## TECHNIQUE OF SEPARATION OF THE COMPLEX ANTIGEN-ANTIBODY FROM THE FREE ANTIGEN ON POLYACRYLAMIDE GEL ELECTROPHORESIS. ITS APPLICATION IN RADIOIMMUNOASSAY AND IN THE DETECTION OF ANTIGENICITY DURING AND AFTER TREATMENT WITH HUMAN GROWTH HORMONE.

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Several methods have been tried for the separation of the complex antigen-antibody (B) formed after incubation, from the free labelled antigen (F). As it is well known this technique is a necessary tool in the determination of an antibody titre and its optimal dilution to be used with a certain labelled antigen before setting up a radioimmunoassay, and also in the detection of antibody occurrence, titre and binding capacity, before, during and after hormone therapy.

Polyacrylamide gel electrophoresis (PAGE) was preferred to other techniques (electrophoresis on paper and cellulose acetate, chromatoelectrophoresis, adsorpion on activated charcoal, gel filtration) because, even being more laborius, it gives a complete separation of the two peaks, presents minimal absorption phenomena, it is a quali- and quantitative system and providing the way of calculating independently both B and F, opens the way to a complete separation between the bound and the damaged antigen, not possible with other techniques.

The original method of Davis had to be adapted, especially for what concerned the application of the sample, that had to be polymerized on top of the stacking gel or of the separation gel directly, to avoid back diffusion of the inevitably large volume of incubate that had to be applied. So the original solutions had to be changed in volume and concentration in order to obtain the polymerization of this gel. The best concentration of the sample gel was found to be at 2% acrylamide. In this way the damaged antigen remained trapped in this gel, while the complex entered stacking gel and the separation gel, still keeping well separated from the free antigen that runs close to the tracking dye.

The application of the present technique to a radioimmunoassay of human growth hormone (HGH) provides a method that does not need a second antibody, reaches a sensitivity of 0,1 ng/ml. (5 pg in absolute!) and allows to visualize the type of complex formed. In this respect, running an experiment at different antigen-antibody ratios, it was obtained that a decrease in this ratio (that is an increased antibody concentration) meant a decrease in Rm, confirming the hypothesis of the formation of more highly polymerized complexes, up to the formation of the lattice.

This same technique has been successfully applied to the detection and measurement of naturally formed antibodies in patients treated with different HGH extracts, representing to this purpose a better tool than the traditionally used paper electrophoresis or chromatoelectrophoresis. It permits in fact a higher number of assays to be run simultaneously, which is very useful especially in the determination of titre and binding capacity.

## APPLICATION OF THE POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) TECHNIQUE TO MOLECULAR WEIGHT DETERMINATION; DEGREE OF PURIFICATION AND AGGREGATION OF DIFFERENT PREPARATIONS OF HUMAN GROWTH HORMONE: NIH, KABI IEA.

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After having first controlled the molecular weight of our HGH preparation throung the gel-filtration technique on Sephadex G-100 (P. Andrews), obtaining a value of about 22,000, the PAGE technique was applied following the method of J. L. Hedrick.

It is known that relative mobilites (Rm) of a certain protein band present decreasing values when plotted against increasing concentration of acrylamide in gel. This plot originates a family of parallel lines when the bands are relative to "charge isomers", because migration for molecules having same weight and different charge is independent of the sieving action of the polyacrylamide. On the contrary it produces a family of non-parallel lines extrapolating to a common point in the vicinity of 0% gel concentration, when the bands are relative to "size isomers", which are molecules having the same charge and different molecular weight. The last case provides a method for the determination of molecular weight based on plotting the slopes of these last curves versus the correspondent molecular weights.

Our runs were perfomed at gel concentration of 7,10,12,14% in acrylamide. This gave for HGH-IEA a value around 21.500 and for Bovine Growth Hormone (WHO) a higher value, both in good agreement with those reported by E.V. Cheever.

Very interesting was also the presence of minor bands due to charge and size isomers of the fundamental HGH molecule. In this respect we compared the electrophoretical behaviour of HGH-IEA to similar preparations from NIH and KABI.

NIH—HGH presented a major band whose slope corresponded to a M.W. of 22,000 and only a very faint faster band, presumably due to a deamidated form.

KABI-HGH showed several slower and faster bands together with a central main one, exactly in the same position of that showed by the NIH preparation. The faster migrating bands must be due to charge isomers, since they gave parallel lines. The slower bands are probably due to size isomers (HGH polymers), though a precise, definitive evaluation of the slopes was not made yet.

HGH-IEA showed two major bands, both with the same slope and perfectly parallel to the main NIH and KABI bands, but a little more advanced in the gel. This could mean that our hormone had a higher negative charge, presumably due to deamidation. Two very faint slower bands, also present, are probably due to size isomers.

No one of these hormone preparations showed significant bands due to contamination from other proteins, and all presented good biological activities. Further studies are carried on, especially on the nature and moment of occurrence of this deamidation, employing now also the SDS-gel electrophoresis technique.

## SEQÜENCIAMENTO PARCIAL DA RIBITOL DESIDROGENASE, MUTANTE F.*

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Ribitol desidrogenase-F (RDH-F) foi purificada a partir de Klebsiella aerogenes, após mutagênese induzida na cultura nativa por nitrosoquanidina e crescimento posterior num quemostato alimentado com xilitol como única fonte carbonada. A enzima mutante purificada apresentou banda eletroforética única em gel de poliacrilamida. A razão X/R, entre a atividade sobre xilitrol 500 mM e ribitol 50 mM, mostrou-se função do pH e igual a 0.15 em pH = 7.0. Para a enzima nativa (RDH-A) essa razão é igual a 0.03 no mesmo pH.

Isolamento dos peptídeos para seqüenciamento foi realizado através de hidrólise parcial com termolisina e tripsina, seguida de cromatografia dos peptídeos em coluna de DEAE-celulose e desdobramento dos "pools" resultantes por eletroforese de alta voltagem em papel.

Os peptídeos purificados foram submetidos à análise N-terminal por dansilação e à determinação da composição em aminoácidos e, em seguida, seqüenciados pelo método manual Edmandansil. Peptídeos contendo de 3 a 19 resíduos de aminoácidos foram assim seqüenciados diretamente em escala de nmoles. Um novo método para identificar, sem destruição, o Trp na seqüência foi por nós desenvolvido. Vários peptídeos foram seqüenciados também ou apenas por espectrometria de massa (Drs. H.R.Morris e A.Dell). Peptídeos obtidos a partir da mutante D (RDH-D) foram seqüenciados em paralelo para comparação.

Duas alterações foram detectadas nas mutantes F e D em relação à seqüência conhecida do RDH-A (*Biochem. J.*, *141*, 701 (1974)). A possibilidade de erro nos "overlappings" realizados para o seqüenciamento do RDH-A não pôde porém ser excluída e está sendo investigada.

Um dos peptídeos do RDH-F, contendo 19 resíduos, mostrou ainda uma troca em relação ao RDH-D (mas não ao RDH-A); $Ala_{196}$ substituindo $Pro_{196}$.

Evidência de outras mutações no RDH-F em relação ao RDH-A foi dada pela determinação da composição em aminoácidos das enzimas globais. Essas mutações devem ser responsáveis pelo aumento da atividade enzimática sobre o substrato não natural.

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## PROTROMBINA DE CARNEIRO: PURIFICAÇÃO E CARACTERIZAÇÃO PARCIAL*

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Protrombina de plasma de carneiro foi parcialmente purificada por método por nós anteriormente descrito (*Biochimie:* (1976) *58,505-512*) até o fracionamento por sulfato de amônio e, em seguida, cromatografada em coluna até apresentar banda eletroforética única em gel de poliacrilamida e atividade específica igual a 1600 unidades de coagulação/mg de proteína (614 vezes em relação ao plasma original).

O produto final revelou Ala como único resíduo N-terminal. Carboxipeptidases A ou B não liberaram o resíduo C-terminal. O pH isoelétrico, determinado por focagem isoelétrica, deu 4.9 a 5.0 (banda única).

O zimogênio não cromatografado pôde ser convertido em trombina ativa por citrato de sódio a meia saturação, semeado com trombina, a 22°C. A protrombrina pura, porém, não se mostrou ativável no mesmo meio nem por tripsina.

Outras determinações, realizadas no zimogênio puro, foram os seguintes: açúcares neutros: 5,6% composição em aminoácidos: $Ala_{35}$, $Arg_{44}$, $Asx_{54-55}$ $Cys_{24}$ $Glx_{72}$, $Gly_{53-54}$ $His_8$, $Ile_{19}$, $Leu_{45}$, $Lys_{31}$, $Met_7$, $Phe_{23}$, $Pro_{36}$, $Ser_{34}$, $Thr_{28-29}$, $Trp_{16}$, $Tyr_{19}$, e