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THE PRESENCE OF TWO IgG SUBCLASSES IN WATER-BUFFALO IMMUNOGLOBULINS

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Summary

The immunoglobulins of a water-buffalo have been fractionated by ammonium sulphate precipitation and DEAE-cellulose chromatography into eight pools. These pools were analysed by polyacrylamide gel electrophoresis, analytical isoelectric focusing, hexose determination, and chemical typing. The results suggested that water-buffalo IgG consists of two subclasses, fast (IgG1) and slow (IgG2).

In addition, the light chains were shown to be entirely of the lambda type. The C-terminal tryptic peptide was purified and shown to contain an extra proline when compared to the equivalent peptide from bovine light chains.

Introduction

There are three major classes of immunoglobulins, i.e, IgG, IgA and IgM, found in most mammals and distinguishable on the basis of gross properties such as molecular weight or carbohydrate content.[1] In addition, each class may be divided into antigenically distinct subclasses, such as the four subclasses of human IgG. Amino acid sequence studies of myeloma proteins in men[2-5] and mice[6-10] have shown that the IgG subclasses differ in their γ-chain *C*-region sequences, most notably in the sequences around the interchain disulphide bonds. These differences in interchain disulphide bridge sequences have formed the basis for the 'chemical typing' of IgG subclasses in man[11] and mice[12]. This procedure involves selective reduction of interchain disulphide bonds, labelling with iodo-^{14}C-acetic and, followed by digestion with pepsin and trypsin, and high voltage paper electrophoresis at pH 6.5 or pH 3.5.

However, the detection of subclasses in a species lacking myeloma proteins is often a difficult undertaking, even with classical immunological techniques. We

have therefore sought to test the potential of the chemical typing and other physico-chemical techniques in studying the immunoglobulins of a previously uncharacterized species, the water-buffalo *(Bubalis bubalis)*, where myeloma proteins are unknown.

Materials and Methods

Fractionation of serum

Blood from a water-buffalo was obtained from the local slaughterhouse and allowed to clot at room temperature overnight. Serum was centrifuged at 12,000 g for 30 min and stored frozen at-20°C until used. Precipitation with ammonium sulphate was carried out with 40-100 ml batches of serum by adding an equal volume of 0.9% NaCl-10 mM sodium phosphate, pH 7.2 (PBS) followed by saturated ammonium sulphate to a final concentration of 43% saturation. The precipitate was collected by centrifugation at 12,000 g and washed three times by resuspending in PBS and repeating the precipitation step. The final precipitate was resuspended in 10 mM Na_2HPO_4 and dialysed exhaustively against the same solvent at 4°C.

The resuspended and dialysed precipitate was applied to a DEAE-cellulose (Cellex-D) column (2.5 cm x 40 cm) equilibrated with 10 mM Na_2HPO_4. The column was eluted with 500 ml of starting buffer, followed by a gradient (600 ml + 600 ml) of 10 mM Na_2HPO_4 + 300 mM NaH_2PO_4. Fractions of 7 ml were collected. The OD_{280} of alternate tubes was measured using a Zeiss PMQII spectrophotometer, and the pH and conductivity of certain tubes were monitored. Eight pools (Fig. 1) containing immunoglobulins, were made on the basis of an analysis of selected tubes by polyacrylamide gel electrophoresis. Each pool was dialysed against distilled water, lyophilized and further analysed by gel electrophoresis, isoelectric focusing, ultracentrifugation and chemical typing.

The immunoglobulin preparation used in the preparation of heavy and light chains was derived from the breakthrough peak of a DEAE-cellulose column equilibrated with 17.5 mM sodium phosphate buffer, pH 6.5 and loaded with a dialysed ammonium sulphate precipitate of serum, obtained as described above.

Selective reduction was carried out after the procedure of Frangione *et al*[11], which has been shown to reduce only the interchain disulphide bonds of human and mouse IgG. Proteins were dissolved as a 10 mg/ml solution in 0.5 M Tris-HCl, buffer, pH 8.2 and reduced with 0.65 mM dithiothreitol for 45 min at 37° under N_2. Alkylation was then carried out with 2 mM iodo-$^{14}C_1$-acetic acid (specific activity 0.5 mCi/mmole) for 1 h at 37° C under N_2. The reaction was stopped by adding β-mercaptoethanol to a concentration of 0.15 M.

Chemical typing

Selectively reduced and^{14}C-carboxymethylated immunoglobulins were dialysed against 5% (v/v) formic acid, digested with pepsin (E:S ratio 1:40) overnight at 37°C and lyophilized. Trypsin digestion was then carried out in 0.5% NH_4HCO_3 for 4 h at 37°C (E:S ratio 1:50). The digest was lyophilized and fractionated by high voltage paper electrophoresis at pH 6.5 or pH 3.5.

Separation of light and heavy chains

140 mg of selectively reduced and ^{14}C-carboxymethylated immunoglobulins were fractionated by gel filtration on a Sephadex G-100 column (3.8 cm x 80 cm) in 1 M propionic acid. Fractions of 7 ml were collected at the OD_{280} of alternate tubes measured. Heavy and light chain peaks were pooled and lyophilized, For tryptic digestion, light chains were first totally reduced[3] with 3.25 mM dithiothreitol and alkylated with 9 mM non-radioactive iodoacetate in 6.6 M guanidine-HCl-0.1 M Tris-HCl buffer, pH 8.1 and then dialysed against 0.5% NH_4HCO_3.

Animalytical Techniques

Polyacrylamide gel electrophoresis was carried out in 7.5% gels in 0.37 M Tris-glycine, pH 9.5[13]. The purity of isolated heavy and light chains was checked by SDS-polyacrylamide gel electrophoresis. Analytical isoelectric focusing in the pH range 3 to 10 was performed according to the procedure of Karlsson *et al.*[14] Analytical ultracentrifugation was carried out in a Beckman Model E ultracentrifuge at 20°C. Proteins were dissolved as 4-5 mg/ml solutions in PBS. Sedimentation values were estimated from photographs taken at 52,000 rpm using Schlieren optics.

High voltage paper electrophoresis[15] was carried out at pH 6.5 or pH 3.5 : electrophoretic mobilities at these pHs ($\mathbf{m}_{6.5}$ and $\mathbf{m}_{3.5}$ respectively) were measured relative to aspartic acid. Radioactive peptides were detected by autoradiography on Kodak NS-2T no-screen X-ray film.

Amino acid analyses were kindly performed by Ms. Suparb Suanparn of the Division of Nutrition, Dept. of Health, Ministry of Public Health, on a Hitachi model KLA-3B amino acid analyser. Peptides were hydrolysed with 6N HCl (Merck Pro Analysi grade) for 20 h under vacuum at 105°C. Analyses of hexose content were carried out by the orcinol procedure[16] and protein assays by the Lowry method[17].

Results

The precipitate of serum, obtained at 43% saturation of ammonium sulphate as described in Materials & Methods, was fractionated by DEAE-cellulose chromatography (Fig.1). On the basis of an analysis of selected tubes on polyacrylamide gel electrophoresis at pH 9.5 eight pools were taken. Each of these pools showed slow-moving diffuse bands characteristic of normal immunoglobulins, entirely free of other contaminants. Pools 1-3 also appeared to have slower electrophoretic mobilities than pools 5-8. This is more clearly seen on analytical isoelectric focusing (Fig. 2), where pools 1-3 have a distinctly higher isoelectric points than pools 5-8 in agreement with their elution patterns on DEAE-cellulose. Pool 4 appears to contain elements of both pools 1-3 and pools 5-8. Although pool 8 is eluted out in a later peak on DEAE-cellulose, the pattern of bands obtained on isoelectric focusing is very similar to that of pool 6 but some bands of lower isoelectric points are visible.

Further analysis of pools 2,4,6 and 8 were made by ultracentrifugation (Fig. 3). At least 85% of the material in each pool had sedimentation values of 6.7 S-7.0 S

(6.8 S for pool 2, 7.0 S for pool 4, 6.9 S for pool 6 and 6.7 S for pool 8). Some faster sedimenting contaminants were also evident : 10.0 S components in pools 2,4 and 6, and also 19.8 S component in pools 6 and 8. The 19.8 S component is consistent with the pentameric form of IgM and the 10.0 S components are consistent with dimeric IgA.

The results of hexose analysis are shown in Table 1. The low hexose contents of pools 1, 2, 3, 4, 5 and 6 (in the range 0.8-1.4%) are consistent with the low values found in most IgG[1]. The higher hexose content of pool 8 (2.5%) may be explained by the relatively large content of IgM (Fig. 3) present, since IgM typically has a high hexose content of over 5%.

TABLE I HEXOSE CONTENT OF DEAE-CELLULOSE POOLS

Pool No.	% Hexose
1	0.8
2	1.1
3	1.0
4	1.3(5)
5	1.0(5)
6	1.4
7	1.8
8	2.5

TABLE II AMINO ACID COMPOSITION OF PEPTIDE Ll

	Residues/mol	Nearest Integer
CMCys	0.52	1
Asp	0.20	–
Thr	0.93	1
Ser	1.87	2
Glu	1.20	1
Pro	1.97	2
Val	1.01	1
Lys	0.99	1

The results of chemical typing of these eight pools by selective labelling of interchain half-cystines, pepsin and trypsin digestion followed by paper electrophoresis at pH 6.5 are shown in Fig. 4. Two peptides are obtained from light chains (peptides Ll and L2). The major light chain peptide Ll has an electrophoretic mobility ($m_{6.5}=0.25$) identical to the radioactive peptide isolated from a tryptic digest of totally reduced light chains. The presence of peptide Ll in all pools indicated thal they all derive from immunoglobulins. Pools 1,2 and 3 clearly differ from pools 5-8 in possessing the characteristic major peptide Hl and minor peptides H2 and H3. While the most distinctive feature of pools 5-8 appears to be the absence of the major peptide Hl, pools 5-8 also share the common feature of possessing the minor peptides H4 and H5. On the basis of the presence of the major peptide Hl and possibly also H2, pool 4 probably falls into the same group as pools 1,2 and 3. But pool 4 may also possess peptide H5 and could thus contain both material similar to some components in pools 5-8 and material similar to some components in pools 1,2 and 3.

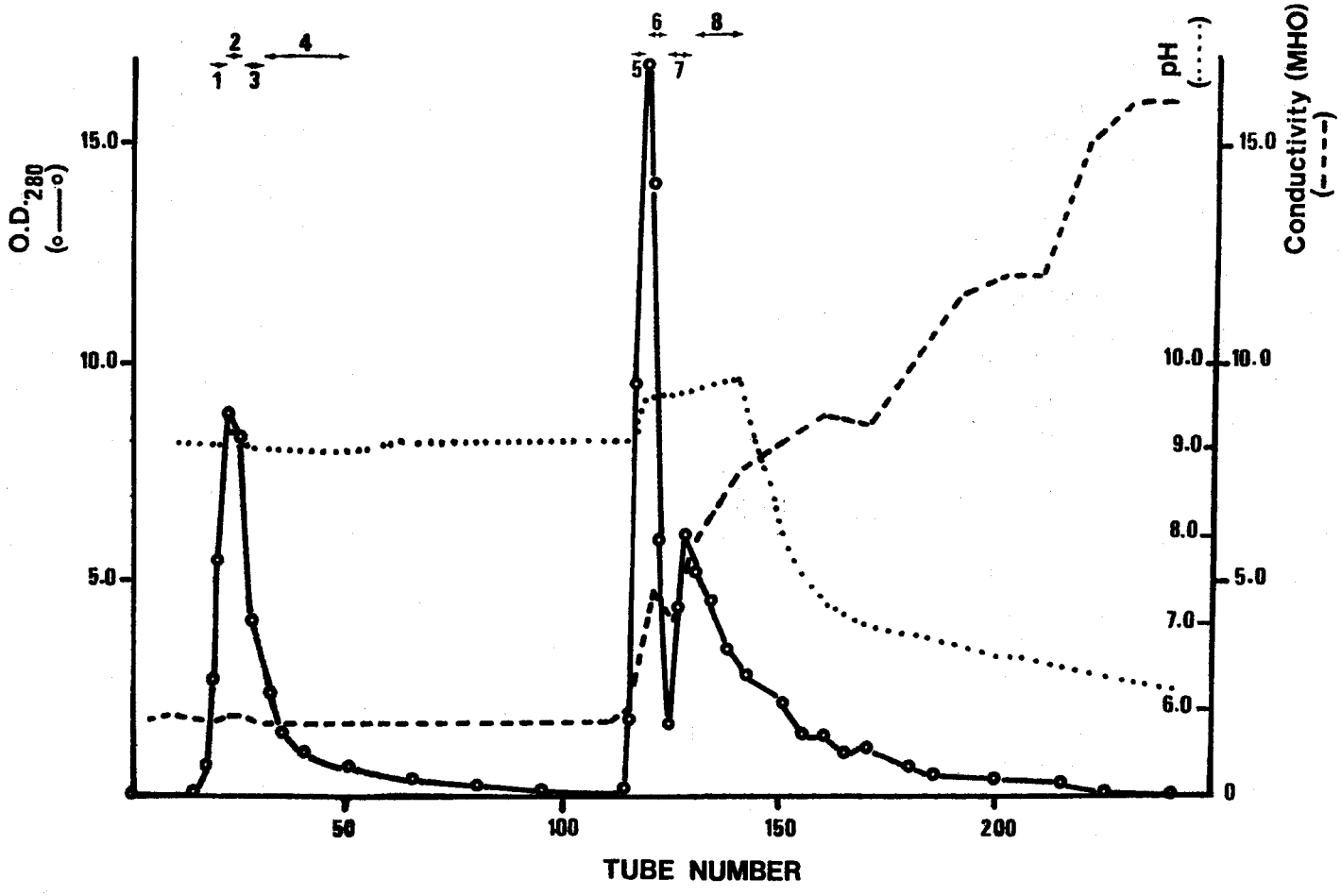

Fig. 1. DEAE-cellulose chromatography of an ammonium sulphate precipitate of water buffalo serum. The dialysed precipitate was applied to a 2.5 cm × 40 cm column of DEAE-cellulose equilibrated with 10 mM Na_2HPO_4. The column was eluted with 500 ml of starting buffer followed by a gradient (600 ml + 600 ml) of 10 mM Na_2HPO_4 + 300 mM NaH_2PO_4. Fractions of 7 ml were collected, Eight pools (1–8) were collected as shown. $O.D._{280}$ o——o; pH .....; conductivity– – –.

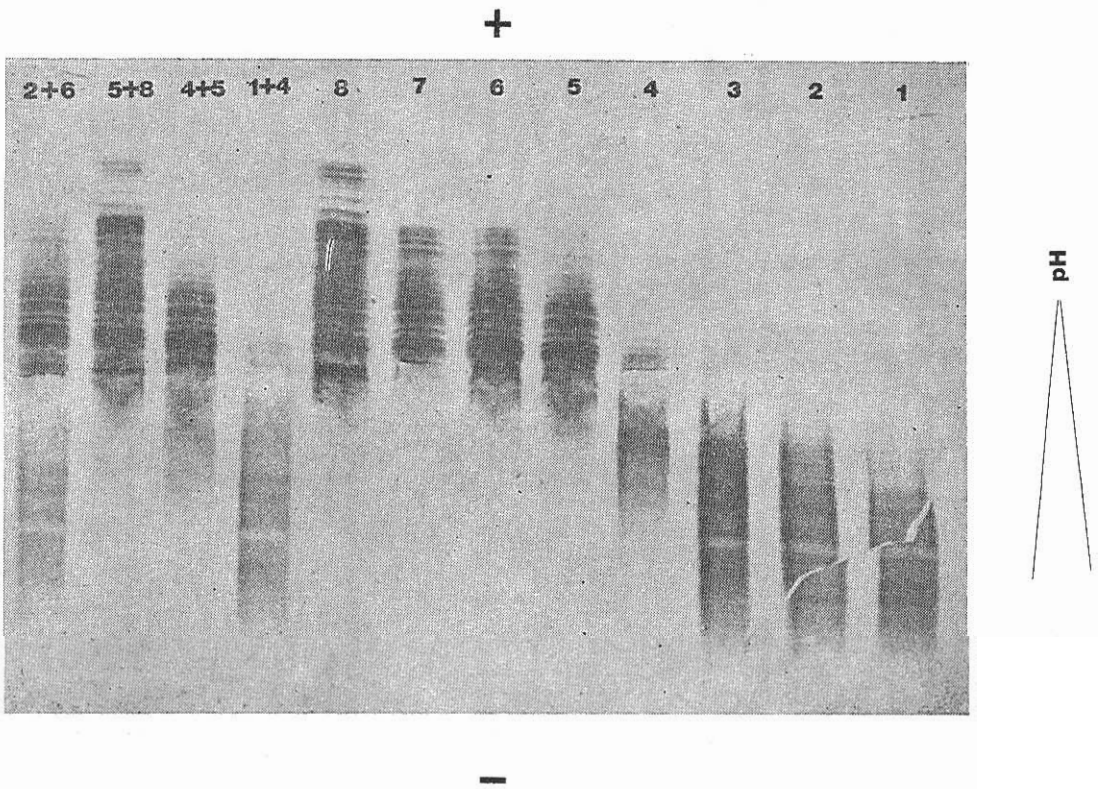

Fig. 2. Analytical polyacrylamide gel isoelectric focusing (pH range 8.5–9.5) of DEAE-cellulose pools 1-8 and of mixtures of pools 1 + 4, 4 + 5, 5 + 8, 2 + 6.

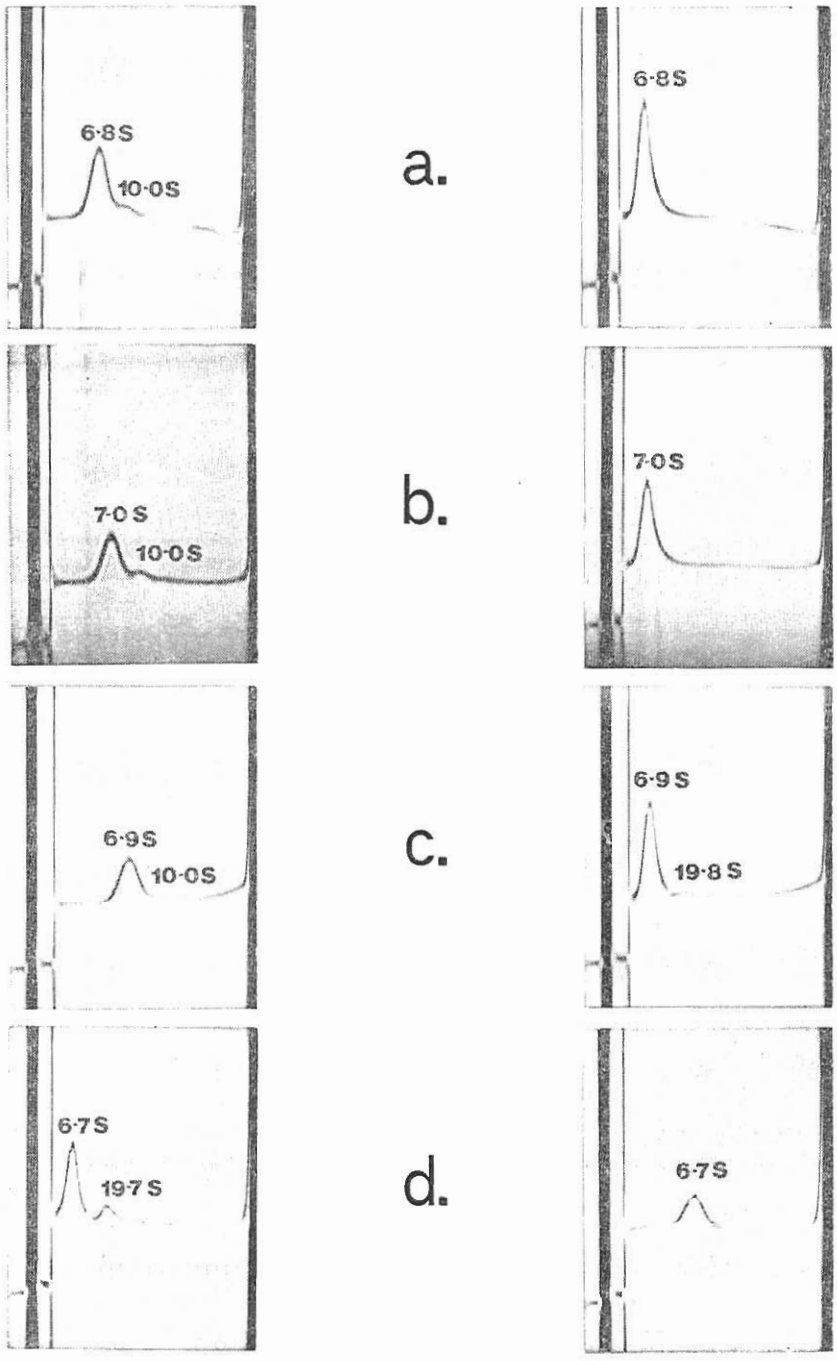

Fig. 3. Analytical ultracentrifugation of DEAE-cellulose pools: (a) pool 2 (b) pool 4 (c) pool 6 (d) pool 8. Two photographs are shown for each pool, taken at approximately 20 min. and 60 min. after reaching 52,000 r.p.m. The s_{20} values of each peak are also shown.

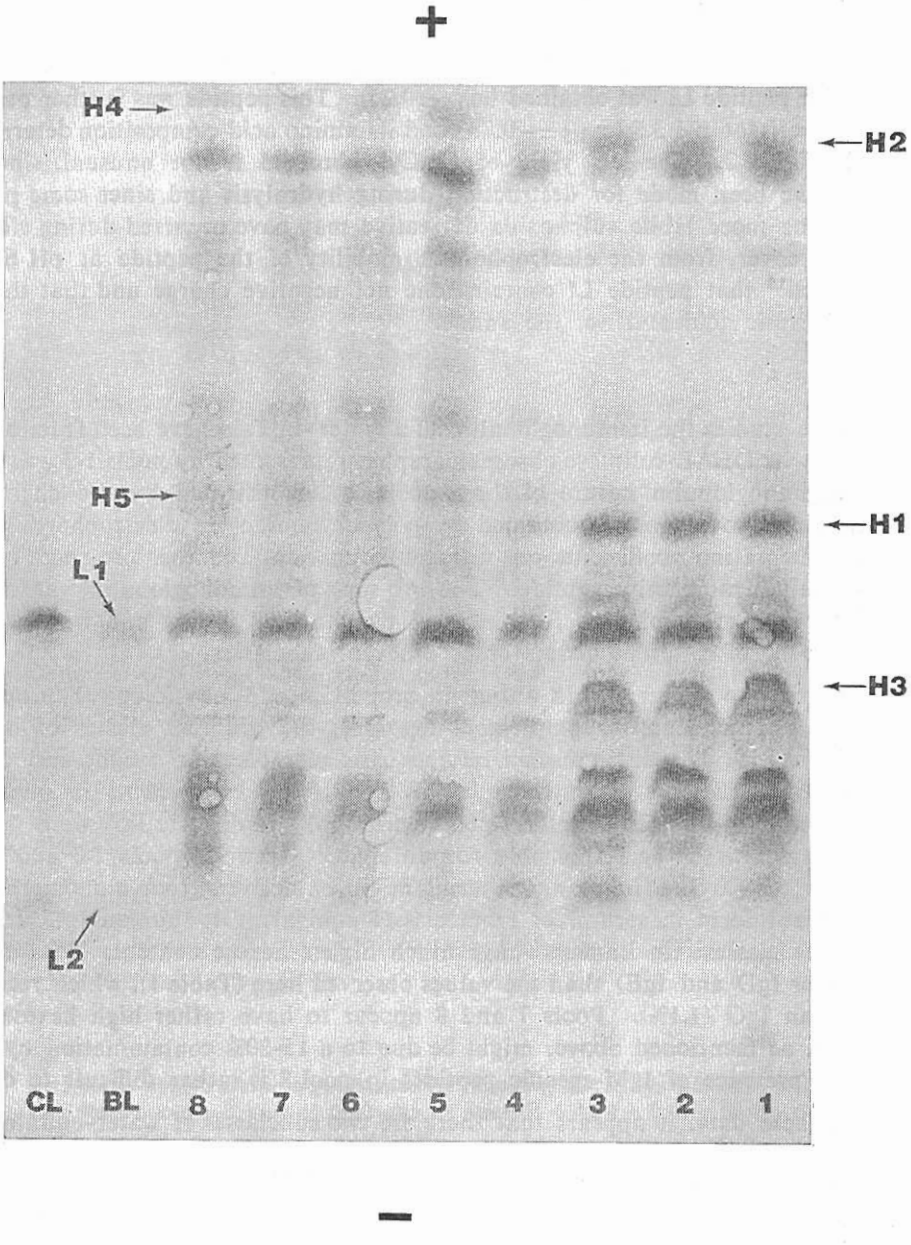

Fig. 4. Chemical typing of DEAE-cellulose pools 1–8 by high voltage paper electrophoresis at pH6.5 (radioautograph). Markers of cow light chains (CL) and of water buffalo light chains (BL) are also included. Peptides L1, L2 are characteristic of water-buffalo light chains. Peptides H1, H2, H3, H4 and H5 distinguish pools 1–3 from pools 5–8.

The light chain peptide containing the half-cystine residue involved in heavy chain binding was further studied using isolated light chains, selectively labelled with[14] C-iodoacetate followed by total labelling with non-radioactive iodoacetate. When the tryptic digest was fractionated by paper electrophoresis at pH 6.5, only one radioactive peptide Ll was obtained ($m_{6.5}=0.25$). This peptide was further purified by electrophoresis at pH 3.5 ($m_{3.5}=-0.78$) and its amino acid composition determined as shown in Table 2. The low yield of CmCys obtained is not unusual, since no corrections have been made for destruction during hydrolysis and since some partial oxidation to the more labile sulphoxide derivative may have occurred during electrophoresis. Moreover, from the electrophoretic mobility of the peptide at pH 6.5, it can be deduced[18] that peptide Ll contains one net negative charge and that the Glx residue is glutamic acid and not the amide.

Discussion

In these studies the immunoglobulins of a water-buffalo heve been fractionated into two sorts by DEAE-cellulose chromatography, represented by pools 1-3 and pools 5-8. The immunoglobulin nature of these pools is demonstrated by the characteristic diffuse slow moving bands obtained by polyacrylamide gel electrophoresis, the 6.7-7.0 S sedimentation coefficients on ultracentrifugation and the presence of the λ-chain C-terminal radioactive peptide. The two sorts of immunoglobulin were shown to differ by analytical isoelectric focusing and by chemical typing and would therefore appear to be of different classes or of different subclasses.

In addition to differences in antigenic properties and physiological properties (such as placental transfer or complement fixation), the different classes of human immunoglobulins may be distinguished on the basis of molecular weight and carbohydrate content[1]. The low amounts of 19.8 S and 10.0 S components in pools 1-8 of the present study would indicate that pentameric IgM and dimeric IgA were present in too small amounts to be responsible for differences between pools 1-3 and pools 5-8. However 6.7-7.0 S sedimenting material might be derived from monomeric IgA (the predominant form in serum) or from classes equivalent to human IgD or IgE. However, these classes[1] (in humans) have much higher hexose contents (3% for IgA, and over 5% for IgD and IgE) than the values observed here (Table I), which resemble those of human IgG (1.1%). Pools 7 and 8 appear to have rather high hexose contents but this, as mentioned above, might be due to a 15-20% contamination by IgM. However, the presence of IgM-specific peptides in pool 8 is rather difficult to detect.

From these data, it appears that there are two subclasses of water-buffalo IgG, which we will call IgG2 (pools 1-3) and IgG1 (pools 6-8) in an analogous manner to the names given to the two subclasses of bovine IgG[19, 20]. The decision to classify different immunoglobulins as belonging to different classes or to different subclasses must remain tentative until more detailed sequence data are available, since the terms class and subclass are most significant in an evolutionary sense. Thus on the basis of a γ1 electrophoretic mobility[21], relatively high carbohydrate content[22] and distinct antigenic determinants[23], horse IgT has been regarded as equivalent to human IgA. Subsequent amino acid studies[24, 25], however, later suggested that horse IgT is probably

analogous to IgG rather than to IgA. Furthermore, unlike the recently evolved subclasses of human IgG, the IgG subclasses in certain species, such as mouse[9, 10] may be of quite ancient evolutionary origin. Such a phenomenon tends to blur the meanings of the terms class and subclass.

Concerning the light chains of water-buffalo immunoglobulin the radioactive peptic-tryptic or tryptic peptides obtained after selective labelling and[14] C-carboxymethylation are characteristic of the λ-type as found in cow immunoglobulin. Since the *C*-terminal radioactive peptide characteristic of κ-chains could not be detected, water-buffalo light chains appear to be entirely of the λ-type. The amino acid composition of the radioactive tryptic peptide (Table II) shows the presence of an extra proline residue when compared to the equivalent peptide[26] from cow light chains. This contrasts strongly with the considerable similarities observed between the tryptic peptide maps of the λ-chains from the two species (Svasti, J., unpublished data).

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บทคัดย่อ

เราได้แยกอิมมูโนโกลบูลินของควายออกเป็นแปดส่วน โดยอาศัยดีอีเออีเซลลุโลสโครมาโตกราฟี จากการเปรียบเทียบแต่ละส่วนด้วยเทคนิคต่าง ๆ ปรากฏว่า IgG ของควายมีอยู่สองชนิด คือ IgG 1 และ IgG 2 นอกจากนี้ เราได้พิสูจน์ว่า หน่วยย่อยเบาของควายเป็นชนิดแลมบ์ดา แต่มีระดับการเรียงตัวของกรดอะมีโนที่ปลายคาร์บอกซิล ไม่เหมือนกับสายแลมบ์ดาของวัว