979; Krangel et al., 1979; Algranati et al., 1980). Such studies suggest that β_2m associates with the heavy chain in the endoplasmic reticulum. The protein complex is then transported via the Golgi complex, where terminal glycosylation occurs, to the cell surface.

The lymphoblastoid cell line DAUDI does not manufacture β_2m (Nilsson et al., 1974), but it seems to produce the HLA antigen heavy chain (Östberg et al., 1975; Ploegh et al., 1979). In a preliminary report, Ploegh et al. (1979) suggested that the newly synthesized HLA antigen heavy chain displayed normal glycosylation but no cell surface expression. In this study, we have examined the biosynthesis of HLA antigens in DAUDI cells and, for comparison, in the lymphoblastoid cell line RAJI. We conclude that the HLA antigen heavy chains in DAUDI cells are normally synthesized but not terminally glycosylated. These data have been presented in preliminary form (Sege & Peterson, 1980).

Materials and Methods

Antisera. Two rabbit anti HLA-antigen sera have been used. (i) Antiserum 286 was raised against a mixture of highly purified, papain-solubilized HLA antigens [see Rask et al. (1976)]. The antiserum was extensively absorbed on a β_2m-coupled Sepharose 4B column [see Cuatrecasas (1970)] until all reactivity against β_2m was eliminated. (ii) Antiserum 303 was obtained by immunization with a mixture of papain-solubilized HLA antigen heavy chains. Dissociation of the HLA antigen chains was achieved by gel filtration in guanidine hydrochloride (Trägårdh et al., 1979a) and the heavy chain was injected in its reduced and carboxymethylated form. A detailed study of the reactivity of this serum will be given elsewhere.[3] The rabbit antiserum against human β_2m (serum 190) was the same as described earlier (Trägårdh et al., 1979a).

The rabbit anti HLA-DR antigen serum (311) was raised against highly purified papain-solubilized HLA-DR antigens derived from cadaveric spleens.[4] The rabbit anti-human immunoglobulin serum, used for preprecipitations, was obtained by repeated injections of pooled human κ and λ light chains.

Cells. The human lymphoblastoid cell lines DAUDI and RAJI (Nilsson et al., 1974) were kindly provided by Professor K. Nilsson (Department of Pathology, the Wallenberg Laboratory, University of Uppsala, Sweden). The cells were cultured in RPMI 1640 medium (Flow Laboratories, Irvine, United Kingdom) supplemented with 10% normal calf serum (Flow Laboratories), 100 units/mL penicillin, and 50 μg/mL streptomycin (Sigma Chemical Co., St. Louis, MO).

Radioactive Labeling of Cells. The cells were harvested when growing in log phase and were washed 3 times in cold methionine-free RPMI 1640 medium. The viability, as assessed by trypan blue exclusion (Gorer & O'Gorman, 1956), always exceeded 95%. When pulse-labeled, the cells were taken up in methionine-free RPMI 1640 medium, supplemented with 10% dialyzed fetal calf serum (Flow Laboratories)

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[1] Abbreviations used: β_2m, β_2-microglobulin; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; BPB, bromphenol blue; Cl_3CCOOH, trichloroacetic acid.

[2] For brevity HLA-A, -B, and -C antigens are called HLA antigens.

[3] L. Trägårdh, K. Sege, L. Rask, and P. A. Peterson, unpublished experiments.

[4] K. Wiman, L. Trägårdh, L. Rask, and P. A. Peterson, unpublished experiments.

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and 6 mM glutamine, at a concentration of 6×10^6 cells/mL. Before the addition of [^{35}S]methionine (usually 150 μCi/mL, New England Nuclear, Dreieich, West Germany), the cells were preincubated for 30 min at 37 °C in 5% CO_2. After the addition of the radioactivity, the incubation was continued for 10 min, after which time the cells were diluted with 10 volumes of RPMI 1640 medium, containing 5 times the normal concentration of methionine, supplemented with 10% fetal calf serum and 6 mM glutamine. The cell suspension was thereafter divided into portions, which were incubated at 37 °C in 5% CO_2, and subjected to various periods of chase. The chase was terminated by centrifugation of the cells at 4 °C, followed by rapid freezing of the cell pellet at −70 °C. When cells were labeled without a chase period, usually when labeling times of 6–18 h were employed, a slightly different protocol was followed: the preincubation was omitted, and the cells were kept at a concentration of 6×10^5 cells/mL. Otherwise the procedure was the same. When radioactive amino acids (New England Nuclear) other than [^{35}S]methionine were used, RPMI 1640 medium containing methionine but lacking the particular amino acid was employed.

When cells were labeled with L-[^{3}H]fucose (New England Nuclear), RPMI 1640 medium, containing 10% dialyzed fetal calf serum and 6 mM glutamine, was used. The cells were seeded at a density of 4×10^6 cells/mL, and L-[^{3}H]fucose was added to a final concentration of 100 μCi/mL. Labelings proceeded for 6 h.

DAUDI and RAJI cells were also surface labeled by the lactoperoxidase-catalyzed iodination technique (Hubbard & Cohn, 1976). Usually 1×10^7 DAUDI or RAJI cells, after three washings, were suspended in 0.1 mL of phosphate-buffered saline containing 0.5 mCi of ^{125}I (New England Nuclear). Oxidation was carried out by using the lactoperoxidase–glucose oxidase system. After the labeled cells were lysed, the solubilized molecules were passed over a lentil lectin column (Hayman & Crumpton, 1972) to eliminate free iodine. Immunoprecipitations were carried out as described below.

Labeled cells were solubilized by incubation on ice for 30 min in 0.02 M Tris-HCl buffer (Trizma grade, Sigma Chemical Co.), pH 8.0, containing 0.15 M NaCl, 1% Triton X-100 (Scintillation grade, Eastman-Kodak, Rochester, New York), 1 mM PMSF,[1] and 1% trasylol (Bayer AG, Leverkusen, West Germany). Usually 4×10^6 cells were lysed in a volume of 400 μL. Nuclei and cellular debris were removed by centrifugation at 100000g for 30 min, and the supernatants were used for immunoprecipitations.

Immunoprecipitations. The radiolabeled cell lysate (5×10^5–2×10^6 cell equiv) was preprecipitated over night at 0 °C with 25 μL of a rabbit-anti human immunoglobulin serum, before precipitation with the relevant antiserum. A 20% solution (100 μL) of formalin-fixed, heat-killed *Staphylococcus aureus* (Cowan I-strain) in lysis buffer was added the next day. After 10 min at room temperature the bacteria were removed by centrifugation at 10000g for 2 min in an Eppendorf microcentrifuge (Eppendorf, Hamburg, West Germany). Supernatants were subsequently treated with the relevant antiserum or normal rabbit serum for 4 h on ice. Immune complexes were harvested with the use of the bacteria. The resulting immunoprecipitates were washed 3 times with ice-cold 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 0.3% Triton X-100, and 0.1% $NaDodSO_4$[1] (British Drug House, Poole, England). The immunoprecipitates were eluted by heating at 95 °C for 3 min in $NaDodSO_4$–polyacrylamide gel electrophoreses sample buffer: 0.08 M Tris-HCl buffer, pH 8.8, containing 3.6% $NaDodSO_4$, 28.8% sucrose (Ultrapure, Schwarz/Mann, Orangeburg, New York), 0.01 M DTT[1] (Sigma Chemical Co.), and 0.008% BPB.[1] The bacteria were removed by centrifugation and, after the mixture cooled to room temperature, 0.05 M iodoacetamide (Sigma Chemical Co.) was added.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoreses was essentially carried out as described by Blobel & Dobberstein (1975) on 10–15% gradient slab gels. All chemicals used were of highest purity available, and stock solutions were filtered through a 0.22- or 0.45-μm Millipore filter (Millipore Co., Bedford, MA). Electrophoresis was carried out at 20 mA constant current for 12–14 h.

The gels were fixed in 10% trichloroacetic acid, then treated with Enhancer (New England Nuclear) for 1 h, soaked for 30 min in water, and finally dried on a Whatman 3 MM paper. Radioactive bands were detected by fluorography (Bonner & Laskey, 1974) on Kodak XR-film. $NaDodSO_4$–polyacrylamide gel electrophoresis in cylindrical rods was carried out according to the method of Laemmli (1970).

When radioactive samples destined for amino acid sequence analysis were run on $NaDodSO_4$–polyacrylamide gel electrophoresis, riboflavin was used as the catalyst to prevent oxidization of the proteins. ^{14}C-Labeled molecular weight marker proteins, used for $NaDodSO_4$–polyacrylamide gel electrophoresis analyses, were obtained from New England Nuclear.

Tunicamycin Treatment. Tunicamycin (a kind gift from Dr. R. Hamill, E. Lily Co., Indianapolis, IN) was dissolved in NaOH at pH 11–12. The concentration was 1 mg/mL. This stock solution was stored in aliquots at −70 °C. Labeling in the presence of tunicamycin was performed at a concentration of 3 μg/mL of the antibiotic.

For determination of the degree of inhibited glycosylation, the cell lysates were routinely passed over *Lens culinaris* coupled Sepharose 4B columns (Hayman & Crumpton, 1972), and the content of HLA antigens present in the breakthrough and eluate was estimated. The amount of HLA antigens bound to the lectin never exceeded 1–2%, when the labeling had been performed in the presence of tunicamycin. The breakthrough was used for subsequent immunoprecipitations.

Enzyme Digestions. Endo-β-*N*-acetylglucosaminidase H (endo-H) (Seikagaku Kogyo Co., Tokyo, Japan) was dissolved in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl at a concentration of 1 unit/mL and stored in aliquots at −70 °C. Samples subjected to endo-H digestion were first immunoprecipitated and released from the bacteria by incubating them for 3 min at 95 °C in 50 μL of 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 1% $NaDodSO_4$, and 10 mM DTT. The bacteria were removed by centrifugation.

The endo-H digestion was carried out by dividing the immunoprecipitates in two equal parts each of which was diluted with 9 volumes of 0.15 M sodium citrate buffer, pH 5.0. The enzyme was added to a final concentration of 0.1 unit/mL, and the reaction proceeded for 16 h at 37 °C. Mock incubations were run in parallel. Enzyme digestions were terminated by precipitation of the proteins by 15% ice-cold Cl_3CCOOH for 2 h on ice. The precipitates were washed once with ether/ethanol (1:1), and the dried samples were then dissolved in $NaDodSO_4$–polyacrylamide gel electrophoresis sample buffer.

Neuraminidase (*Vibrio cholerae*, 500 units/mL, Behring Diagnostics, Marburg/Lahn, West Germany) treatment was performed by diluting a 100000g supernatant of a cell lysate with 1 volume of 0.1 M sodium acetate buffer, pH 5.5, con-

taining 1.8% NaCl, and 0.2% $CaCl_2$. Incubations were carried out at 37 °C. The enzyme (20 μL) was added at zero time, and the same amount of enzyme was also added at 30, 60, and 120 min after initiation of the digestion. The digestions were allowed to proceed for 120 min after the last addition of the enzyme. The reactions were terminated by raising the pH to 8.0 with 1 M Tris-HCl buffer, pH 8.0. Concomitantly, the samples were placed on ice. The neuraminidase-treated samples were then subjected to immunoprecipitation.

Partial Amino Acid Sequence Determination. Amino acid sequence analyses were carried out on immunoprecipitated material recovered from disc $NaDodSO_4$–polyacrylamide gel electrophoresis. The gels were segmented and minced by an automatic gel slicer (Gilson Medical Electronics, Villiers le Bel, France) and the protein was extracted from the gel by soaking the gel pieces in 0.3 mL of distilled water containing 1 mg/mL urease (type IX, Sigma Chemical Co.) and 0.1% $NaDodSO_4$. The radioactivity was localized by liquid scintillation counting of aliquots from each fraction. The gel pieces were removed. Tris-HCl buffer, pH 8.0, was added to a final concentration of 0.2 M, and the protein was reduced with 0.01 M DTT for 30 min and alkylated with 0.05 M iodoacetic acid. After 30 min of alkylation, protein was precipitated with ice-cold Cl_3CCOOH at a final concentration of 20% (w/v) and collected by centrifugation after 4 h at 0 °C. Cl_3CCOOH and $NaDodSO_4$ were removed from the precipitates by ion-pair extraction (Henderson et al., 1979). The reduced and alkylated protein was dissolved in 0.7 mL of heptafluorobutyric acid and placed in the cup of a Beckman 890 C protein sequencer. After drying, a wash cycle omitting the addition of the cleavage acid was carried out. The Fast Protein Quadrol Program with 0.5 M Quadrol was used in all analyses (Trägårdh et al., 1979a). The anilinothiozolinones were dried under a stream of N_2 and converted to phenylthiohydantoins in 0.2 mL of 1 M HCl containing 0.1% ethanethiol at 80 °C for 10 min. The phenylthiohydantoin derivatives were extracted with two 0.7-mL portions of ethyl acetate, dried under N_2, and dissolved in toluene containing 5% of 2.5-diphenyloxazole (New England Nuclear) and 0.0625% of *p*-bis[2-(5-phenyloxazolyl)]benzene (New England Nuclear). The radioactivity was measured in a Packard Tri-carb liquid scintillation counter (Downers Grove, IL).

Results

Biosynthesis of HLA Antigens in DAUDI Cells. Despite the fact that two laboratories have presented data suggesting that synthesis of HLA antigen heavy chains occurs in DAUDI cells (Östberg et al., 1975; Ploegh et al., 1979), other laboratories have failed to disclose the presence of HLA antigen heavy chains in these cells. Therefore, it seemed essential to establish whether DAUDI cells manufacture the HLA antigen heavy chain. To this end, DAUDI and RAJI cells were separately labeled with [^{35}S]methionine either for 10 min or for 16 h. After solubilization aliquots of the labeled lysates were separately mixed with antisera directed against native HLA antigen heavy chains (286), denatured heavy chains (303), and β_2m (190). Immune complexes were isolated and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1). After a 10-min labeling period the anti-HLA antigen heavy-chain sera precipitated molecules with molecular weights of ~43 000 from both types of cells. Neither antiserum precipitated labeled β_2m (mol wt 12000). However, the anti-β_2m serum precipitated the 43 000-dalton chain from RAJI cells (Figure 1A, lane D).

From DAUDI cells labeled for 16 h with [^{35}S]methionine both anti-heavy-chain sera precipitated a 43 000-dalton component (Figure 1B, lanes E and F). Occassionally, a band with an apparent molecular weight of ~70 000 occurred (Figure 1B, lanes D–F). Since this band was not always apparent (cf. Figure 3B, lane E) and was precipitated in the absence of the nominal antigen (Figure 1B, lane D), it most probably has no relationship to the HLA antigens. The same sera reacted mainly with a 45 000-dalton chain when long-term labeled RAJI cell lysates were analyzed (Figure 1A, lanes E and F). In the latter case also, anti-β_2m serum precipitated the 45 000-dalton chain. Figure 1A (lanes E and F) shows that after long-term labeling the amount of HLA antigen heavy chain precipitated with the antiserum raised against native heavy chain far exceeds the amount precipitated with the antiserum against the denatured chain.

In some experiments the labeled DAUDI and RAJI lysates were separately enriched for glycoproteins on a *Lens culinaris* hemagglutinin column (Hayman & Crumpton, 1972). The

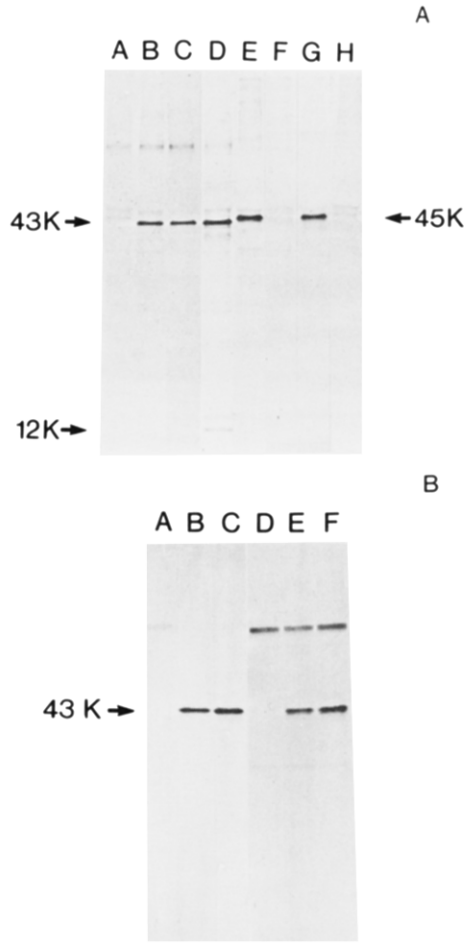

FIGURE 1: Pulse-labeled and long-term-labeled HLA antigen heavy chains derived from RAJI cells (A) and DAUDI cells (B). The cell lysates of ~5 × 10^5 cells were immunoprecipitated with 5 μL of various antisera. In (A) lanes A–D depict HLA antigen heavy chains labeled for 10 min with [^{35}S]methionine, and lanes E–H depict heavy chains labeled for 16 h with the same amino acid. Immunoprecipitations were carried out with normal rabbit serum (lanes A and H), an antiserum (303) against denatured HLA antigen heavy chains (lanes B and F), an antiserum (286) against native heavy chains (lanes C and E), and an antiserum (190) against β_2-microglobulin (lanes D and G). In (B) lanes A–C depict HLA antigen heavy chains labeled for 10 min with [^{35}S]methionine, and lanes D–F depict heavy chains labeled for 16 h with [^{35}S]methionine. Immunoprecipitations were generated with antiserum 190 (lanes A and D), 303 (lanes B and E), and 286 (lanes C and F). Radioactive molecular weight markers were phosphorylase B (92 500), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000), lactoglobulin A (18 400), and cytochrome *c* (12 300).

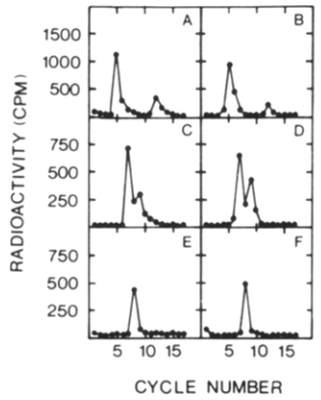

FIGURE 2: NH_2-Terminal amino acid sequence of DAUDI (A, C, and E) and RAJI (B, D, and F) HLA antigen heavy chains. The two types of cells were separately labeled with [^{35}S]methionine (A and B), [^{3}H]tyrosine (C and D), and [^{3}H]phenylalanine (E and F). After NaDodSO_4–polyacrylamide gel electrophoresis, HLA antigen heavy chains were eluted from the gels and subjected to automatic amino acid sequence determination. The phenylisothiohydantoin derivatives were subjected to radioactivity measurements.

43 000–45 000-components from cells labeled either for 10 min or for 16 h bound to the column. After 10 min of labeling little if any ^{35}S-labeled β_2m was bound. However, anti-β_2m serum precipitated the 43 000-dalton chain from RAJI cells (Figure 1A, lane D), suggesting that unlabeled β_2m was associated with the labeled heavy chain.

DAUDI and RAJI cells were separately labeled for 14 h with various radioactive amino acids to obtain further evidence for DAUDI cells producing HLA antigen heavy chains. After solubilization the lysates were either passed over a column containing covalently bound human β_2m, or the lysates were subjected to indirect immunoprecipitation with an anti-HLA antigen heavy-chain serum. Immune complexes as well as material bound and desorbed from the β_2m column were subjected to disc NaDodSO_4–polyacrylamide gel electrophoresis. In all cases only a single, labeled band of ~44 000 daltons was visualized. Material in this band was extracted from the gels and subjected to automatic amino acid sequence determination. Figure 2 shows that in five out of the five positions available for comparison the amino acid sequence of the DAUDI material was identical with that of the RAJI cells. Apart from the methionine in position 12, the amino acid sequence is identical with that previously found for a mixture of HLA antigen heavy chains (Trägårdh et al., 1980). It should be noted that some HLA antigen heavy chains do have methionine in position 12 (Ballou et al., 1976).

Synthesis of HLA Antigen Heavy Chain in the Presence of Tunicamycin. Results described in the previous section demonstrated that after long-term labeling DAUDI and RAJI cell HLA antigen heavy chains differed as to apparent molecular weight (see Figure 1A, lane E and Figure 1B, lane E). To explore if this difference was dependent upon variations in the glycosylation, we labeled DAUDI and RAJI cells separately with radioactive methionine for 10 min and 16 h, respectively, in the presence and absence of tunicamycin. The tunicamycin was added to the cultures 2 h before the addition of the labeled methionine. After solubilization of the labeled cells and indirect immunoprecipitation with the use of anti-HLA antigen heavy-chain serum, the material was analyzed by NaDodSO_4–polyacrylamide gel electrophoresis (Figure 3). As expected DAUDI HLA antigen heavy chains labeled in the absence of tunicamycin displayed an apparent molecular weight of 43 000 regardless of whether the [^{35}S]methionine had been present for 10 min or for 16 h. In the presence of

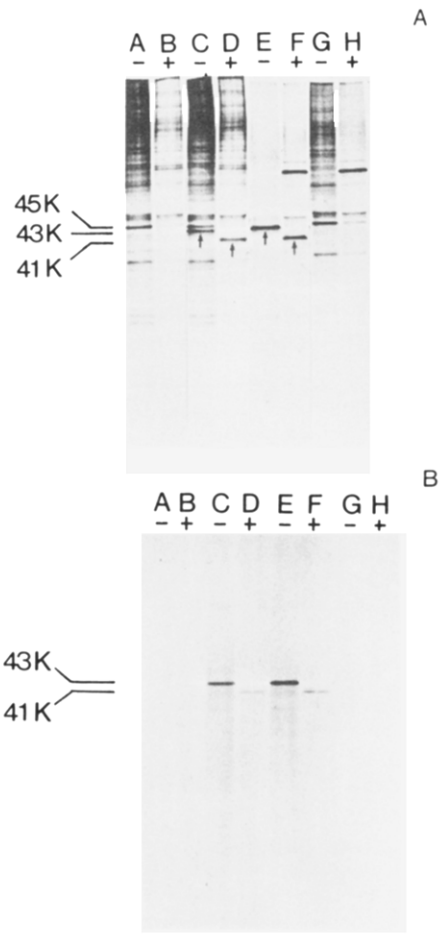

FIGURE 3: Effect of tunicamycin on the biosynthesis of HLA antigen heavy chains from RAJI (A) and DAUDI (B) cells. Approximately 1×10^6 cells were labeled with [^{35}S]methionine in the presence (+) or absence (–) of 3 μg/mL tunicamycin for each immunoprecipitation. The cells were labeled either for 10 min (lanes A–D) or for 16 h (lanes E–H). The solubilized cells were subjected to immunoprecipitation with normal rabbit serum (lanes A, B, G, and H) and with an antiserum (286) against native HLA antigen heavy chains (lanes C–F). Due to the rather high background in the RAJI experiments, the arrows indicate the HLA antigen heavy chain bands. The same molecular weight markers as described in Figure 1 were used.

tunicamycin the molecular weight of the HLA antigen heavy chain decreased to 41 000 regardless of the labeling time. Figure 3 also depicts the results obtained with the RAJI cells. Also, HLA antigen heavy chains obtained from these cells had an apparent molecular weight of 41 000 provided the chains had been synthesized in the presence of tunicamycin. Thus, due to the tunicamycin the RAJI heavy chains decreased their molecular weights to 41 000 from 43 000 during pulse labeling and from 45 000 during long-term labeling. Since tunicamycin inhibits the formation of dolichol-bound oligosaccharides (Kuo & Lampen, 1974), thereby preventing asparagine-linked core glycosylation [see Robbins et al. (1977)], the results of Figure 3 suggest that the difference noted between DAUDI and RAJI HLA antigen heavy chains may be due to differences in glycosylation.

Analysis of HLA Antigen Heavy-Chain Carbohydrate Moiety. The effect of tunicamycin on the apparent molecular weight of the HLA antigen heavy chains of DAUDI and RAJI cells prompted a detailed analysis of the biosynthesis of the heavy-chain prosthetic group. To this end, newly synthesized heavy chains were digested with the enzyme endo-H, which cleaves the glycosidic bond between the core *N*-acetylglucosamines of asparagine-linked carbohydrate moieties

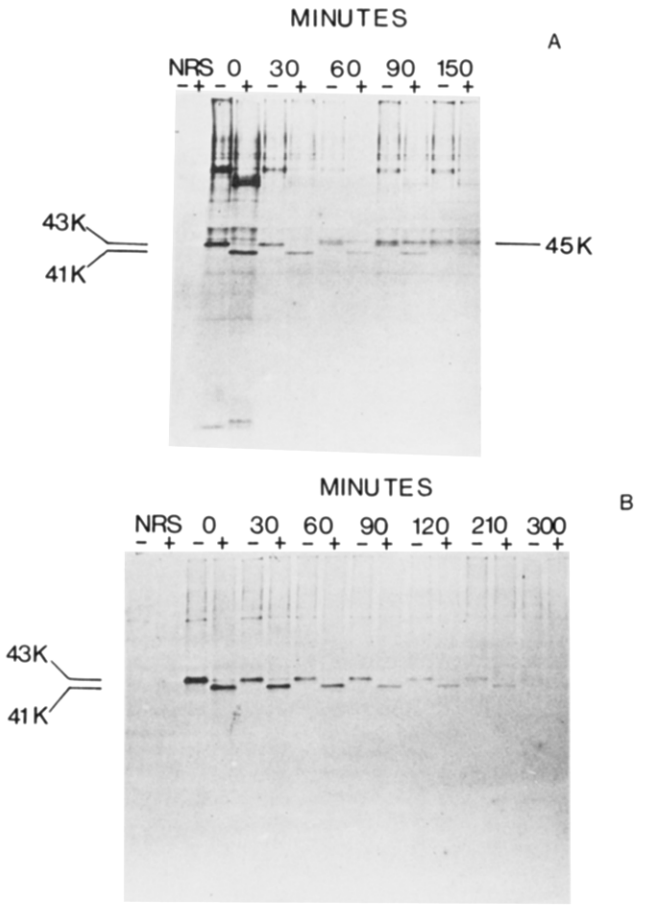

FIGURE 4: Endo-H treatment of newly synthesized HLA antigen heavy chains from RAJI (A) and DAUDI (B) cells. Approximately 2 × 10^6 cells were used for each immunoprecipitation. The cells were labeled for 10 min with [^{35}S]methionine. Subsequently, the labeled molecules were chased with an excess of unlabeled methionine for the periods of time indicated. Endo-H treatment (+) and control incubations (−) were carried out as described under Materials and Methods. The immunoprecipitations were achieved with normal rabbit serum (NRS) and with an anti-heavy-chain serum (286). Only the NRS precipitation of material chased for 0 min is shown. The molecular weight markers are the same as those described in Figure 1.

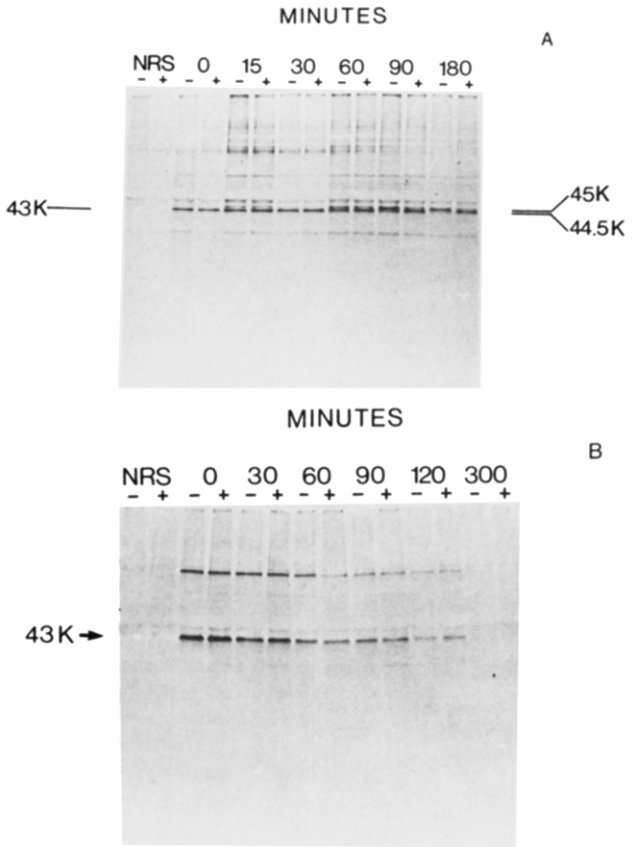

FIGURE 5: Effect of neuraminidase treatment on newly synthesized HLA antigen heavy chains from RAJI (A) and DAUDI (B) cells. Neuraminidase digestions (+) and control incubations (−) were carried out on lysates from ~2 × 10^6 cells, which had been labeled for 10 min with [^{35}S]methionine and chased for the indicated periods of time. Immunoprecipitations were performed with normal rabbit serum (NRS) and with an anti-HLA antigen heavy-chain serum (286). Only the NRS precipitation of material chased for 0 min is shown. The molecular weight markers are the same as those described in Figure 1.

(Tarentino & Maley, 1974). However, this cleavage only occurs provided the oligosaccharide chain is in the "high-mannose" form, i.e., before trimming and terminal glycosylation have occurred in the Golgi complex [see Hunt et al. (1978)].

Consequently, DAUDI and RAJI cells were separately labeled for 10 min in the presence of [^{35}S]methionine. Thereafter, the cells were cultivated in the presence of an excess of unlabeled methionine, and cells were harvested at various times. After solubilization and indirect immunoprecipitation with an antiserum specific for the HLA antigen heavy chain, isolated immune complexes were either mock incubated or incubated with endo-H. Figure 4 depicts the $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of a typical experiment. As can be seen in Figure 4A, at the end of the labeling period (time 0 min), the RAJI HLA antigen heavy chain had a molecular weight of 43 000 which on digestion with endo-H was reduced to 41 000. The situation was the same after chase periods of 30 and 60 min, respectively. However, after a chase period of 90 min a new HLA antigen heavy-chain band with an apparent molecular weight of 45 000 was visualized. This occurred concomitantly with part of the heavy chain being resistant to endo-H digestion. This was even more pronounced after a chase period of 150 min when none of the HLA antigen heavy-chain material was sensitive to endo-H digestion. The situation as regards the DAUDI HLA antigen heavy-chain material was distinctly different (Figure 4B). Thus, the DAUDI heavy chain was sensitive to endo-H digestion regardless of the chase period, indicating that it does not undergo terminal glycosylation.

Since neuraminic acid is added to the carbohydrate chain in the Golgi complex, it appeared of interest to examine whether DAUDI and RAJI HLA antigen heavy chains obtained this monosaccharide during intracellular processing. Consequently, DAUDI and RAJI cells were separately labeled with [^{35}S]methionine for 10 min. After addition of unlabeled methionine in excess to chase the labeled molecules, the cells were cultivated for various periods of time. The solubilized macromolecules were digested with neuraminidase, or mock incubated, prior to indirect immunoprecipitation and $NaDodSO_4$–polyacrylamide gel electrophoresis analyses. Figure 5A shows that after 30 min of chase the RAJI HLA antigen heavy chain, as expected, has an apparent molecular weight of 43 000 regardless of whether it has been subjected to neuraminidase treatment or not. However, after 60 min or longer periods of chase, a new heavy-chain band with an apparent molecular weight of 45 000 emerges. This species of the heavy chain is sensitive to neuraminidase treatment since its apparent molecular weight decreases to ~44 500 upon enzymatic treatment. Figure 5B shows that the DAUDI HLA antigen heavy chain is insensitive to digestion with neuraminidase

Table I: Incorporation of [^{35}S]Methionine and L-[^{3}H]Fucose in HLA-A, -B, and -C and HLA-DR Antigens from DAUDI and RAJI Cells

	antigen precipitated[a] (dpm)			
	HLA-A, -B, and -C[b]		HLA-DR[c]	
cell line	[^{35}S]Met	L-[^{3}H]fucose	[^{35}S]Met	L-[^{3}H]fucose
DAUDI	16 100	<25	6 900	2 100
RAJI	15 400	5 200	10 200	15 800

[a] DAUDI and RAJI cells (1.2×10^7) were separately labeled with [^{35}S]methionine (200 μCi) for 10 min and with L-[^{3}H]fucose (200 μCi) for 6 h. After labeling, the cells were solubilized, glycoproteins were isolated on a lectin column, and identical aliquots were immunoprecipitated with either an anti-HLA-A, -B, and -C antigen serum (286), an anti-HLA-DR antigen serum (311), or normal rabbit serum. After $NaDodSO_4$–polyacrylamide gel electrophoresis, analyses of the immunoprecipitates HLA-A, -B, and -C and HLA-DR antigen chains were eluted from the gels and subjected to radioactivity measurements. Material precipitated by normal rabbit serum did not display electrophoretic mobilities similar to those of HLA-A, -B, and -C and HLA-DR antigen chains. [b] *Only the radioactivity incorporated into the heavy chains was* measured. [c] The figures given represent the sum of radioactivity in all HLA-DR antigen chains resolved on the gel (cf. Figure 6).

regardless of the chase period.

Taken together, the data described above clearly suggest that DAUDI HLA antigen heavy chains do not become terminally glycosylated. However, it cannot be ruled out from these analyses that a small fraction of the heavy chains reaches the Golgi complex to become terminally glycosylated. To directly investigate this possibility, we labeled DAUDI and RAJI cells separately for 6 h with L-[^{3}H]fucose, a monosaccharide known to be added in the Golgi complex [see Beyer et al. (1979)]. After solubilization, L-[^{3}H]fucose-labeled HLA-A, -B, and -C antigens and HLA-DR antigens (see below) were separately immunoprecipitated and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The amounts of radioactivity present in the HLA-A, -B, and -C antigen heavy chains and in the HLA-DR antigen chains, respectively, were estimated (Table I). In parallel experiments the two cell lines were labeled with [^{35}S]methionine for 20 min, the two types of transplantation antigens were isolated and separated by $NaDodSO_4$–polyacrylamide gel electrophoresis, and the ^{35}S radioactivity in the bands was estimated. Table I shows that the DAUDI and RAJI cells incorporated about equal amounts of [^{35}S]methionine into the HLA-A, -B, and -C antigen heavy chains. However, when the same labeled amino acid was used, ~50% more radioactivity was incorporated into RAJI HLA-DR antigens than into DAUDI HLA-DR antigens. Likewise, considerably more L-[^{3}H]fucose was found in the HLA-DR antigens of RAJI cells as compared to the DAUDI equivalents. However, DAUDI HLA-A, -B, and -C antigen heavy chains did not contain any L-[^{3}H]fucose, in contrast to those of the RAJI antigens.

Thus, it can be concluded that DAUDI cells do not manufacture measurable amounts of fucose-labeled HLA-A, -B, and -C antigen heavy chains. Provided fucose labeling of the DAUDI HLA-A, -B, and -C antigen heavy chains had shown the same relationship to the RAJI counterpart as did the DAUDI HLA-DR antigens to the RAJI HLA-DR antigens, ~1000 dpm of L-[^{3}H]fucose should have been expected. Since <25 dpm was observed, at the most, 3% of the DAUDI HLA-A, -B, and -C antigen heavy chains could have become fucose labeled.

Cell Surface Expression of HLA-A, -B, and -C and HLA-DR Antigens. To examine whether DAUDI HLA-A, -B, and -C antigen heavy chains are expressed on the cell surface despite their lack of terminal glycosylation, we subjected DAUDI cells and, for comparison, RAJI cells to lactoperoxidase-catalyzed iodination. After solubilization, HLA-A, -B, and -C and HLA-DR antigens were separately immunoprecipitated and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. HLA-DR antigens from both cell lines were easily demonstrated, but HLA-A, -B, and -C antigens were only precipitated from the RAJI cells (not shown). Despite the use of both anti-HLA-A, -B, and -C antigen heavy-chain sera (286 and 303), we failed to detect any ^{125}I-labeled DAUDI HLA-A, -B, and -C antigen heavy chains. Provided DAUDI HLA-A, -B, and -C antigens incorporate ^{125}I to the same extent as their counterparts on RAJI cells, we would have discovered 0.5% or more of the amount of the HLA-A, -B, and -C antigens present on RAJI cells, should DAUDI cells have expressed such an amount. Thus, whereas DAUDI cells express HLA-DR antigens, they do not seem to have cell surface exposed heavy chains of HLA-A, -B, and -C antigens.

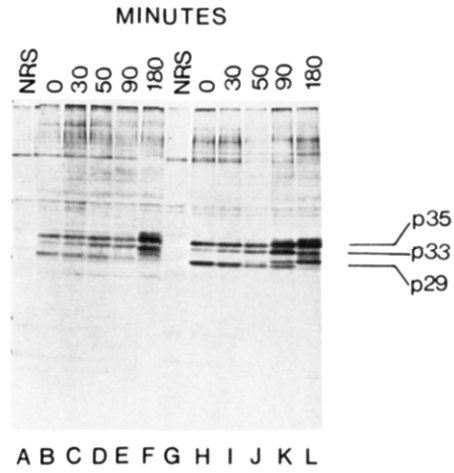

FIGURE 6: Pulse–chase experiment demonstrating the biosynthesis of HLA-DR antigens in DAUDI (A–F) and RAJI (G–L) cells. The cells (~1×10^6 cells for each precipitation) were labeled with [^{35}S]methionine for 10 min and chased with an excess of unlabeled methionine for the time periods indicated. Lysed cells were subjected to immunoprecipitation with normal rabbit serum (*NRS*) and with an anti-HLA-DR antigen serum (311). The NRS precipitations were carried out on material chased for 0 min. The molecular weight standards were the same as those described in Figure 1.

Intracellular Transport of HLA-DR Antigens. To examine whether DAUDI cells may have a general defect in the intracellular processing and transport of membrane glycoproteins, we examined the behavior of HLA-DR antigens in pulse–chase experiments. DAUDI and RAJI cells were separately labeled with [^{35}S]methionine for 10 min. The cells were then cultured for various periods of time in the presence of an excess of unlabeled methionine. After solubilization and immunoprecipitation, HLA-DR antigens were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Figure 6 depicts typical results. As can be seen in the figure three distinct polypeptide chains were observed at the end of the labeling period. During the chase period all chains were processed identically in RAJI and DAUDI cells.

Discussion

DAUDI and RAJI cells produce about equal amounts of HLA antigen heavy chains, as measured by incorporation of [^{35}S]methionine. In both cell lines the newly synthesized heavy chains become core-glycosylated as evidenced by their changed electrophoretic mobility after tunicamycin treatment, endo-H digestion, and binding to a *Lens culinaris* hemagglutinin

column. However, only heavy chains from RAJI cells form a complex with β_2m since DAUDI cells do not manufacture this protein (Nilsson et al., 1974). In RAJI cells β_2m is bound to the labeled heavy chain already at the end of a 10-min labeling period. This was ascertained by the observation that anti-β_2m serum precipitated the heavy chain. However, in RAJI cells the labeled HLA antigen heavy chain forms a complex with unlabeled β_2m since the pulse-labeled β_2m does not bind to the lectin column and is not precipitated by anti-heavy-chain sera. Accordingly, the endoplasmic reticulum of RAJI cells must contain an excess of unlabeled, free β_2m which dilutes the newly synthesized molecules. The observation that β_2m binds at an early time to the HLA antigen heavy chain corroborates similar findings by Dobberstein et al. (1979) in regard to H-2 antigens. Krangel et al. (1979) conclude that β_2m only becomes associated with the HLA antigen heavy chain at a later stage. The difference between our results and those of Krangel et al. (1979) may entirely depend on the use of different lymphoblastoid cell lines in the two studies.

RAJI HLA antigen heavy chains are processed and transported inside the cell like many other cell surface proteins [see Robbins et al. (1977), Kornfeld et al. (1978), and Jokinen et al. (1979)]. Thus, endo-H digestion and neuraminidase treatment of the heavy chains isolated from pulse–chase experiments clearly demonstrated that the HLA antigen heavy chains become terminally glycosylated, which most probably occurs in the Golgi complex [see Schmidt & Schlesinger (1980)]. These data are in agreement with previously published studies on the intracellular processing and transport of H-2 (Dobberstein et al., 1979) and HLA antigens (Krangel et al., 1979; Algranati et al., 1980). DAUDI HLA antigen heavy chains behave differently. Despite the fact that HLA antigen heavy chains were detected in DAUDI cells, as evidenced by NH_2-terminal amino acid sequence analyses and isolation on a β_2m column, the heavy chains did not change their molecular characteristics during pulse–chase experiments. No resistance to endo-H digestion was encountered, and neuraminidase treatment did not change the electrophoretic mobility of the DAUDI heavy chain. Furthermore, in contrast to the RAJI HLA antigen heavy chain which reacted well with the antiserum raised against denatured heavy chains only during the early period after synthesis, the DAUDI heavy chain reacted as well with this antiserum after a 10-min pulse label as after long-term labeling.

To rule out that the DAUDI HLA antigen heavy chains displayed any defect in their protein structure, we compared the size of the heavy chains with that of the RAJI heavy chains after labeling in the presence of tunicamycin. The heavy chains derived from the two cell lines were indistinguishable in size which suggests that neither the protein moiety nor the core glycosylation of the HLA antigen heavy chains of DAUDI cells is abnormal. Consequently, the lack of terminal glycosylation of the DAUDI HLA antigen heavy chains is most easily explained by the assumption that these chains do not become transported from the endoplasmic reticulum to the Golgi complex. This notion receives further support from the observation that DAUDI cells do not seem to express HLA antigen heavy chains on the cell surface, a finding in agreement with those of other investigators (Arce-Gomez et al., 1978; Ploegh et al., 1979).

In contrast, Östberg et al. (1975) found HLA antigen heavy chains on the DAUDI cell surface, and Ploegh et al. (1979) claimed that DAUDI heavy chains were normally glycosylated but not expressed on the cell surface. The reason for these discrepancies are not obvious.

The reason why DAUDI HLA antigen heavy chains do not become terminally glycosylated is not immediately obvious. Thus, β_2m may play a role in the intracellular processing of the HLA antigen heavy chain, but DAUDI cells may also have some general defect in their transport machinery. To investigate the latter of these possibilities, we examined the behavior of another type of transplantation antigen, the HLA-DR antigens [see Engleman (1980)], which are expressed by DAUDI and RAJI cells. The biosynthesis, intracellular transport, and processing of these glycoproteins were indistinguishable in the two cell types. Thus, there does not seem to be any obvious general defect in the biogenesis of membrane proteins in DAUDI cells.

Provided β_2m is needed for normal intracellular transport and processing of HLA antigen heavy chains, the lack of terminal glycosylation of the DAUDI HLA antigen heavy chain may be due to the absence of β_2m in this cell line. Since β_2m is a water-soluble protein which seems to occur in excessive amounts in the endoplasmic reticulum of RAJI cells and other lymphoblastoid cell lines (Dobberstein et al., 1979; Krangel et al., 1979), one might envision that the HLA antigen heavy chain, if not combined with β_2m during synthesis, may form aggregates or become associated with other proteins in the endoplasmic reticulum. In such events the heavy chain may be broken down so as not to interfere with the biosynthesis of other proteins.

After submission of this article a paper by Owen et al. (1980) appeared. These authors have examined the biosynthesis of HLA-A, -B, and -C antigens and conclude that in DAUDI cells the heavy chains are normally synthesized but that these cells display a defect intracellular transport. Their data are in complete agreement with those presented here.

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References

Algranati, I. D., Milstein, C., & Ziegler, A. (1980) *Eur. J. Biochem. 103*, 197–207.

Arce-Gomez, B., Jones, E. A., Barnstable, C. J., Solomon, E., & Bodmer, W. F. (1978) *Tissue Antigens 11*, 96–112.

Ballou, B., McKean, D. J., Freedlander, E. F., & Smithies, O. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4487–4491.

Berggård, I., & Bearn, A. G. (1968) *J. Biol. Chem. 243*, 4095–4103.

Beyer, T. A., Rearick, J. I., Paulson, J. C., Prieels, J.-P., Sadler, J. E., & Hill, R. L. (1979) *J. Biol. Chem. 254*, 12531–12541.

Blobel, G., & Dobberstein, B. (1975) *J. Cell Biol. 67*, 835–851.

Bonner, W. J., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059.

Dobberstein, B., Garoff, H., & Warren, G. (1979) *Cell (Cambridge, Mass.) 17*, 759–769.

Engleman, E. G., Ed. (1980) *J. Exp. Med. 152, 2:2*, 3s–326s.

Gorer, P. A., & O'Gorman, P. (1956) *Transplant. Bull. 3*, 142.

Hayman, M. J., & Crumpton, M. J. (1972) *Biochem. Biophys. Res. Commun. 47*, 923.

Henderson, L. E., Oroszlau, S., & Konigsberg, W. (1979) *Anal. Biochem. 93*, 153–157.

Hubbard, A. L., & Cohn, L. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., Ed.) pp 427–501, Chapman and Hall, London.
Hunt, L. A., Etchison, J. R., & Summers, D. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 754–758.
Jokinen, M., Gahmberg, C. G., & Andersson, L. C. (1979) *Nature (London) 279*, 604–607.
Kornfeld, S., Li, E., & Tabas, I. (1978) *J. Biol. Chem. 253*, 7771–7778.
Krangel, M. S., Orr, H. T., & Strominger, J. L. (1979) *Cell (Cambridge, Mass.) 18*, 979–991.
Kuo, S. C., & Lampen, J. O. (1974) *Biochem. Biophys. Res. Commun. 58*, 287–295.
Laemmli, V. K. (1970) *Nature (London) 227*, 680.
Möller, G., Ed. (1974) *Transplant. Rev. 21*, 3–143.
Nilsson, K., Evrin, P.-E., & Welsh, K. I. (1974) *Transplant. Rev. 21*, 53–84.
Orr, H. T., Lopez de Castro, J. A., Parham, P., Ploegh, H. L., & Strominger, J. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4395–4399.
Östberg, L., Rask, L., Nilsson, K., & Peterson, P. A. (1975) *Eur. J. Immunol. 5*, 462–468.
Owen, M. J., Kissonerghis, A.-M., & Lodish, H. F. (1980) *J. Biol. Chem. 255*, 9678–9684.
Peterson, P. A., Cunningham, B. A., Berggård, I., & Edelman, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1697.
Ploegh, H. L., Cannon, L. E., & Strominger, J. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2273–2277.
Rask, L., Lindblom, J. B., & Peterson, P. A. (1976) *Eur. J. Immunol. 6*, 93–100.
Robbins, P. W., Hubbard, S. C., Turco, S. J., & Wirth, D. F. (1977) *Cell (Cambridge, Mass.) 12*, 893–900.
Schmidt, M. F. G., & Schlesinger, M. J. (1980) *J. Biol. Chem. 255*, 3334–3339.
Sege, K., & Peterson, P. A. (1980) *Prog. Immunol. Int. Cong. Immunol., 4th*, Abstr. 8.2.29.
Snary, D., Barnstable, C. J., Bodmer, W. F., & Crumpton, M. J. (1977) *Eur. J. Immunol. 8*, 580–585.
Tarentino, A. L., & Maley, F. (1974) *J. Biol. Chem. 249*, 811–816.
Trägårdh, L., Curman, B., Wiman, K., Rask, L., & Peterson, P. A. (1979a) *Biochemistry 18*, 2218–2226.
Trägårdh, L., Rask, L., Wiman, K., & Peterson, P. A. (1979b) *Scand. J. Immunol. 10*, 597–600.
Trägårdh, L., Rask, L., Wiman, K., Fohlman, J., & Peterson, P. A. (1979c) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5839–5842.
Trägårdh, L., Rask, L., Wiman, K., Fohlman, J., & Peterson, P. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1129–1133.
Walsh, F. S., & Crumpton, M. J. (1977) *Nature (London) 269*, 307–311.

Purification of the Two Major Forms of Rat Pituitary Corticotropin Using Only Reversed-Phase Liquid Chromatography†

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ABSTRACT: Reversed-phase liquid chromatography has been used to extract and purify to homogeneity the two major forms of corticotropin (ACTH) from 60 rat anterior pituitaries. Tissue was homogenized in a medium designed to minimize peptidase activity and maximize solubilization of peptides. The supernatant obtained from the tissue homogenate was extracted in a batch procedure with octadecylsilylsilica (ODS-silica). Elution of the ODS-silica gave rise to a desalted and essentially protein-free preparation, which was enriched in peptides. From this initial extract, the two major forms of rat ACTH were purified to apparent homogeneity by reversed-phase high-performance liquid chromatography (RP-HPLC), using solvent systems containing either trifluoroacetic acid or heptafluorobutyric acid as hydrophobic counterions. The recovery of ACTH immunoreactivity through the tissue extraction and chromatography stages was close to 100%. In control experiments, it was observed that the structural integrity of synthetic tritiated human ACTH was maintained throughout the extraction and purification procedures. The two forms of rat ACTH were found in approximately equimolar amounts, with very similar amino acid compositions which indicated a close similarity to other mammalian corticotropins. Both forms were found to have biological activities and molecular weights comparable to standard synthetic human $ACTH_{1-39}$. Trypsin digestion indicated that the two peptides were identical except for a modification of one form in the carboxyl-terminal tryptic peptide. Initial radiolabeling experiments, using cultured rat anterior pituitary cells, have shown that the more polar form of rat ACTH is O-phosphorylated on the serine residue at position 31.

Reversed-phase high-performance liquid chromatography (RP-HPLC)[1] is proving to be a very useful technique in the preparation of both natural and synthetic polypeptides (Rivier, 1978; Hancock et al., 1978). High resolution of closely related compounds can be achieved with the chromatographic supports which are now available. However, RP-HPLC is almost invariably used in the final purification step which follows more conventional ion-exchange and gel-filtration chromatographic procedures. For instance, such methods have been used to purify several natural peptide hormones, including α-endorphin and γ-endorphin from a rat hypothalamic–hypophyseal extract

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[1] Abbreviations used: RP-HPLC, reversed-phase high-performance liquid chromatography; ACTH, corticotropin; α-MSH, α-melanotropin; β-LPH, β-lipotropin; RIA, radioimmunoassay; $NaDodSO_4$, sodium dodecyl sulfate; F_3CCOOH, trifluoroacetic acid; HFBA, heptafluorobutyric acid.

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