AN ACCURATE RADIOIMMUNOASSAY OF HUMAN GROWTH HORMONE WITH SEPARATION ON POLYCRYLAMIDE GEL ELECTROPHORESIS OF FREE ANTIGEN, ANTIGEN ANTIBODY COMPLEX AND DAMAGED LABELLED ANTIGEN

FURTHER STUDY OF DAMAGED LABELLED ANTIGEN TO OBTAIN LONG LASTING LABELLED PRODUCTS

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CENTRO DE APLICAÇÕES BIOMÉDICAS DE RADIOISÓTOPOS E DE RADIAÇÕES
CABRR AAMRR 009

INSTITUTO DE ENERGIA ATÔMICA
SÃO PAULO – BRASIL

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ABSTRACT

The purpose of this work was to obtain a radioimmunoassay that would be sufficiently accurate and precise to provide a suitable means of determining human growth hormone (hGH) in both extracts and physiological fluids for specific research purposes rather than for routine clinical assays where the labelled products could be used as long as possible. The only technique found that could satisfy these requirements was polycrylamide gel electrophoresis (PAGE) though in some respects it is more laborious than other techniques. By introducing some modifications to the original method of Davis it was possible with 11 cm tubes to separate the free the antibody bound and the damaged labelled antigen on the same gel. The method being able to detect separately and independently these three components and to give a better control of the analytically dangerous damaged antigens furnished accurate and reproducible curves. An example of a determination is the one on KABI Crescormon which compares the results obtained with the present technique with those presented by another laboratory. Thanks to the method the labelled antigen could be used for up to one month after which re-purification on Sephadex enabled the same labelled product to be used profitably for two more months. Parallel to this work a study has been performed on the various components originating in this so-called process of damaging and particular importance has been given to a more precise knowledge of the amount of antigen in terms of mass present in an assay.

In setting up this radioimmunoassay technique which had to be applicable to the determination of human hormone (hGH) in extracts and in physiological fluids there were three main requirements:

1) The determination had to provide accurate and absolute values (an absolute which is of course relative to the reference laboratories using the same standard. The difficulty in achieving this has already laboratories using the same standard. The difficulty this has already been mentioned[1,2].

2) The determination of bound to free ratio (B/F) had to be very precise and as free as possible from misclassification errors due to overlap or incomplete separation of the free antigen from the antigen antibody complex and from the so-called 'damaged antigen' the three components normally involved in this assay.

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3) Use of the labelled hormone (^{125}I hGH) had to be prolonged as much as possible, without affecting too much the accuracy precision and sensitivity, so that the technique would be less dependent on regular shipments of ^{125}I and would not involve new labelling and standardization procedures every week or second week

Only polycrylamide gel electrophoresis (PAGE) could in our opinion satsify these requirements though it was in some respects more laborious than other techniques The sample containing antigen, antigen antibody complex and always a certain amount of the damaged antigen was added in gel polymerized on top of the separation gel according to a modification[3] of the original method of Davis[4] Separation of the free hGH (which moves ahead close to the tracking dye) from the complex with the antibody just entering the first part of the separation gel and from the damaged trapped in the polymerized sample gel was performed in 8 cm or better 11 cm gel tubes The gel was cut with a gel slicer segments 0 5 or 0 7 cm long and the radioactivity was measured in a well type gamma counter (Figure 1)

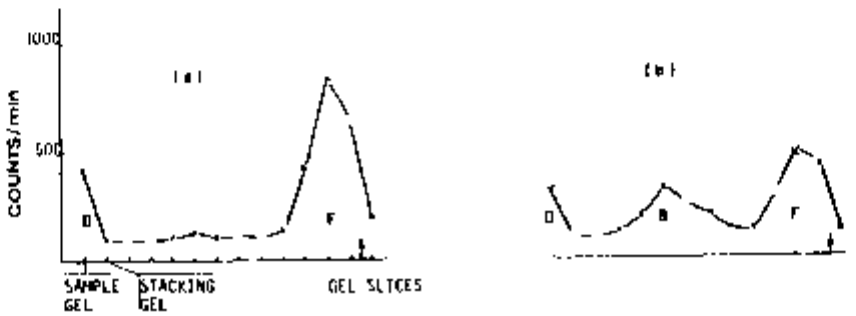

Figure 1 — Polyacrylamide Gel Electrophoresis using 11-cm Long Tubes and 5% Acrylamide Separation Gel (a) Purified Labelled Hormone (Incubation Blank) (b) The same Incubated with Antibody at a Dilution of 1 10^6
D = damaged antigen B = antigen bound to antibody F = free antigen

For the first requirement the technique was tested on a hGH preparation of declared immunoactivity determined in another laboratory using the same standard (WHO hGH 1st IRP 66/217 for immunoassay) but with a different separation technique For clinical use KABI Crescormon was chosen with an activity (4 IU/ampoule 2 IU/mg) that had been determined by the double-antibody method in the KABI Laboratories of Stockholm[1] The technique presented here had two definite advantages over the KABI method the possibility of a separate independent detection of the three peaks involved (antigen, complex and damaged antigen) and no need a second antibody which as already stated could introduce a factor of uncertainty in the determination itself[2]

Before running the radioimmunoassay the same PAGE technique was used to determine the optimal antibody dilution for the assay the detection range and the sensivity of the method One of the curves produced by the present technique is shown in Figure 2, and results of determinations carried out with it on KABI Crescormon are presented in Table I

After this the exact amount in terms of mass (picograms) of labelled antigen present in each incubation was determined under the prefixed assay conditions This parameter which is seldom mentioned in the literature but whose importance cannot be overlooked[5], especially for comparing different labellings, was determined by a method based on the work of Berson et al [6] The values were then compared with those obtained by the method used by Greenwood et al [7] A curve, like the one in Ref [7] was used to calculate the amount in picograms of labelled hGH that was present in each incubation Of course the same purified hGH for the labelling (in our case NIH RGH HS 2002 F) had to be used for the standard curve Instead of checking the overlaping of the two curves, we used the standard curve to calculate directly the amount of ^{125}I hGH present (Figure 3), finding this method more reproducible than the one based on the monitoring of radioactivity at each step in the labelling

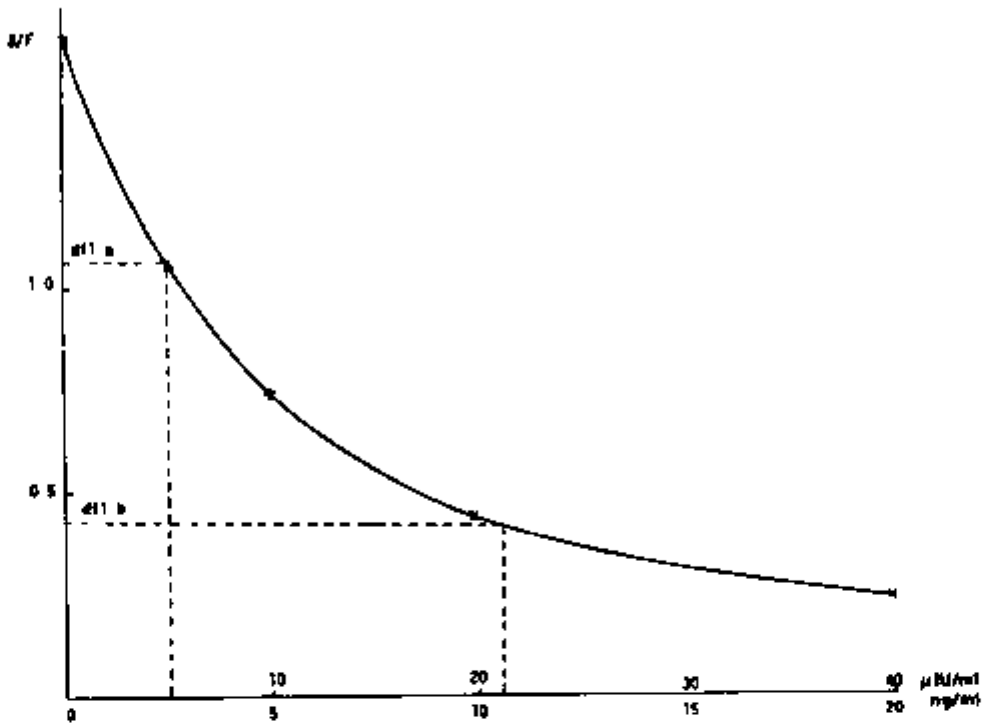

Figure 2 — Example of Radioimmunological Determination using PAGE as a B-F Separation System For the Standard Curve, WHO hGH 1st IRP 66/217 was used for Immunoassay The two Dilutions Correspond to Experiment No 1 Table I

Table I

Radioimmunoassay Determinations on Kabi Crescormon

Experiment No	Stated Content (ng/ml)	Found Content (ng/ml)	IU/mg
1	2 5	2 50	2 00
	10 00	10 60	2 12
2	5 0	6 30	2 12
3	6 0	6 60	2 24
4	2 0	2 40	2 40
	4 0	4 36	2 17
	8 0	8 40	2 10
		average	2 125
		S D ± 0 1267	
		S E ± 0 0478	
		fiducial limits (P = 0 95)	
		2 008 — 2 242	

and purification Though the average value of 84 pg per incubation found by this method is of the same order of magnitude as that furnished by the other approach it is still not in good agreement

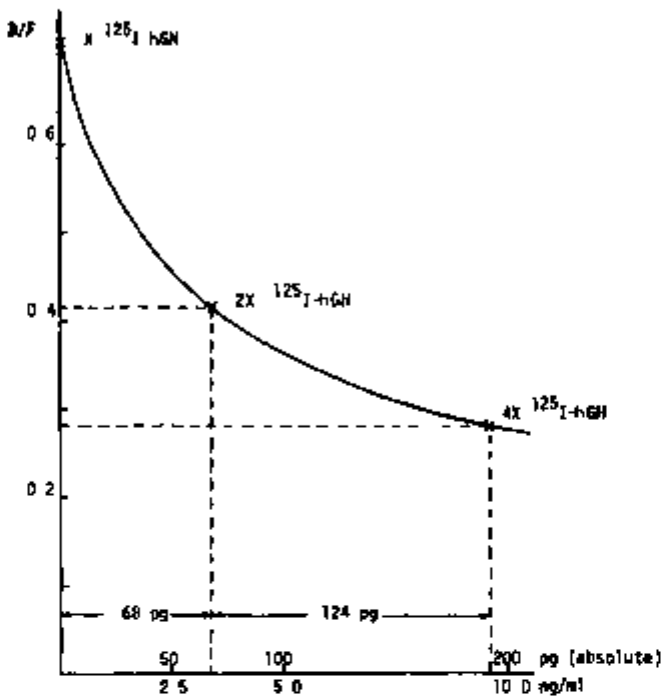

Figure 3 — Determination of the Amount of ^{125}I hGH in Terms of Mass Present in a Radioimmunoassay Sample The Curve was Pre-constructed using Various Amounts of the same hGH that was used for the Labelling (X = 64 pg Average)

These results were obtained with a very newly labelled antigen The possibility of extending the application of the same ^{125}I hGH to subsequent assays for a certain time without the need of a new labelling reaction depended mainly on the behaviour of the damaged antigen and on the possibility of avoiding its interference in the calculation of B/F Here again the PAGE technique proved to be one of the best tools available offering the possibility for separating and at the same time studying the behaviour of a certain unknown component

It is known that when a good labelled antigen has been used for about a month or even less that the damaged antigen which in our PAGE technique is trapped in the sample gel increases to values higher than 20% of the total and creates problems in the traditional assays In our case the first positive approach was to repeat the same purification procedure on Sephadex G 75 as is carried out after the labelling This re-purification produced exactly the same peaks as those obtained after the first passage which we have called PI PII PIII and PIV The identity of only two of these is known for sure PII which is undamaged hGH and PIV which is undoubtedly free iodide (Figure 4) The re-purified antigen was of course at a higher dilution (about 1 10 with respect to the first passage) but this did not affect the assay technique in any way since the labelled antigen was used at a much higher dilution (1 100 or higher) What was important was that the specific activity (in μCi/μg of ^{125}I hGH) had not changed except for the obvious decay of the ^{125}I The capacity of binding to the antibody and consequently the various assay parameters based on a certain antibody dilution did not change In fact, a second purification one month after the labelling at the same antibody dilution gave the same B/F but with a little less ^{125}I hGH activity in counts/min After the second purification, the amount of damaged

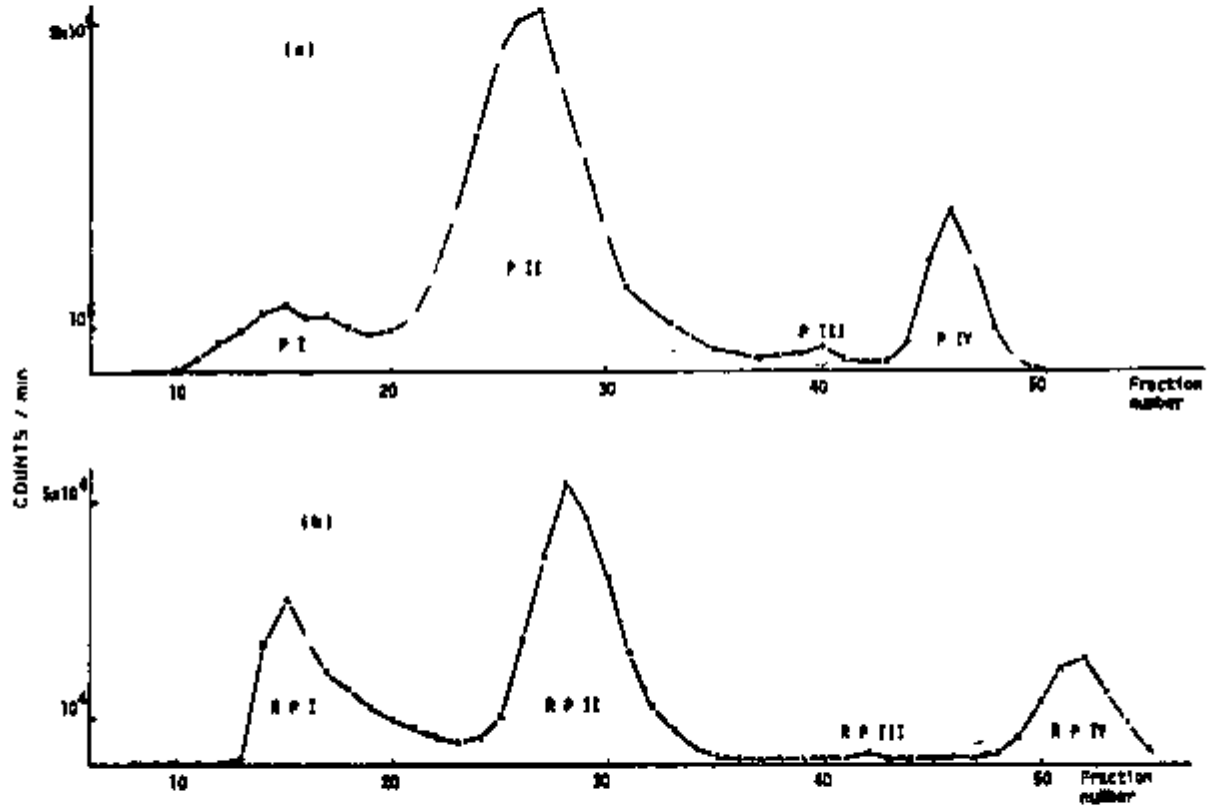

Figure 4 – Elution Pattern from Sephadex G 75,2 cm x 35 cm (a) Purification immediately after Labelling (b) Re-purification on the same Type of Column one Month after Labelling

antigen was insignificant and it was still lower than 10% after two months of profitable use from the time of re purification (Figure 5)

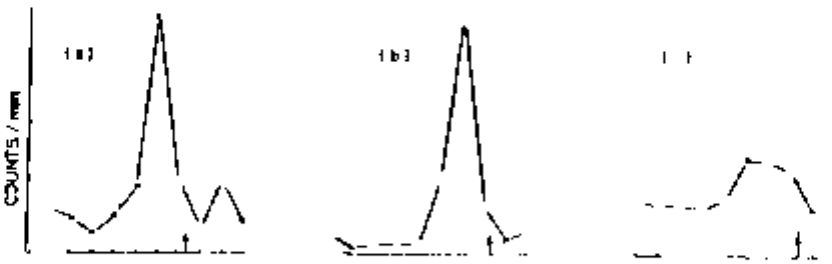

Figure 6 – Electrophoretograms on PAGE of (a) PII one Month after Labelling (b) R PII Immediately after Re purification (c) R PII two Months after Re purification

More recently two other types of controls have been introduced into the assay technique Together with the blank of labelled hGH (incubated simply with buffer and guinea pig serum and without antibody) a second control is run in which a large excess of standard hGH is added to the already pre-determined antibody dilution This gaves information about the minimum B/F we are working with confirms the amount of free and displaceable ^{125}I hGH and consequantly reveals the presence of what has been called unspecific binding This last control is very valuable especially in the determination of hGH in serum and in the evaluation of hGH treatment and we intend to investigate its use more thoroughly

We have recently developed and are still studying a more systematic approach to the problem of damaged antigen The four peaks (PI PII PIII PIV) from the first purification on Sephadex run on PAGE give the results such as shown in Figure 6 As can be seem four electrophoretic peaks can appear but they do not necessarily correspond to the four Sephadex peaks The first one in the sample gel that had always been considered the only one representing damaged hGH we have called D1 The second one in the position of the undamaged hGH also appears, strongly enough in a predominent amount in the electrophoretogram of PI Later it was shown that the incubation of PI with an excess of antibody (1 10^5) presented, on electrophoresis practically no binding of this "fake undamaged" antigen whereas the incubation of PII under the same conditions gave almost complete binding (Figure 7) This led us to call it D2 to distinguish it from the pure electrophoretic peak that is in the same position and that, coming from PII, is certainly good undamaged ^{125}I hGH The third Sephadex peak (PIII), though very small and practically non interfering was constantly found after purification and re purification The application of a large volume of this low activity material gave rise to a third peak on PAGE which we have called D3 and which moved togheter with the tracking dye whereas hGH and D2 were always two segments behind Free ^{125}I appearing as PIV on Sephadex always gave a band on PAGE that was two segments ahead of the tracking dye and is the only component that does not create problems in terms of its identification and exclusion A source on uncertainty could however be the behaviour of different shipments of Na ^{125}I In fact of two lots of Na ^{125}I received in two different months one showed a small peak (about 6% on the total radioactivity) in the D1 position whereas the other like peak IV of Na ^{125}I purified on Sephadex did not show any radioactive component in the sample gel (Figure 8)

A further characteristic of these fractions in that with or without purification, with or without thawing whether stored at 4 or at -20°C the process of damaging consistently occurs with liberation of free Iodide The transformations of these samples with time followed on PAGE and the last

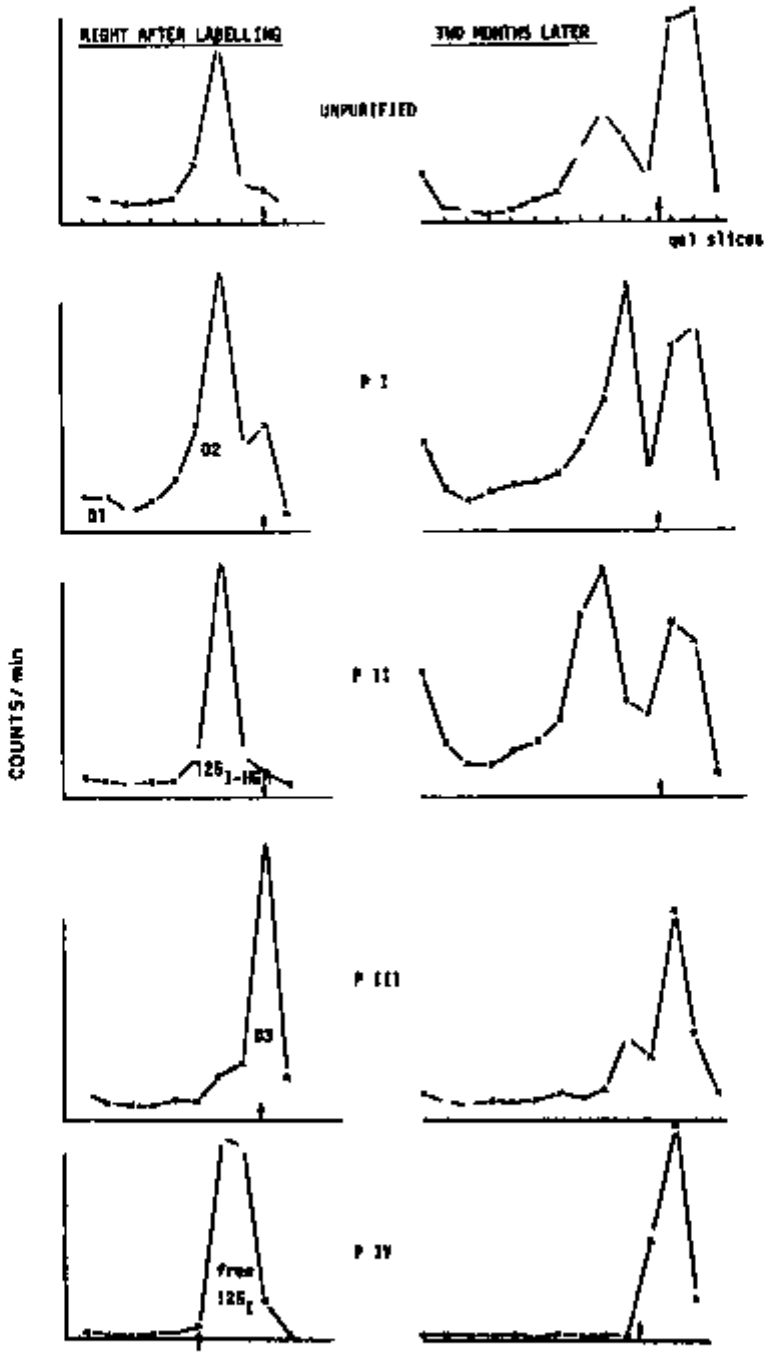

Figure 6 — A Comparison of PAGE Electrophoretograms of the various Peaks from Sephadex Immediately after Labelling and two Months Later

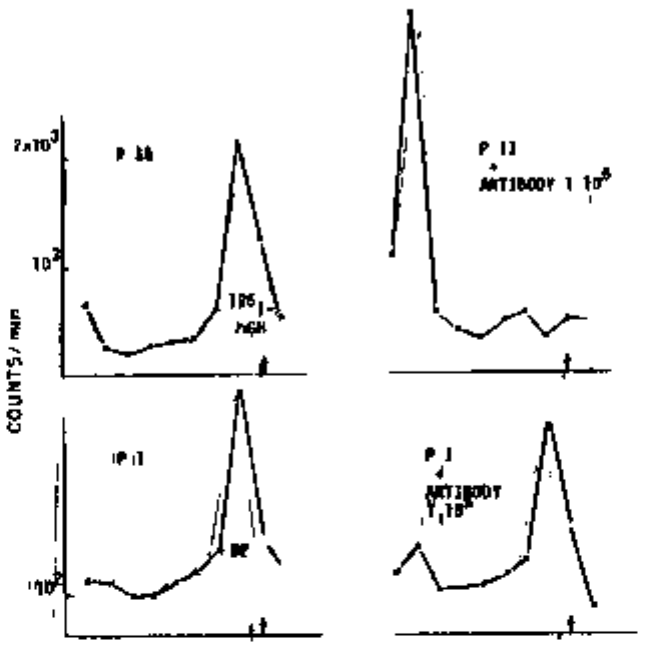

Figure 7 -- Detection of the Immuno-Inactivity of the Apparently Undamaged D2

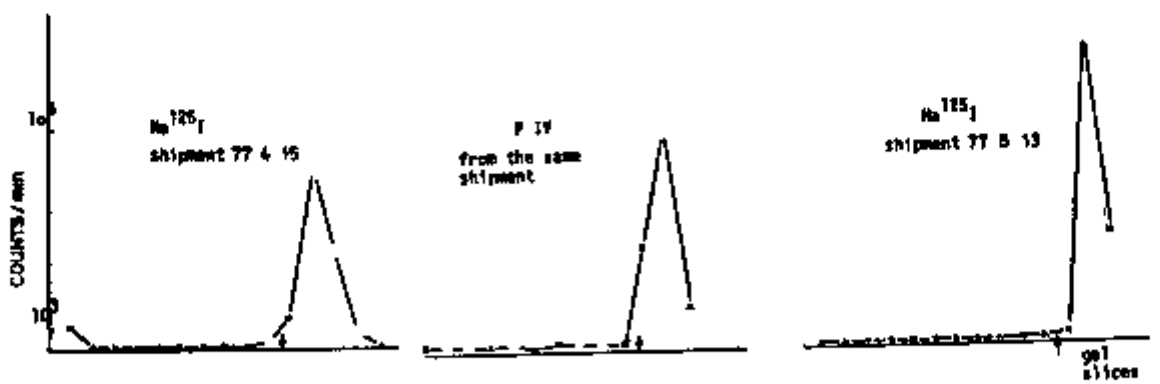

Figure 8 - Detection of the Presence of a Kind of Impurity in Certain Shipments of ^{125}I that Affected D1 Position

electrophoretograms obtained two months after labelling are shown in Figure 6 Only PIII is unaltered but a better determination of its rate of mobility (R_m) is needed A quantitation of this phenomenon for PII gave more than 50% of the radioactivity liberated as free iodide even two months after the labelling

With regard to the other components one could guess that D1 is an aggregate (as has been mentioned by several authors) and not simply ^{125}I hGH bound to gamma globulins because it would enter then the separation gel as does the complex that D2 is uncleaved but immuno inactive antigen (or even something cleaved but carried by albumin) and that D3 are small fragments of labelled of peptides or amino acids but no other test has yet been performed in our laboratory to identify them Only the other control was done which in fact is done regularly in every assay namely the incubation not only of a blank of ^{125}I hGH without antibody but also of the same amount of labelled antigen with an excess of antibody This is done to ensure that the assay is carried out in the absence of significant hidden D2 if such is present to evaluate it so that its effect may be subtracted Further work is being done to try to better determine the moment and reason for the production of this D2 There is some hint that the relatively long Sephadex column used to according to Cerasi et al [2] might have some influence on its formation

Thus requirement[3] of the radioimmunoassay technique has practically been reached through re purification of the labelled antigen or by complete separation on gel of the interfering components or by both procedures In this way the same labelled preparation has been used for up to four months and has still given good reproducible curves

DISCUSSION

P G MALAN Have you considered incorporating free radical inhibitors in the storage medium of your labelled samples? In our laboratory we have found that substances which are capable of removing free radicals can protect labelled protein and peptide hormones during storage for periods of up several months It is assumed that the radioactive decay produces free radicals which may cause coupling via the ortho positions of two tyrosyl residues to yield a bityrosyl residue either within the protein or alternatively between different molecules which are then excluded from gel filtration columns

P BARTOLINI No we did not consider this possibility but we would like to test this type of inhibitor In fact we are still in doubt whether the damaging process is due to chemical enzymatic or radioactive effects A positive action of the free radical inhibitors would of course indicate the presence of such radicals.

K von WERDER Four years ago at the symposium on the same subject held in Istambul we reported some studies similar to yours At that time we described three different peaks of labelled hGH two eluting on a Sephadex G 75 column before ^{125}I hGH and the third being ^{125}I hGH We found as you have found in one of your labelled fractions that all the labelled products – the so called damaged hGH and perhaps the labelled big hGH – were bound by our antibody but that this binding could not be inhibited or could be inhibited only ineffectively by increasing doses of unlabelled hGH This incidentally explains the reduced steepness standard curve if the larger molecular fractions are incorporated in the tracer used for the RIA Did you investigate whether your larger fractions which bound to the hGH antibody could be displaced by the addition of cold hormone?

P BARTOLINI We found only slight binding (perhaps 10% or less) to antibody in the case of the so-called damaged fraction from Sephadex It is only PII (the undamaged ^{125}I hGH) which binds almost completely to the antibody while PI binds very little at the same excess I think it would be interesting to carry out the test you mention on this partial binding and to see if it is displaceable We have performed the test on various occasions but only to check displacement on bound undamaged fractions

P VITINS We can confirm the appearance of free iodide with time in radioiodinated growth hormone Have you followed this phenomenon to determine whether is linear with time? Would you care to speculate whether the radioactivity observed is from a degradation product or from non specifically bound iodine which has been dissociated from the hormone?

P BARTOLINI We did not check the linearity of this phenomenon with time although we observed that iodine release was continuous and increased in five or six months to 80% or more of the total radioactivity

I would be surprise to find non specifically bound iodine though I cannot a priori exclude this possibility In fact PAGE has always separated free iodide after incubation in the presence on guinea pig serum or even cold hGH carrier Moreover the release of ^{125}I is always accompanied by the appearance of other damaged components which should not occur in the case of simple dissociation of unspecifically bound iodine

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INSTITUTO DE ENERGIA ATÔMICA
Caixa Postal 11049 — Pinheiros
CEP 05508
01000 — São Paulo — SP

Telefone 211 6011
Endereço Telegráfico — IEATOMICA
Telex — 011 23592 IENA BR