A CARBOHYDRATE-CONTAINING MOUSE LIGHT CHAIN-PROTEIN

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The immunoglobulins are glycoproteins composed of two heavy chains (H-chains)* (M.W.~50,000) and of two light chains (L-chains)* (M.W.~20,000) (Fleischmann, Pain and Porter, 1962; Edelman and Gally, 1964). Carbohydrate is found covalently linked to the papain produced Fc fragment, the carboxyl terminal half of the H-chain (Fleischmann, Porter and Press, 1963). The L-chains usually are free of carbohydrate, although traces are sometimes found.

We have examined the product of one of the mouse plasma cell tumors, (MOPC46)** which seems in all ways tested to be an L-chain and to have attached carbohydrate. Moreover, this L-chain shows electrophoretic heterogeneity in starch gels. This work reports a successful attempt to resolve the protein into its separate components and to find the chemical basis for the heterogeneity.

RESULTS AND DISCUSSION

Purification of MOPC46-protein and its identification as an L-chain.

Urine was collected from female mice directly into a bottle and quickly frozen for storage. The protein was precipitated from urine in 10^{-3}M EDTA by adding $(NH_4)2SO_4$ to 70% saturation, dissolved in H_2O and dialyzed 36 hours against 3-5 changes of H_2O. Insoluble material was spun out and the supernatant protein used for identification and separation. The main protein in the urine of BALB/c mice bearing the MOPC46 tumor has been identified serologically by Liebermann, Dray and Potter (1965) as a K-type* L-chain. We have in addition identified it as an L-chain by reconstitution with an H-chain to make an IgG-protein, by serological studies and by the "fingerprint" of its tryptic peptides (Melchers, Lennox and Facon, in preparation).

Separation of MOPC46-protein. MOPC46-protein could be separated into fractions on DEAE-cellulose at pH 8.0 (Fig.1). Bands I and II (fractions d and e) were

* Terms recommended by the Committee on Nomenclature of Human Immunoglobulins, Bull.Wld.Hlth.Org. (1964). 30, 447.

** The plasma-cell tumor, MOPC46, was grown in BALB/c-mice. It was given to us by Dr. Michael Potter, NIH, Bethesda, in passage numbers 30 and is now in passage 46.

were purified by rechromatography on DEAE-cellulose at pH 8.0. Fraction f was further purified by passing it through Sephadex G100 (Fig.2). Rechromatography of fraction f-3 on DEAE-cellulose at pH 8.0 then separated bands II and III. Evidence that fractions d, e and f-3 are monomer L-chains and fractions g to j and f-2 are dimerized L-chains will be presented elsewhere. (Melchers, Lennox and Facon, in preparation).

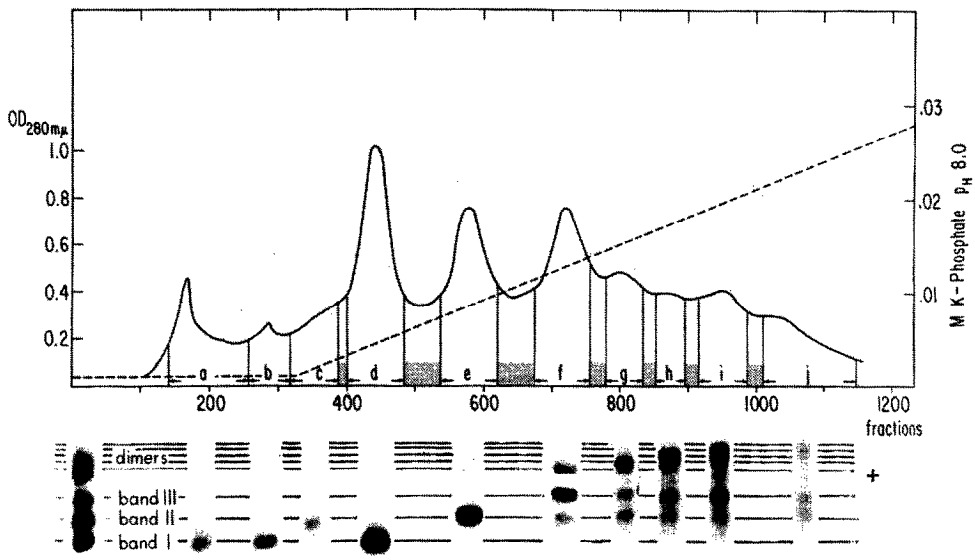

Fig.1 Chromatography of 3600 OD_{280}-units MOPC46-protein in 0.001M K-phosphate-buffer, pH 8.0 on a 4.5 x 109 cm DEAE-cellulose-column (Serva Entwicklungslabor, Heidelberg, Germany, 0.68 meq/g, Batch B 2573) using a linear gradient of 5000 ml each 0.001M to 0.04M K-phosphate-buffer, pH 8.0. Fraction volume was 5.6 ml. Electrophoresis was performed in 8M urea-formate starch gels (Edelman and Poulik, 1961).

Characterization of the separated bands. The separated MOPC46-monomer proteins are identical by serological tests with antiserum prepared against crude MOPC46 proteins (Melchers, Lennox and Facon, in preparation) and in amino acid composition. (Table 1). This suggests that the protein of MOPC46 is homogeneous with a defined amino acid sequence.

First hint of carbohydrate in MOPC46 protein was a ninhydrin positive peak between Leu and Tyr on analysis for amino acids which is attributable to hexosamines. Hydrolysates of an H-chain from a γG-myeloma-protein also showed this peak, while its L-chain did not. The occurrence of carbohydrate in MOPC46-L-chain-protein was shown directly by incorporation of C^{14}-glucose, C^{14}-mannose and C^{14}-galactose into a suspension of MOPC46 tumor cells. More than 80% of the C^{14} radioactivity bound to the purified MOPC46-protein was found to exist

in sugars which remained attached to the L-chain after chromatography and dialysis in 1N propionic acid, in 8M urea and in 5M guanidine. Tryptic digestion of the reduced and alkylated protein yielded a hexose-positive peak cochromatographing on Dowex 1x2 columns with ninhydrin-positive material. These experiments indicate that the carbohydrate portion is covalently attached to the L-chain. (Melchers and Lennox, 1966 and in preparation). Qualitative analysis of the carbohydrate portion of MOPC46 protein by paper chromatography after acid hydroly-

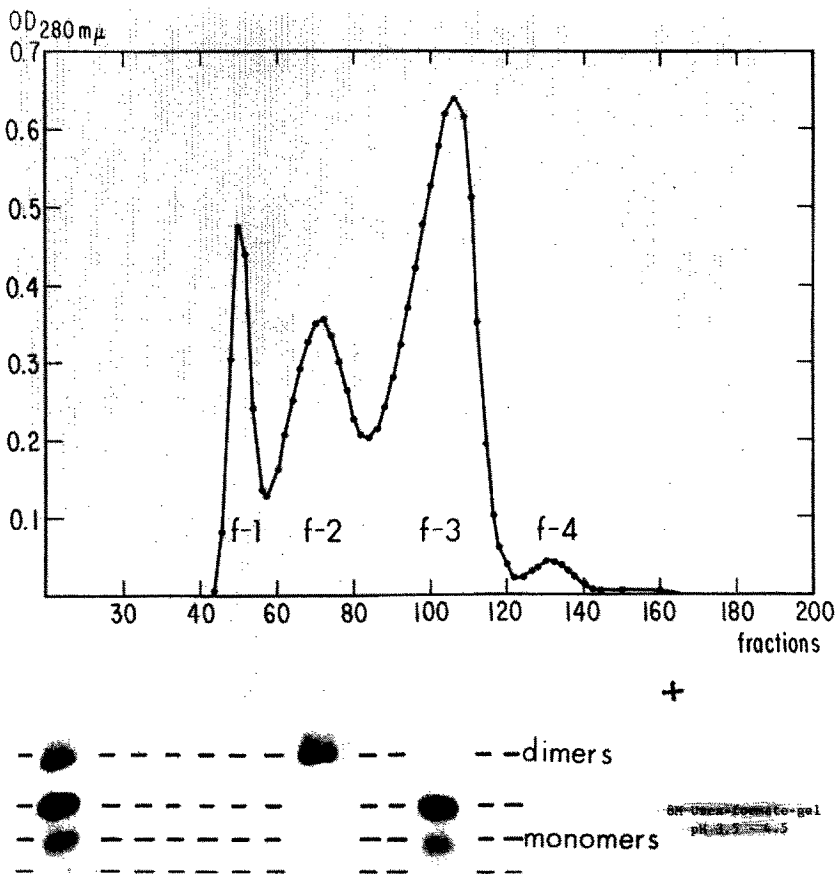

Fig.2 Chromatography of 300 OD_{280}- units DEAE-cellulose-fraction f (Fig.1) on a 5.5 x 30 cm G100-Sephadex-column in 0.2N propionic acid. Fraction volume 5.0 ml.

sis revealed mannose, galactose, fucose and glucosamine* (See legend to Table 1).

Quantitative determination of the carbohydrate composition of the separated monomer proteins was done in duplicate. The amounts of mannose, galactose, fucose and glucosamine* were the same in all three monomer proteins of MOPC46-L-chain. Differences among them were found in their content of sialic acid. (Table 1). Sialic acid was identified by paper chromatography (n-butanol/n-propanol/0.1N HCl 1:2:1 (v/v), Svennerholm and Svennerholm, 1958) as N-glycolyl-neuraminic acid (R_F=0.35) using N-acetyl- and N-glycolyl-neuraminic acid (R_F= 0.44 and 0.35) as standards. Thus heterogeneity in the banding of MOPC46-L-chain protein on electrophoresis in urea starch gel is due to different amounts of sialic acid attached to the same protein.

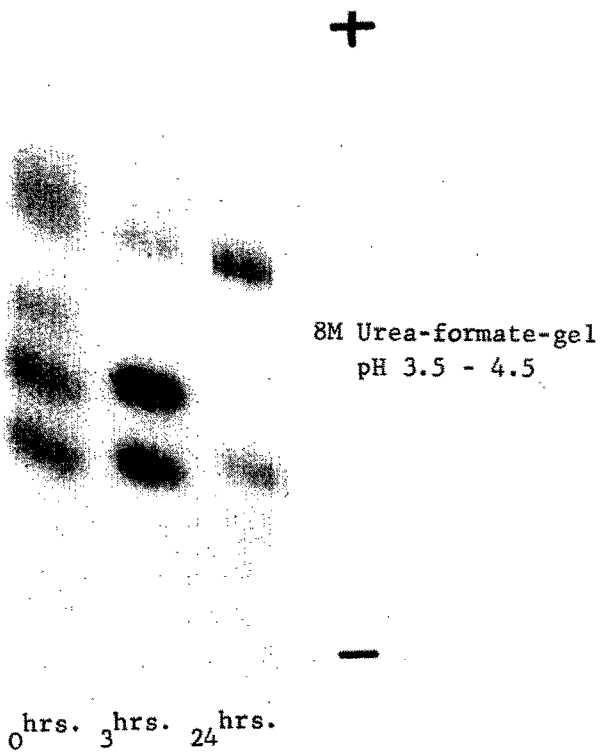

Fig.3 Neuraminidase treatment of MOPC46 protein. Enzymic digestion with neuraminidase was carried out in 0.05M Na-Acetate pH 5.5, 10^{-2}M $CaCl_2$ using 1000 RDE-units/25 mg protein. Neuraminidase (RDE) of Cholera vibrio was a gift from Dr. S. Fazekas de St.Groth.

* It is not clear whether glucosamine is present in part or totally as N-acetyl-glucosamine, since the hydrolysis conditions applied remove the acetyl-groups quantitatively from hexosamines.

Moreover, one could demonstrate interconversion of the monomer bands of MOPC46-protein by treating them with 0.025N H_2SO_4 at 80° for 1 hour or by enzymic digestion with neuraminidase (Fig.3). Both experiments indicated that the differences in mobilities in starch gel electrophoresis might be explained by variation in the amount of sialic acid bound to terminal position(s) of the carbohydrate portion of the MOPC46-L-chain-protein. The removal of negatively charged sialic acid gives the protein an increased mobility towards the negative pole. Fig. 3 also shows sharpening of the dimer bands into the first dimer band indicating that neuraminidase also acted on the dimers. The conversion of band I + II + III into band I + II was completed in 2 - 3 hours; the conversion from band I +II into band I required approximately 24 hours, even after further addition of fresh neuraminidase. This may indicate a difference either in the nature of the bond of the sialic acids to their next sugar residues, a difference in the sugars next to the sialic acids, or a difference in the molecular structure of the sialic acids.

Normally the L-chains of γ-globulin have been found not to contain significant amounts of carbohydrate. The MOPC46-L-chain is amongst the first clear exceptions. Its carbohydrate composition and the size of its carbohydrate portion is similar to the one analyzed in two myeloma γG-globulins from BALB/c-mice (Melchers, unpublished). Carbohydrate on L-chains has recently also been found in other laboratories (Marshall and Potter, 1966; Edmundson, 1966). The carbohydrate-containing L-chain producing plasma cell tumor is a good system to study where in the cell the carbohydrate portion of a glycoprotein is attached (Melchers and Lennox, 1966), how it is attached to a specific point within the polypeptide chain and what the biological functions of the carbohydrate group are. The tryptic glycopeptide from MOPC46-L-chain and the two myeloma γG-globulins are presently under study to determine the nature of the carbohydrate-protein bond and to establish the sequence of amino acids around the attachment point of carbohydrate.

Table 1 Amino acid* and carbohydrate** composition of the three separated monomer-bands of MOPC46-L-chain-protein.

	Band I	Band II	Band III
Lys	9.4 ± 0.5	9.4 ± 0.4	10.2 ± 0.3
His	4.1 ± 0.3	4.1 ± 0.35	4.0 ± 0.2
Arg	5.8 ± 0.6	5.8 ± 0.4	5.6 ± 0.6
Asp	24.6 ± 0.3	24.6 ± 0.3	25.3 ± 0.4
Thr	19.3 ± 0.4	18.4 ± 0.5	18.9 ± 0.4
Ser	34.6 ± 0.8	33.7 ± 0.9	34.7 ± 0.9
Glu	19.7 ± 0.5	20.9 ± 0.7	19.7 ± 0.4
Pro	10.1 ± 0.2	10.0 ± 0.3	10.4 ± 0.2
Gly	12.4 ± 0.3	11.8 ± 0.4	11.4 ± 0.3
Ala	10.0 ± 0.5	10.8 ± 0.5	9.9 ± 0.6
Val	6.9 ± 0.2	7.2 ± 0.3	7.5 ± 0.3
Met	4.0 ± 0.4	4.0 ± 0.4	3.9 ± 0.3
Ilu	8.4 ± 0.2	8.0 ± 0.2	8.7 ± 0.4
Leu	15.2 ± 0.2	15.2 ± 0.2	15.4 ± 0.2
Tyr	6.2 ± 0.3	6.5 ± 0.3	5.7 ± 0.4
Phe	7.8 ± 0.2	8.1 ± 0.2	7.8 ± 0.1
Carboxy-methyl-Cys	4.4	4.5	4.6
Try	5.5	5.6	5.4
Mannose	4.01	3.85	3.92
Fucose	2.08	2.02	2.04
Galactose	4.22	3.92	3.85
Glucosamine	6.04	5.86	6.00
Sialic acid	0.15	1.04	1.77

* No. of Amino acids/200 Amino acid for total MOPC46-L-chain (uncorrected for losses during hydrolysis). Determined with the Beckman 120 Amino Acid Analyzer using the modified elution system by Dus, Lindroth, Pabst and Smith (1966) five times for each band using two separate hydrolysates for each band. Cysteine was determined after reduction of the protein in 8M urea, 0.5M Tris-HCl, pH 8.5, 10^{-3}M EDTA, 0.05M Dithioerythritol (Cyclo Chem.Co.) and alkylation with 0.15M Iodoacetamide. Tyrosine and Tryptophan were determined spectrophotometrically by the method of Goodwin and Morton (1946). Tryptophan was also determined by the method of Ramanchandran and Witkop (1959) measuring spectrophotometric changes after oxidation by N-bromo-succinimide (Fisher Sci. Co.)

** Residues/MOPC46-L-chain (M.W. 23,700). Corrected for losses during hydrolysis and separation. MOPC46-protein was hydrolyzed in 1N H_2SO_4 for 8 hours at 100°C. H_2SO_4 was neutralized by $Ba(OH)_2$. Amino sugars were separated from each other and from neutral sugars on Amberlite CG-120 (Mallinckrodt, 100-200 mesh, 0.33N HCl as eluent at 50°C) (Jolles, Samour and Lederer, 1963). The fractions of the Amberlite-column were dried over P_2O_5 and NaOH and assayed for aminohexoses by the method of Boas (1953). Neutral hexoses in aliquots of the hydrolyzed samples were separated and identified using the method of Walborg and Christensson (1965a) and Walborg, Christensson and Gardell (1965b). All sugar fractions detected on Amberlite CG120 and Dowex 2 x 8 columns were further identified by paper chromatography (n-butanol/pyridine/H_2O 5:3:2 (v/v), Ethyl-acetate/pyridine/H_2O 3.6:1:1.15 (v/v)). Reducing sugars were detected by

the $AgNO_3$-method (Trevelyan, Procter and Harrison, 1950), or by the Ferricyanide method (Mowery, 1957), reducing and non-reducing sugars by the periodate method of Mowery (1957). Removal of sialic acid by mild acid hydrolysis was done by incubating the protein in 0.025N H_2SO_4 for 1 hour at 80°. Released sialic acid was isolated by the method of Gyorky and Houck (1965). The thiobarbituric acid assay (Aminoff, 1961) was used for the quantitative determination of sialic acid.

SUMMARY - A K-type L-chain protein from a plasma cell tumor in BALB/c mice (Potter, MOPC46) shows electrophoretic heterogeneity by banding in urea starch gel. The protein bands can be purified on DEAE-cellulose at pH 8.0. The L-chain protein contains carbohydrate; neuraminidase converts the three protein bands into one. Amino acid analysis of the three separated monomer proteins detects no differences. Analysis of the carbohydrate portion gives 4 mannose, 4 galactose, 2 fucose, 6 glucosamine residues in each of the three protein components. The three separable proteins differ in their sialic acid content, having respectively 0, 1 or 2 residues per molecule. The sialic acid is identified as N-glycolylneuraminic acid. The results explain the banding of this K-type-L-chain protein by electrophoresis in urea starch gel as single negative charge differences due to different amounts of sialic acid perhaps at the terminal position(s) of the carbohydrate portion of the L-chain.

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REFERENCES

Aminoff, D., (1961). Biochem. J., 81, 384.
Boas, N.F., (1953). J. Biol. Chem., 204, 553.
Dus, K., Lindroth, S., Pabst, R., and Smith, R.M., (1966). Anal. Biochem., 14, 41.
Edelman, G.M., and Gally, J.A., (1964). Proc. Nat. Acad. Sci. U.S., 51, 846.
Edelman, G.M., and Poulik, M.D., (1960). J. Exper. Med., 113, 861.
Edmundson, A.B., (1966). Personal communication.
Fleischmann, J.B., Pain, R.H., and Porter, R.R., (1962). Arch. Biochem. Biophys. Supplement 1, 174.
Goodwin, T.W., and Morton, R.A., (1946). Biochem. J., 40, 628.
Gyorky, G., and Houck, J.C., (1965). Canad. J. Biochem., 43, 1807.
Jolles, P., Samour, D., and Lederer, E., (1963). Biophys. Biochim. Acta, 18, 342.

Liebermann, R., Dray, S., and Potter, M., (1965). Science, 148, 640.
Marshall, R.D., and Potter, M., (1966). Abstract 3rd Symp. Feder. Europ. Biochem. Societies, Warsaw. p.258.
Melchers, F., and Lennox, E.S., (1966). Feder. Proc., 25, 654.
Mowery, D.F., Jr., (1957). Anal. Chem., 29, 1560.
Ramachandran, L.K., and Witkop, B., (1959). J. Amer. Chem. Soc., 81, 4028.
Svennerholm, E., and Svennerholm, L., (1958). Nature, 181, 1154.
Trevelyan, W.E., Procter, D.P., and Harrison, J.S., (1950). Nature, 166, 444.
Walborg, E.F., Jr., Christensson, L., (1965a). Anal. Biochem., 13, 186.
Walborg, E.F., Jr., Christensson, L., and Gardell, S., (1965b). Anal. Biochem., 13, 177.