(^{14}C-acetate) portions of the γ-globulin molecule and assaying for specifically co-precipitable material in the extracellular fluid, it might be possible to determine whether secretion had been prevented by blocking attachment of the carbohydrate.

Our results indicate that at concentrations comparable to those effective in other cell systems[4], hexosamine inhibitors, under these experimental conditions, do not alter the secretion of γ-globulin. Only very high concentrations appear to reduce the amount of specifically co-precipitable carbohydrate without affecting the amount of labelled protein present in the extracellular fluid. This would imply that some γ-globulin was secreted without attached carbohydrate. We cannot exclude the possibility that the ^{14}C-acetate was incorporated into substances other than γ-globulin which co-precipitated under the present conditions.

2.5 ml cultures of 25×10^6 chicken spleen cells	H^3-leucine (cpm)		C^{14}-acetate (cpm)	
	Total	Co-precipitation	Total	Co-precipitation
Control	4769	2928	710	243
DON 30 µg/ml	5229	2996	798	233
Glucosamine 1 mg/ml	4609	2867	727	264
DON 30 µg/ml Glucosamine 1 mg/ml	4926	2501	745	273
Control	5076	3724	740	267
DON 150 µg/ml	4962	3233	514	118
Glucosamine 1 mg/ml	4701	3413	672	211
DON 150 µg/ml Glucosamine 1 mg/ml	4270	2744	503	105

cpm, counts per minute. The values in this representative experiment showing total glycoprotein and co-precipitable material in extracellular fluid are the means of duplicate cultures.

Zusammenfassung. Hemmung von Hexosamin vermag die Sekretion von γ-Globulin durch antikörperproduzierende Zellen unter in vitro Bedingungen nicht zu verhindern. Dies könnte darauf hinweisen, dass unter den gewählten Bedingungen kohlehydratfreie γ-Globulinmoleküle sezerniert werden.

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Extent of Immunoglobulin Light Chains Heterogeneity Revealed by Isoelectric Focusing

The light polypeptide chains, prepared from normal immunoglobulins of a variety of species, form 8–10 electrophoretic bands on starch or acrylamide gels in urea containing buffers[1]. Each electrophoretic band seems to contain a certain number of different kinds of L-chains, at least 5 as calculated by CHOULES and SINGER[2], so there appear to be 40–50 distinct forms of light chains[3].

In order to prove experimentally that each band consists of a number of components having the same total electric charge under the condition of electrophoresis, we resorted to the isoelectric focusing method (IEF)[4]. This method allows in principle the focusing of L-chains into as many bands as there are kinds of L-chains with distinct isoelectric points. IEF turned out to be efficient at the advanced fractionation of immunoglobulins[5] but it was not found significant in Bence-Jones (B-J) proteins and light chains analyses[6].

We submitted to both IEF and electrophoresis on acrylamide gels containing urea, light chains isolated from rabbit immunoglobulin G (IgG) and from a human IgG myeloma and an urinary B-J protein.

Materials and methods. Rabbit and human myeloma IgG were prepared by DEAE-cellulose chromatography[7]. Light chains were isolated by the method of FLEISCHMAN et al.[8]. The B-J protein(λ-type) was isolated from the urine of an IgD myeloma patient by precipitation with ammonium sulphate and subsequent gel filtration on a column of Sephadex G-75 eluted with 0.1 M phosphate buffer pH 7.5 containing 6 M urea. The first peak contains the B-J protein as dimer and the second peak the monomer. The other chromatographic peaks contain the urinary proteins.

Electrophoresis was carried out by a horizontal technique in 6% acrylamide gels made 6 M in urea[9]. The slides length was 8 cm and the gel thickness was 1.5–3 mm. Electrophoresis was performed for 8 h for the rabbit L-chain and 4 h for the L-myeloma chain and urinary B-J protein, at a potential gradient of 2 V/cm.

The protein extraction from some electrophoretic bands was made by an electro-elution technique similar to that described by LEWIS and CLARK[10]. The bands were preliminarily localized by rapid staining of a strip cut from the length of the gel. The protein eluted from the

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same electrophoretic band cut from 6 slides was analyzed, after concentration by dialysis against a solution of 50% polyvinylpyrrolidone in 6 *M* urea.

The isoelectric separation was performed using a 6% acrylamide gel in 2% ampholyte and 6 *M* urea[11]. The cathodic and anodic vessels were filled with 5% (v/v) ethylenediamine and phosphoric acid solutions. Slides length was 8 cm and the gel thickness 1.5 mm. During the migration, the current gradually decreased from 1.5 mA/cm to 0.1 mA/cm, reaching a constant value after 9 h for a gradient pH 3–10 and after 4 h for a gradient pH 5–8. Gels with ampholytes removed by repeated washing with a 5% trichloracetic solution were stained with amido-black. For rapid staining the technique of AWDEH[12] was used.

Results and discussion. The electrophoretic and IEF separations of the rabbit L-chains are illustrated in Figure 1. The 7–8 bands, which are obtained usually by acrylamide gel electrophoresis in the presence of 6 *M* urea, are separated into 20 bands by IEF realized in the gradient 3–10 in presence of 6 *M* urea. This finding suggests that each electrophoretic band can be separated into about 3 L-fractions which differ in their isoelectric points.

Our results, that the L-chain appears more heterogeneous by IEF, is in agreement with the observation of AWDEH et al.[5] that some rabbit and mouse immunoglobulins can be separated into more than 10 bands by IEF. The isoelectric heterogeneity of immunoglobulins seems to be decided by their Fab fragment which presents a similar isoelectric heterogeneity to that of the L-chains, while the Fc fragment appears more homogeneous[13].

By IEF the proteins eluted from the electrophoretic band 5 or 6 (Figure 1a) show 2–3 close bands. The fact that

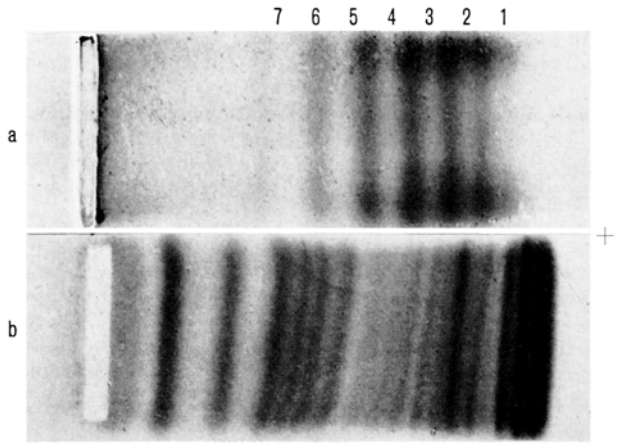

Fig. 1. Electrophoresis and IEF of rabbit L-chains in acrylamide gel. a) Electrophoresis; b) focusing in the gradient pH 3–10.

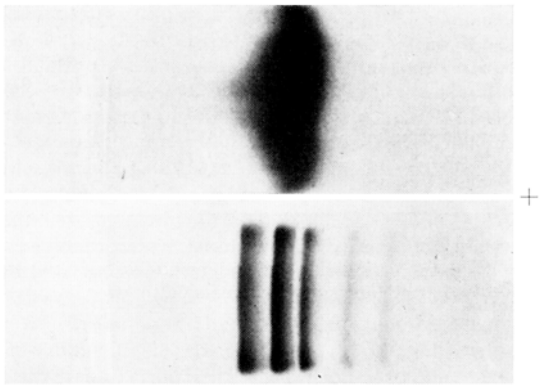

Fig. 3. Electrophoresis and IEF of the urinary B–J protein in acrylamide gel. a) Electrophoresis; b) focusing in the gradient pH 5–8.

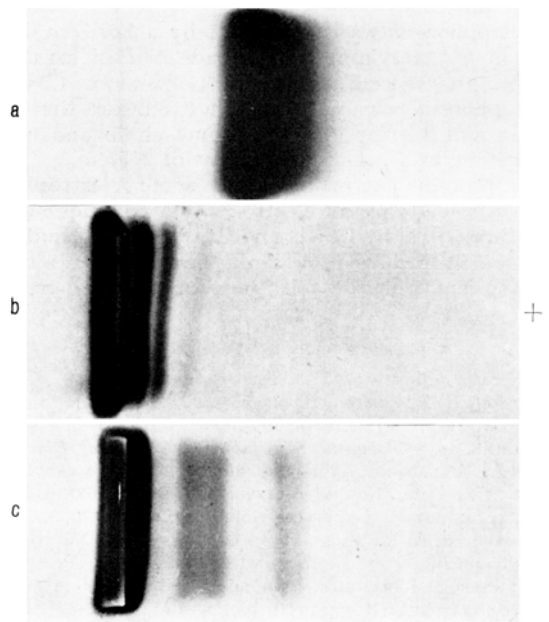

Fig. 2. Electrophoresis and IEF of human myeloma L-chains in acrylamide gel. a) Electrophoresis; b) focusing in the gradient pH 3–10; c) focusing in the gradient pH 5–8.

in a single electrophoretic band there exist 2–3 isoelectric fractions, was confirmed with L-chains isolated from myeloma IgG, which present a limited degree of heterogeneity (Figure 2a). By IEF in the gradient 3–10 this L-chain was separated into 5 bands (Figure 2b). In a narrower gradient (pH 5–8) the myeloma L-chain appears more heterogeneous; except for the very intense band on the start, 4 bands were rather intense by stained and other 4 bands so weakly stained that it was not possible to record them (Figure 2c).

The presence of the very faint bands in the isoelectric pattern can be determined by normal L-chain traces which could exist in the myeloma L-chain. The other 4 more intense bands from Figure 2c, or the 2 intense bands situated before the start from Figure 2b, cannot proceed from the normal L-chain because they represent about 20–30% of the major protein fraction localized at the start, therefore exceeding the normal L-chain percentage which may exist in the analyzed myeloma L-chain preparation. However, to eliminate this suspicion we analyzed a B-J protein which was isolated from urine

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practically free of normal L-chain[14]. In the Figure 3 is shown that the B-J protein, rather homogeneous by electrophoresis, is separated into 5 bands, 3 of them being intensely stained.

Our results show that the isoelectric heterogeneity of the normal and pathological L-chains is considerably grater than is indicated by electrophoresis.

The isoelectric heterogeneity of the L-chains could arise from the alteration of some labile groups on its surface, during the reduction of the disulphide bonds, as AWDEH et al.[15] recently suggested. Because the B-J protein analyzed was not subjected to any reduction, it is not impossible that the charge properties alteration of this protein take place in blood or urine, after its secretion by the cells. AWDEH et al.[16] have demonstrated that, immediately after its synthesis, the mouse myeloma IgG_2 becomes very susceptible to alteration of its charge properties and both inside and outside the cells the IgG_2 presents an increased isoelectric heterogeneity.

The IEF is able to separate those L-chain variants which present differences in their isoelectric points and it seems possible, using this technique, to show differences of heterogeneity between different L-chain preparations[17].

Zusammenfassung. Die Kaninchen-L-Kette wurde bei der Elektrophorese in 7–8 und bei der Isoelektrofokusierung in 20 Fraktionen aufgetrennt. Die mielomatöse L-Kette wurde durch Elektrofokusierung bei pH 3–10 in 5 und bei pH 5–8 in 8–9 Fraktionen aufgetrennt. Fünf Fraktionen weist das bei gewöhnlicher Elektrophorese homogen erscheinende Bence-Jones-Protein bei pH 5–8 auf.

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'Hypothalamic Deafferentation' and Gonadotropin Secretion

FRASCHINI and MOTTA[1] reported in 1967 that in adult, normal, male rats of the Sprague-Dawley strain caged under standard light conditions (14 h of light beginning at 06.30 h, 10 h of dark) there is a diurnal cycle in the levels of gonadotropins stored in the anterior pituitary. A peak of follicle stimulating hormone (FSH) and of luteinizing hormone (LH) concentrations was observed to occur in the afternoon, between 16.00 h and 18.00 h. Several reports of the same laboratory have subsequently confirmed these findings[2,3]. This diurnal cycle apparently does not exist in adult, male rats of the Holtzman strain[4].

It has been proposed that the diurnal variations in the concentrations of pituitary gonadotropins found in male animals might be due to the influence of pineal principles[3]. It is known that the biosynthesis of the pineal principles melatonin and 5-methoxytryptophol is cyclic in nature, and is strictly regulated by the light-dark schedule to which the animals are exposed[5]: the synthesis of pineal methoxyindoles is inhibited during the day and is activated during the night, because light inhibits the activity of the enzyme hydroxy-indole-*o*-methyl-transferase (HIOMT) which is essential for introducing the methoxy group on the indole molecule. Moreover, melatonin seems to be a specific inhibitor of the synthesis of LH[3,6–8], while 5-methoxytryptophol specifically blocks the formation of FSH[3,7–9].

It has recently been demonstrated that rats submitted to a complete 'hypothalamic deafferentation' according to HALÁSZ[10] technique do not show the diurnal cycles of plasma corticosterone levels[10,11] and of pituitary ACTH concentrations[10,12] which are usually found in normal animals. 'Deafferented' rats lose also the ability of releasing gonadotropins cyclically[10].

It was deemed of interest to investigate whether adult, normal male rats of the Sprague-Dawley strain, kept in conditions identical to those described in the papers by the Milan group[1–3], might retain the diurnal cycle in pituitary gonadotropin concentration 8 days after being submitted to a complete 'hypothalamic deafferentation'.

Materials and methods. Mature, Sprague-Dawley male rats were used in this study. They were allowed a standard rat pellet diet and water ad libitum. A complete 'hypothalamic deafferentation' was performed on 4 groups of rats (12 animals per group) using the technique by HALÁSZ[10,11] and a Stoelting stereotaxic instrument. At time of autopsy (8 days following the operation) each group was subdivided into 2 sub-groups: the first one was killed at 10.00 h; the second was sacrificed at 16.00 h.

For the assays of LH and FSH, the anterior pituitary lobes of each sub-group of animals were pooled and homogenized. LH was assayed by the ovarian ascorbic acid depletion method of PARLOW[13] as modified by

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