The Principle and the Method of the Radioimmunoassay※

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＝國文抄錄＝

호르몬 免疫學的 測定法의 原理와 方法

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호르몬의 免疫學的 測定法은 內分泌學的인 立場에서 가장 重要한 硏究方法의 하나이며, 또한 核醫學的 立場에서도 放射性 同位元素를 利用한 試體管內 測定의 하나로 重要한 位置를 차지하고 있다.

이 方法의 一般原理는 放射性 同位元素로 標識된 호르몬이 特定抗體에 對하여 加逆的으로 反應하고, 標識된 抗原—抗體 複合體를 形成하는데 根據를 두고 있다. 單一 system 內에서 標識되지 않은 호르몬이 標識된 호르몬과 免疫學的으로, 特定抗體에 對해 競合的으로 作用하기 때문에, 實際的으로는 一定量의 標識된 호르몬과 一定量의 抗體가 있는 條件하에서 未知試料와 알려진 量의 호르몬을 가진 標準 호르몬을 同時에 處理하여, 標準曲線을 얻고, 여기에서 未知試料의 호르몬 量을 推定하게 된다.

이때 競合的으로 結合한 標識호르몬(bound form)과 結合하지 못한 標識호르몬(free form)을 分離하는 것이 必要하며, 電氣泳動法, 二重抗體法, solid phase 法, 鹽析法 및 酵素에 의한 단백消散法 등이 이 目的을 위해 開發되어 있다. 이 中에서 電氣泳動法은 變性된 호르몬 및 遊離된 放射性沃素를 알 수 있는 特長이 있고, 二重抗體法 등은 多數의 試料를 처리하거나, 時間을 短縮시키는 利點이 있다. 호르몬에 放射性 同位原素를 附着시키는 데에는 ^{131}I 및 ^{125}I 가 主로 쓰이나, 각기 長短點이 있으며, 높은 放射性 活性度를 얻기 위해 chloramine-T 및 Na-metabisulfite 를 사용하는 附着方法이 主로 사용된다. 미량의 호르몬을 실제 測定하기 때문에 標識호르몬의 比放射能度가 높을 必要性이 있으며, 이 경우에는 放射能에 依한 호르몬의 變性이 큰 문제점으로 등장하게 된다.

The measurements of the amounts of various hormones in the body is one of the most important subjects in the field of endocrinology. The result obtained is not only helpful for the basic studies, such as the function studies of each organ or their interrelationship, but also valuable for routine clinical diagnosis. For most of peptide hormones or protein hormones, a chemical measurement is very difficult. Biological methods are mostly utilized for this purpose but are not always satisfactory. The use of labeled hormone in combination with its antiserum led to the highly specific and sensitive measurement of the hormone in human plasma. This method is based essentially on the principle of isotope dilution method and is called radioimmunoassay. From the nuclear medical aspect, this is now one of the major fields of "in vitro" assay with radioisotope. With this method, less than 1 micro unit of insulin per ml of serum can be detected. In this lecture, I would like to talk about the principle and the method of the radioimmunoassay.

The general principle involves reacting reversibly

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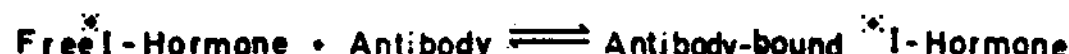
a radioisotopically labeled hormone with a specially prepared specific antibody to form a labeled antigen-antibody complex. The radio-immunoassay exploits the ability of unlabeled hormone in plasma or in standard solutions to compete with the labeled hormone for the antibody(Fig 1). As a consequence the higher the concentration of unlabeled hormone in the mixture, the less antibody there is available for the labeled hormone. The lower the raio of bound to free labeled hormone—the B to F ratio—the higher must be the concentration of the unlabeled hormone in the mixture. Thus the B to F ratio must be determined.



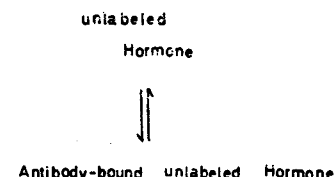


Fig. 1. Principle of radioimmunoassay.

In practice, unknown plasma samples, which are diluted according to need, are incubated at 4°C for several days in a mixture containing labeled hormone and an appropriate dilution of antiserum. At the same time, a set of standard solutions containing increasing known amounts of hormone is incubated with the same concentration of labeled hormone and antiserum under the same conditon. Ideally, these standard hormones should be dissolved in plasma solution known to be devoid of hormone to exclude non-specific effects of plasma on the immune reaction. If buffered solution is to be used without hormone-free plasma, control experiment should be carried out. After the separation of B and F with an appropriate method, B to F ratio should be determined. Then, one can graph B/F ratio (or B%) versus hormone concentration to obtain a standard curve. The B/F ratio(or B%) observed in the unknown samples can be applied to the standard curve for determination of hormone concentration (Fig 2).

Std. Hormone added mμg	B Observed %
0	75
0.78	67
1.56	55
3.12	47
6.25	32
12.5	21
25	14
50	8
100	4

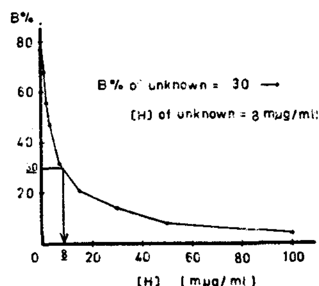


Fig. 2. Assay procedure.

The antibody should be diluted according to its titer to a molar concentration of the same order of magnitude as that of the plasma hormone in sample solution. The optimal dilution of antiserum is one that permits binding of about 50 to 60% of the smallest workable concentration of ^{131}I-labeled hormone. The normal plasma concentrations of insulin, parathyroid hormone, and human growth hormone are about 10^{-10}M and that of adrenocorticotropic hormone (ACTH) is about 0.5×10^{-10}M. In general, the assay is carried out with plasma at a dilution of 1 : 10. Therefore, the hormone concentrations to be measured are of the order of 10^{-12} to 10^{-11}M. It is evident from the principle that the concentration of labeled hormone should be of the same order as that of unlabeled hormone for the precise of the assay.

Several methods of separating the free from the bound hormone have been developed. Among these are the paper electrophoresis method of Yalow and Berson,[1] the single antibody precipitation technique of Grodsky,[2] and the double antibody precipitation technique of Hale & Randle,[3] the ion exchange method of Meade,[4] and enzymatic proteolysis technique of Mitchell[5].

Berson and Yalow first described the separation of bound and free in sulin-^{131}I by chromatoelectrophoresis on filter paper[1]. In this mothod, bound hormone moves on the filter paper with antibody but unbound or free insulin remains at the site of origin. Hydrodynamic flow chromatography with-

out electrophoresis is almost as satisfactory. Adequate separation of the two components may be achieved within one hour by either of these methods. After the incubation of the reactants for 3 to 4 days at 4°C a small portion of the mixture is applied on a paper strip. Whatman 3 MC or Toyo Roshi No. 525 is believed to be best for this purpose. During the storage of labeled hormone and the incubation with the plasma to be analyzed, the labeled hormone may be altered. This chromatographic method has the advantage of permitting recognition of iodide and damaged labeled components from free undenatured hormone or bound hormone. This can not be done by other separation methods and "the damaged" part of the radioactivity mostly contributes to the error involved in radioimmunoassay methods that is, when we try to measure B to total ratio(B%), the damaged products are generally not bound by antibody and therefore are excluded from the reaction system. Consequently, B% decreases and falsely high estimate of hormone concentration can be obtained. For this reason, this method continues to have enduring value in spite of its rather cumbersome procedure. However, when plasma concentration of a hormone is very low, or when labeling of a hormone to have sufficiently high specific activity is difficult, we must limit the concentration of the labeled hormone in the assay system. To provide enough counting rate, it may be desirable to count larger volume. In paperchromatographic method, the volume of sample which can be applied on a paper strip is limited. For these reasons, several workers have used a variety of alternative method for separation of bound and free, as I mentioned previously. Criteria for suitability of a separation technique are related to completeness of separation, flexibility of conditions, volume of incubate that can be employed, and convenience.

Before we discuss the double antibody system, which is most widely applied in our country, I would like to introduce briefly other methods. When we try to separate protein or protein-bound substance from plasma in the laboratory it is one of the most easily conceivable method to precipitate it with a chemical precipitant. In 1960 Grodsky and Forsham applied this method for radioimmunoassay.[2] Since trichloroacetic acid was proved to precipitate both the free and bound hormone simultaneously, they used sodium sulfite to salt out the globulins with bound insulin. Ethanol and dioxane were also experimentally used as precipitants by other investigators. This concept has been further developed by Beck et al.[5] in 1964 and by Mitchell and Byron in 1967[6]. They use insulinase to solubilize the free fraction of insulin in trichloroacetic acid. This technique is called enzymatic proteolysis. Mitchell utilized the catalytic properties of glutathione-activated ficin to separate bound from free insulin.

Another approach to the separation of the free from the bound is to adsorb the free hormone on a solid substance. Meade described the attachment of free insulin but not bound insulin to an anion exchange resin.[4] In this method, they use low-specific-activity insulin and several ml of serum for assay. Herbert et al. used dextran coated charcoal for this purpose and separated by centrifugation.[7] In our country, Dr. Fukuchi of Tohoku Universty has successfully applied this method to Angiotensin.[8] This hormone is known to be very unstable in aqueous solution and, therefore, a considerable amount of damaged hormone is formed during long incubation periods. For this reason, double antibod system does not seem to be applicable and quick separation of B and F is required. In this method, quality of dextran and charcoal, and their concentration should be carefully chosen for each hormone to be assayed.

Berson et al. utilize talcum tablet or precipitated silica for the same purpose.[9] It is reported that

alcum powder has proved to be a very useful ıdsorbent for separation in the radioimmunoassay ɔf ACTH, parathyroid hormone, thyrocalcitonin, nsulin, growth hormone and glucagon. A talc :ablet of a size appropriate to the individual hormone and the incubation mixture is added to the test tube at the completion of the incubation period. The powder is dispersed by a brief shake and the free hormone is adrorbed on it. On centrifugation the talc packs well and the supernatant fluid containing the bound hormone is easily decanted. In Fig. 3, Berson illustrates the separation of free and bound ACTH and the relationship of quantity of adsorbent to adsorption per cent, plasma dilution and so on. 25mg of talc is sufficient for measurement of ACTH in this experimental condition. I am grateful to Dr. Berson for his permission to use this figure. He also investigated another adsorbent, "Quso silica" which are microfine particles of precipitated silica and are manufactured from sand by Philadelphia Quartz Co in U.S.A. In his laboratory, the talc is used for measurement of ACTH, insulin, HCH etc. and Quso is used for parathyroid hormone, ACTH etc.

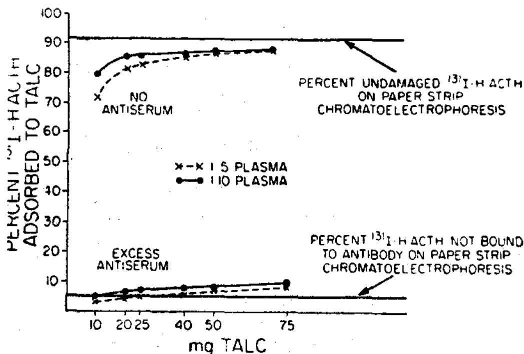


Fig. 3. ^{131}I-H-ACTH adsorption to talc from 2.5ml of diluted plasma.

The third approach to the separation of the free from the bound is to utilize antibody adsorbed on a solid substance (Fig. 4). The antibody is coupled to insoluble polymers and labeled hormone with unknown sample serum is added to it and

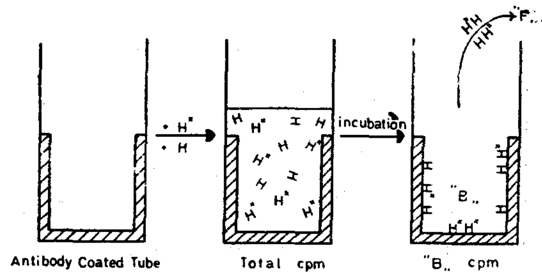


Fig. 4. Solid phase technique.

incubated. In this solid phase technique, the free hormone is decanted after incubation and the bound fraction is counted. Catt et al. used plastic discs or disposable tubes as adsorbents for antibody.[10] The two most commonly available types of disposable plastic tube are manufactured from polypropylene or polystyrene, both of which give satisfactory results in the assay.

Hales and Randle, in 1963, had precipitated the insulin-^{131}I-antibody complex with a second antibody from rabbit serum directed to the guinea-pig gamma globulin.[3] This technique is called "two, or double antibody system" and is now most widley accepted in our country because of its simplicity of technique for the treatment of large number of samples(Fig. 5). The second

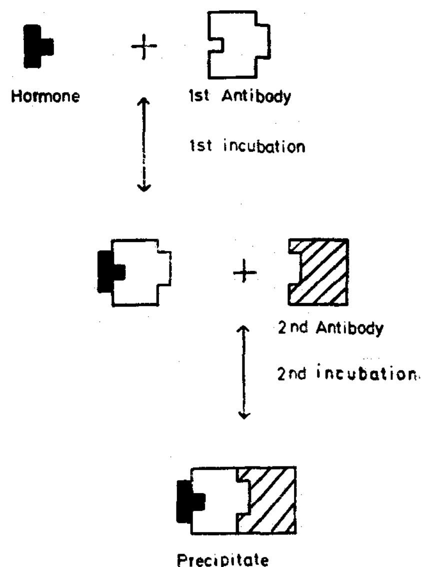


Fig. 5. Double antibody system.

antibody is added to the incubation tube after the first incubation and they form precipitable complex after the second incubation. The second antibody of goat serum directed to guinea-pig or rabbit is commercially available from our laboratory. The standard curve should be obtained each time with the same testing materials so that the error which may be caused from the damaged fraction should be eliminated. The experimental procedure for insulin assay by double antibody system will be discussed in rather detail. To perform the assay, we must have labeled hormone, standard hormone, 1st and 2nd antibodies, normal serum and buffer solution for dilution. Of course, these proteins and hormones should be kept frozen during storage and thawing of those should not be repeated. We recommend using 0.5% of bovine serum albumin in 0.1M boroate buffer of pH 8.6, which prevents the hormone and antibody from adsorbing on the test tube during the incubation. The accuracy of pipetting is the most essential in the assay. There are several pipets commercially available, which permit us quick and accurate transferring of small volume liquid. Among those, we recommend usage of Eppendorf type pipet. When we use a common graduated glass pipet, care should be taken to choose sharper point one, or the outside of the pipet will touch the inside of the test tube during transferring, which causes an error.

It is evident from the immunoassay principle shown previously that if the antibody is present in a large excess over the hormone to be assayed, the binding of the labeled hormone cannot be significantly inhibited by unlabeled hormones. Therefore, in practice, the most optimal dilution should be determined for each batch of antiserum as is shown in Fig. 6. After appropriate dilutions of 1st antibody, standard hormone and labeled hormone, they are dispensed into test tubes, mixed and incubated for 24 hrs at 4°C. Then, the second antibody and guinea-pig serum are added to each tube. The guinea-pig serum is used to increase the

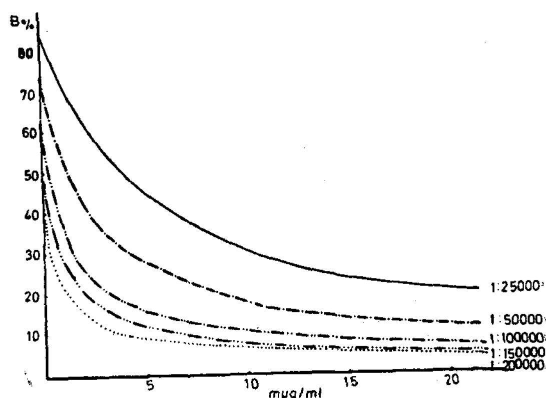


Fig. 6. Comparision of various dilution of antiserum (GP-34).

precipitates of first and second antibody complex. After another 16 hr incubation at 4°C, total counts are measured. On the consideration of pipetting and other random experimental errors, 10,000 counts per each tube, which give a statistical counting error of 1%, will be quite sufficient. Then, the tubes are centrifuged for 30min at 3,000 revolutions per minute, and the supernatants are decanted, and remaining radioactivity of the precipitates are again counted. The standard curve can be obtained by plotting bound % versus standard hormone concentration and hormone concentration in each sample serum can be obtained by application of bound % of the serum to the standard curve.

I would like to discuss labeled hormone. What is the best labeled hormone which gives the most precise and sensitive assay? The first requisite is high specific activity to allow sensitive competitive reaction with hormone to be assayed and to give enough counting accuracy. The second is of course preservation of the original immunochemical reactivity. The third requisite is the purity. The radioactivity of any other chemical form than labeled hormone lowers the accuracy of the assay. In practice, a damaged fraction is easily formed by chemical treatment or radiation effect during labeling, storage, and incubation.

Let me first discuss specific activity. We must

dilute an antiserum sufficiently to reduce the concentration of antibody-combining sites to the same order of magnitude as the concentration of plasma hormone. To perform sensitive assay by competing with the plasma hormone for the diluted antiserum, the concentration of labeled be kept within the same range. As is shown in Fig. 7, the lower the concentration of the labeled insulin, the sharper is the initial slope of the standard curve. On the other hand, we should have a high enough radioactivity for each sample mixture for accuracy of the assay. Those two requirements can be effected by increase in specific activity of labeled hormone.

However, this has a limit in relation to the preservation of the immunoreactivity. Since there is no suitable radioactive isotope of the chemical elements which constitute hormone, we must introduce a radioisotope of a foreign element into a hormone to label it. Radioisotope of iodine are mostly used for this purpose. Therefore, the

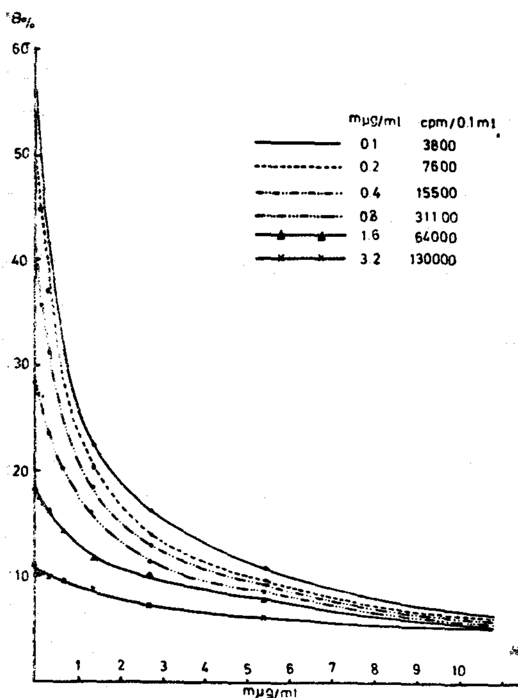


Fig. 7. Comparision of various dilution of insulin-125 (GP-34 1 : 100000 dilution).

labeled hormone is a chemically different compound from the original hormone and if the radioactive label is attached to the hormone near an antigenic site, alteration of immunochemical reactivity can be anticipated. The degree of the alteration is, in most cases, related to the degree of chemical modification of the original hormone. To obtain higher specific activity, we must introduce more iodine atoms into a molecule. We must be very careful about this when we attempt to use a labeled protein as a tracer to study its metabolism. Fortunately, in radioimmunoassay, it is not essential that the labeled hormone retains the identical immunochemical behavior as the unlabeled hormone, but only that the labeled hormone should retained the ability to bind to the specific antibody. It is reported that, iodinated insulins from 2 to 7 iodine atoms per molecule retain the same degree of immunoreactivity. However, during its storage, when a radioactive iodine atom decays, the hormone molecule to which it is attached experiences some shock, which is called "decay catastrophe" by Dr. Berson. If the molecule contains no other radioiodine atoms, it is no longer of interest. However, if the molecule has other radioactive iodine atoms, a still radioactive labeled molecule which may suffer other alterations is left behind. Even at an average of one iodine atom per molecule significant fraction of iodine is present in molecules containing 2 or more iodine atoms. For example, when human serum albumin is iodinated at an average of a half iodine atom per molecule, it is shown by simplified calculation that 40 per cent of total iodine atoms are present in multi-labeled molecules. From experience, we usually label hormone at an average of one iodine atom per molecule.

Here, I would like to explain briefly about the principle of iodination of protein. When an iodine solution is added to protein, the reaction with sulfhydryl always occurs first. However, it

does not result in stable bonding of iodine. Whenever geometrical factors permit, S-S bonding is formed, liberating all of the odine as iodide ion. The reaction with tyrosyl residues is the basis of all iodine tagging of proteins. With incomplete iodination, both mono and diiodo tyrosyl residues are to be expected. In general, proteins are easily iodinated to a maximum of 2 iodine atoms per tyrosine residue with equal readiness. Therefore, the maximum attainable counting ratio per mg of the labeled hormone depends on the number of tyrosine residue per gram of the individual hormone, and on the isotopic abundance ratio of the radioactive iodine used for labeling, and on the counting efficiency of the nuclide with the instrument used.

^{131}I and ^{125}I are mostly used for labeling. The number of radioactive atoms per curie is in inverse proportion to its half life. Therefore, ^{131}I theoretically has a 7.5-fold advantage over ^{125}I. However, this advantage is not practically realizable, since carrier-free ^{131}I is not available. As shown in the Fig. 8, ^{131}I is mostly produced by neutron bombardment of tellurium target. The isotopic abundance ratio of tellurium-130 in natural tellurium is only 35% and the stable iodine is inevitably produced during the irradiation. The isotopic abundance of ^{131}I depends on the conditions of the irradiation and the processing of the target and on the time elapsed after the processing. In Fig. 9 is shown the comparision of number of

Comparison between ^{131}I and ^{125}I

^{125}I	60 α	0.27 γ	no β^-
^{131}I	8 α	0.61 γ etc.	β^-

Carrier Formation in Te(n, γ) I Reaction

^{126}Te (18.7%) $\longrightarrow$ ^{127}Te $\xrightarrow{9\,h}$ ^{127}I (stable)

^{128}Te (31.8%) $\longrightarrow$ ^{129}Te $\xrightarrow{72\,m}$ ^{129}I ($1.7\times10^7\gamma$)

^{130}Te (34.5%) $\longrightarrow$ ^{131}Te $\xrightarrow{25\,m}$ ^{131}I

Fig. 8

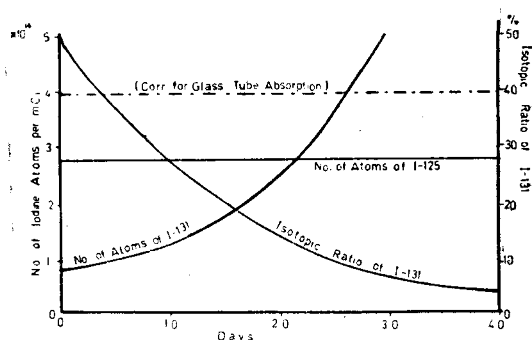


Fig. 9. Number of atoms per mCi.

iodine atoms per mCi between ^{131}I and ^{125}I, assuming the isotopic abundance of ^{131}I at day zero is 50% and ^{125}I is carrier free. After 22 days, both have almost the same number of atoms per mCi. Most of the scintillation well counter available in our country is directed to measurement of ^{131}I. The crystal is covered with rather thick aluminum plate. With this type of instrument, counting efficiency for ^{131}I is almost the same as about 50% or less. If we use an instrument with thinner aluminum cover, the counting efficiency for ^{125}I can be increased to about 70%. Because of the necessary consideration of counting geometry and absorption of weaker radiation, ^{125}I may not be easier to count. When we consider the decay catastrophe, ^{131}I is more favorable at a given iodine to molecule ratio. On the other hand, the longer half life and lower radiation exposure are the advantages for ^{125}I Therefore, it is hard to place one above the other in general. Each investigator should decide in due consideration of the instrument and technician available, and the procurement of radioisotope or labeled hormone.

At the end of this paper, I would like to explain about labeling of hormone briefly. The commercially available labeled hormones for radioimmunoassay from Japan or any other country as far as I know are only human growth hormone and insulin. For radioimmunoassay of other hormones, we must label them by ourselves. It must be helpful for us to know a routine procedure of

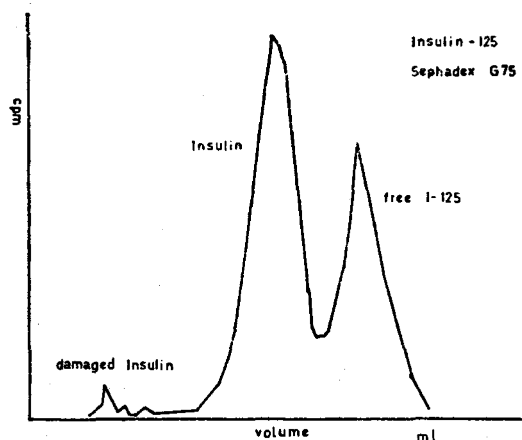


Fig. 10. Elution patt ern.

labeling hormones and problems involved. Even when the labeled hormone is available, this kind of knowledge is helpful to establish a good assay system and to the interpretation of the interpretation of the result. Most of radioactive iodine commercially available is in iodide form. The first step in iodination is to convert the iodide to a higher oxidation state, since iodide cannot form stable linkage with protein. This is done by the addition of an oxidation reagent such as chloramine-T to the mixture of radioactive iodine and hormone. After 10 to 15 seconds mixing, the reaction is stopped by adding a reducing reagent such as sodium metabisulfite. The longer reaction period will give more damaged fraction. The addition of the reducing agent to stop the reaction is not only for protect the hermone from damaging with oxidating reagent, but also for removing loosely bound iodine from molecule. The purification of the labeled hormone is done usually by Sephadex column. The kind of Sephadex should be chosen according to the molecular weight of the hormone. We use G-75 for insulin and G-100 for human growth hormone. Each elution fraction is collected into a test tube containing buffer solution of 0.5% bovine serum albumin to protect labeled hormone from adsorbing on glass wall and self radiation damage. Fig. 10 shows a typical elution curve of purification of ^{125}I insulin with Sephadex G-75. The damaged fraction comes down first and free iodine comes last.

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