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Summaries of the papers presented at the conference on

Problems connected with the preparation and use of labelled proteins in tracer studies

PISA, January, 17-19, 1966

Symposium on : **Fundamental aspects of proteins labelling.**

Chairman : W. L. HUGHES, Tufts University, School of Medicine, Boston.

W. L. HUGHES (Tufts Medical School Boston, Massachusetts) :

The chemical requirements of a satisfactory label for proteins.

Proteins to be used in bio-medical studies are usually labeled by a suitable chemical modification *in vitro*. Results amenable to direct biological interpretation require proteins labeled in conformity with the following precepts : The isotopic label should be *stably bound to the protein* in such a way as to *retain full biological activity* and should cause *minimal changes in physical, chemical and biological properties.* Ideally, the isotope should be uniformly bound to the same position in each protein molecule to give a *homogeneous product.* The mechanism of release of label during degradation of the protein should be known and the subsequent fate of the isotope relative to *reutilization* should be known. In addition, certain technical considerations must be met for practical work : The label must be *easy to introduce* with subsequent ready *removal of unbound activity.* Labeling should proceed at *very low concentrations* of the reactants with the production of *high specific activities* and conditions should exist for storing the product with *no appreciable destruction from self-irradiation.*

This paper will discuss the iodination of specific proteins and enzymes in the above framework with possible digressions to other labeling techniques.

U. ROSA, G. F. PENNISI (Sorin, Saluggia); L. DONATO, R. BIANCHI, G. FEDERIGHI (Clinica Medica, Pisa); C. A. ROSSI, I. COZZANI (Istituto di Chimica Biologica, Pisa); C. AMBROSINO (Istituto di Chimico-fisica, Torino) :

Factors affecting protein iodination.

The effect of various parameters involved in the iodination of proteins have been studied, with special reference to human serum albumin and insulin iodination.

Efforts have been made to separately evaluate the importance of the various steps involved in the preparation of a labeled protein in determining its final biological behaviour. Extraction and purification of the protein, role of SH groups oxidation,

type of labeling procedure employed, effect of the distribution of the labeling iodine within the molecule are considered.

Attempts are made to correlate the above factors' modifications with the biological behaviour of labeled proteins.

T. FREEMAN (National Institute for Medical Research, Mill Hill, London) :

The assessment of homogeneity of iodine labelled plasma proteins.

Valid data can only be obtained from turnover experiments with labelled plasma proteins if the protein for labelling is of adequate purity and if it is known that the labelled protein is handled by the body in the same way as the native protein.

Evidence that the controlled introduction of limited amounts of iodine into the protein molecule does not produce denaturation has been provided by the comparison of the behaviour of iodine labelled proteins in animals with similar proteins labelled biosynthetically with ^{14}C, and also by a simultaneous comparison in a human analbuminaemic subject of the removal rate of unlabelled albumin with that of a trace dose of the same albumin labelled with ^{131}I.

However, proteins can be readily denatured during the procedures necessary for their isolation in a state of purity and because of variable factors in the labelling technique it is necessary to assess the quality of individual labelled preparations. Even for a human labelled protein this may be done in animals. Since grossly denatured proteins are rapidly removed by the reticuloendothelial cells the amount of activity retained in the RE system of the liver shortly after injection is an index of gross denaturation. Also, minor differences in the behaviour of labelled heterologous proteins can be detected by injecting them into tolerant animals.

Evidence of denaturation is also apparent after injecting labelled proteins into humans. A pure undenatured protein will give a constant rate of breakdown throughout the period of study; thus a falling catabolic rate indicates either denaturation or heterogeneity of the labelled protein. In particular, the breakdown rate, calculated from the urinary excretion rate of the label, should not be greater during the first 24 hours than on subsequent days.

Session on : **Fundamental aspects of proteins labelling.**

Chairman : W. L. HUGHES, Tufts University, School of Medicine, Boston.

E. NIEMANN (Farbwerke Hoechst AG, Frankfurt (Main)-Höchst) :

The location of iodine and chromium atoms in labelled proteins.

Chromium and iodine atoms used for the labelling of proteins are bound to definite amino-acids which are specific for these elements. The following materials were iodinated under identical conditions, varying the amount of iodine over a wide range : insulin, chains A and B of insulin and glucagon. After enzymatic hydrolysis, the ratio of the iodotyrosines to the iodohistidines formed, was determined using paper chromatography and electrophoresis.

The best results in hydrolysing the labelled proteins were achieved with an aminopeptidase from the kidney. Hydrolysis of iodinated insulin yielded iodothyrosines; iodohistidines were formed only under higher degrees of iodination. In the case of glucagon and isolated chain B of insulin, iodohistidines appeared, as well as iodotyrosines, even with an iodination degree of only one atom I per protein molecule.

Albumin was employed to study the reaction of trivalent chromium on proteins. Chromium is not bound to either tyrosine or histidine. It was found, however, to react with another amino-acid in the molecule.

W. P. NASS, H. C. HEINRICH (Institute of Physiological Chemistry, University of Hamburg) :

Chromatographic properties and purification of ^{131}I- and ^{51}Cr-labelled human serum albumin.

It was observed that commercially available preparations of ^{131}I- and ^{51}Cr-labelled human serum albumin are significantly different from native human serum albumin if checked with dextrangel filtration and ion-exchange chromatography and also have unrealistic biological half-lives in the human body. Modern protein-chromatography techniques were applied in order to obtain criteria for pure and still native labelled plasma proteins. The chromatographic properties and metabolic behaviour were compared in the case of several labelled human serum albumin preparations.

S. FITTKAU, U. KETTMANN, H. HANSON (Physiologisch-chemischen Institut der Universität, Halle) :

Specific labelling of crystallized leucineaminopeptidase with manganese-54.

It is possible to crystallize the protein « Leucineaminopeptidase » out of ox-lenses and to activate it by Mg and Mn ions. If ^{54}Mn in 2×10^{-3} M concentration is added at pH 8 to the solution for crystallization the enzyme crystals then formed are radio-active. At a probable leucineaminopeptidase molecular weight of 300,000 a content of 4-5 atoms of manganese per molecule of the enzyme will be found. The metal is bound tightly and can neither be separated by Sephadex G 25 nor by continuous dialysis at pH 8 against Tris-buffer.

The labelling of leucineaminopeptidase with ^{54}Mn may also be carried out by simple incubation of the solution of the enzyme with $MnCl_2$. The superfluous non-protein-bound metal is separated quantitatively on a column of Sephadex G 25. Registration of the protein during the elution is effected continuously by measuring the UV absorption with the aid of a perfusion-cuvette and automatic registration of the extinction. The rate of impulse is synchronically registered by means of a combination of instruments for measuring with integration.

Subsequently the binding capacity of leucineaminopeptidase for Zn and Co ions was stated as a function of pH-value and the concentration of the metal ions.

T. GOSTONYI, J. MARTON, A. KOVACS (Nat. A. En. Com. Isot. Inst. Budapest), L. KOCSAR (Nat. Res. Inst. for Radiobiol. and Hyg., Budapest), S. VIRAG (IV. Dept. of Med., Univ. of Budapest) :

Labelling of proteins with tritium.

Various methods of labelling proteins with tritium are found in the literature. In our laboratories, a simple method was developed for the labelling of several proteins and precursors. The reagent used was the boron trifluoride complex of tritiated aliphatic carboxylic acids and labelling experiments were performed by bringing the target material into contact with the reagent and shaking it. This procedure offered mild reaction conditions.

Preparation of the reagent and labelling reactions are also simple and require no special apparatus.

Human albumin, ovoalbumin, gammaglobulin, ribonucleo-proteides, insulin and beta-lipoproteide were the proteins under study. Purity control and identification of these labelled products were performed by immunoelectrophoresis.

Specific activities obtainable, purity and biological activity of the products and comparison of the new method with other labelling procedures are discussed.

Symposium on : **Labelled plasma proteins for metabolic studies.**

Chairman : A. S. McFARLANE, National Institute for Medical Research, London.

R. BIANCHI, G. FEDERIGHI, L. DONATO (Clinica Medica, Pisa), U. ROSA, G. F. PENNISI, G. A. SCASSELLATI (Sorin, Saluggia) :

Iodinated albumin for metabolic studies.

A selected preparation of HSA has been used to study the effect of progressive iodination on the biological behaviour of the labeled protein, as judged from its catabolic rate.

Iodination up to 24 iodine atoms per albumin molecule has been performed under standardized and controlled conditions. Preparations were tested for their catabolic rate level in normal humans. The amount of MIT and DIT residues at various degrees of iodination were determined.

The existence of a limiting value of iodination degree below which the catabolic rate is little affected by the degree of iodination was observed. This limiting value has been shown to vary widely with the conditions under which iodination is performed, and to be related to the internal distribution of iodine inside the molecule.

S. B. ANDERSEN (Department of Clinical Physiology, Glostrup Hospital, Copenhagen) :

Iodinated gammaglobulin for metabolic studies.

The gamma fraction of human serum contains three different proteins : the immunoglobulins, γG-globulin, γA-globulin and γM-globulin. More than 80 per cent of the protein content of the gamma area is γG-globulin. This communication will be concerned with the metabolism of γG-globulin, particularly with the evidence which can be presented for the validity of such studies. The homogeneity of the labelled preparation should be controlled both by physical and by biological methods. A gentle fractionation method should be used and it is essential that the protein is not iodinated too heavily.

The most important biological control which can be used in a clinical material is the urinary excretion of activity the first day of a metabolic study and the U/P-ratio of activity during the first week or so. Results will be presented which are relevant for the discussion of this problem.

Finally, evidence will be presented that γG-globulin is catabolised in close connection with the circulation. The consequence of this for the calculation of the metabolic parameters will be discussed.

E. ZETTERQVIST, B. BLOMBÄCK, L. A. CARLSSON, S. FRANZÉN (Department of Coagulation Research, King Gustav Vth Research Institute, Stockholm):

The turnover of ^{131}I-labelled fibrinogen in man.

The turnover of ^{131}I-labelled fibrinogen was studied in normal subjects, coronary heart disease, lipemia, congenital coagulation defects, liver cirrhosis and in polycythemia vera. The iodination was essentially according to McFarlane. The labelled fibrinogen contained on an average 1.5 atoms I per molecule fibrinogen. The coagulability of the fibrinogen (92-96 %) was not decreased by more than 3 % after iodination. The administered dose was around 30 μC. The radioactivity decay was followed for about 3 weeks. The half-life time in plasma was determined from the exponential part of the decay curve (days 7 to 21). The half-life time was found to be 4.4 days (3.8-4.9) for normal women and 4.7 (4.0-6.1) days for normal men. The values for patients with coronary heart disease and lipemia were within normal limits. Thus, in these states increased consumption of fibrinogen by clotting can not be demonstrated with this technique. In patients with defective coagulation (hemophilia A, von Willebrand's disease, hypoproconvertinemia and hypofibrinogenemia) the half-life time was also normal. This indicates that the rapid « normal » turnover of fibrinogen only to a small extent may occur via *in vivo* fibrin formation. In 3 out of 6 patients with polycythemia vera and in 3 patients with cirrhosis of the liver the half-life time was definitely shorter than in the normal subjects.

M. A. ROTHSCHILD, M. ORATZ, S. S. SCHREIBER (Radioisotope Serv., N.Y. V. A. Hosp., the Dept. of Med., N. Y. U. Sch. of Med., and the Dept. of Biochem., N. Y. U. Coll. of Dentistry, New York) :

Use of labeled proteins for studying the regulatory processes of plasma proteins metabolism.

The metabolism of albumin and γ-globulin have been studied using proteins labeled with ^{131}I. The balance between serum protein degradation and synthesis has been altered in an attempt to determine the reaction of each to various stimuli. Albumin degradation is increased following the administration of exogenous adrenocortical and thyroid hormones, and in the presence of an elevated albumin pool following albumin infusions. Albumin degradation is depressed when the serum albumin level and/or pool size are low. In contrast albumin synthesis is not sensitive to low levels of serum albumin per se but is capable of significant increments upon the administration of adrenocortical and thyroid hormones. Albumin synthesis is depressed in the presence of increased colloids other than albumin.

Gammaglobulin degradation is elevated when the pool size is increased during hyperimmunization but is unaltered during cortisone administration. Gammaglobulin synthesis is not stimulated by cortisone but is capable of marked increments following an appropriate antigenic stimulation.

Serum protein degradation appears to be related to the concentration of the protein, or to the pool size or both. *In vitro* incubation and perfusion studies indicated that while many tissues possess the potential for degradation of the serum proteins the accessibility to a degradative site may play a limiting role.

The synthetic mechanism is not related to pool size or concentration nor are degradation and synthesis interdependent. A colloid osmotic mechanism affecting control by regulating albumin synthesis is postulated.

P. W. DYKES (Department of Experimental Pathology, the Medical School, Birmingham) :

The influence of the plasma concentration on the rate of albumin catabolism.

Catabolism of plasma proteins has been shown to be related to their concentration, but the nature of this relationship is to date unclear. A study was undertaken in human subjects, in whom hypoalbuminemia was the result of cirrhosis of the liver. The rate of catabolism was found to be unexpectedly low, and well below that anticipated from the plasma concentration. Albumin infusions resulted in the return to normal of both the serum concentration and the fractional rate of catabolism. The data appear to support the hypothesis that the rate of catabolism is dependent upon plasma concentration, by a square law relationship.

Study of catabolism of human γ-globulin in rabbits has shown that an S10 fraction, believed to represent a dimer form, has a plasma disappearance rate indistinguishable from that of the normal S7, suggesting that at least for this protein, the dimer does not represent the normal route of catabolism.

Session on : **Labelled plasma proteins for metabolic studies.**

Chairman : J. COURSAGET, Département de Biologie du C. E. A., Saclay.

A. H. GORDON (National Institute for Medical Research, Mill Hill, London), P. JACQUES (Fonds National de la Recherche Scientifique, Bruxelles) :

Distribution of screened ^{131}I and ^{125}I labelled homologous plasma proteins among subcellular particles of rat liver.

^{125}I labelled rat albumin and ^{131}I rat transferrin were screened separately and injected into rats.

After periods from 5 min. to 6 hrs. the livers were perfused with saline and fractionated by a method similar to that of de Duve *et al* (1955).

As has been previously observed with heterologous proteins the specific activities of the light mitochondrial (lysosome rich) fractions (L.) derived from both ^{131}I albumin and from ^{125}I transferrin were greater than those from any of the other fractions.

In contrast however, to results with heterologous proteins no systematic change in the distribution pattern with time after dosage was seen. Another difference was that much more activity appeared in the 1×10^6 g min. supernatent fraction (S.). As the transferrin to albumin ratio in this fraction was much higher than that in the plasma of the liver donor this high value of the S fraction cannot be explained only by residual plasma. The ratio of the specific activities of the L to S fraction was found to be more than twice as high for transferrin as for albumin. The distribution pattern of non protein radioactivity was in general rather similar to that found for the proteins, thus suggesting proteolysis of homologous proteins inside the granules.

H. C. HEINRICH, E. E. GABBE (Institute of Physiological Chemistry, University of Hamburg) :

Biological half-life of ^{131}I- and ^{51}Cr-labelled human serum albumin in the human body.

The metabolic behaviour of purified preparations of ^{131}I- and ^{51}-Cr-labelled human serum albumin was studied in man, using a 4 π large-volume radioactivity detector with liquid organic scintillators. The biological half life of ^{131}I-human serum albumin is identical in the whole body and in the blood plasma space (11.3 and 11.7 days respectively), if the persons are under complete iodide blockade.

Commercial preparations of ^{131}I- and ^{51}Cr-labelled human serum albumin are not homogeneous and show considerable degrees of protein denaturation in chromatographic systems. The biological half-life of such a denaturated commercial ^{51}Cr-human serum albumin preparation was found to be more than 200 days in the whole human body and also affords evidence of denaturation. Using dextrangel-filtration and ion-exchange chromatography as a purification procedure, it was possible to demonstrate that only a very small fraction of this commercial ^{51}Cr-human serum albumin preparation has a biological half-life of about 22 days in the whole body and 14 days in the blood-plasma space.

The whole-body metabolism of purified ^{131}I- and ^{51}Cr-human serum albumin was studied in normal volunteers and patients with gastrointestinal protein loss. The metabolic and diagnostic aspects of the results will be discussed.

C. REUGE, C. BLATRIX, J. P. BREVET, M. STEINBUCH (Centre National de Transfusion Sanguine, Paris) :

Labelling and study of half-life of isolated α_2-macroglobulin.

The labelling of a pure protein, α_2-macroglobulin, was effected by two different techniques using chloroform or chloramine T.

This protein was isolated from human plasma by a technique based on the use of Rivanol and adsorption of the impurities on Bentonite and DEAE cellulose. The purity of this preparation of α_2-macroglobulin was checked by several techniques — electrophoretic and immuno-electrophoretic — and was shown to be homogeneous by ultracentrifugation study.

The half-life of various preparations of this protein was studied in human subjects. The results obtained by various labelling techniques are also reported.

M. A. ROTHSCHILD, M. ORATZ, S. S. SCHREIBER (Radioisotope Service, N. Y. V. A. Hosp., the Dept. of Med., N. Y. U. Sch. of Med., and the Dept. of Biochem., N. Y. U. Coll. of Dentistry, New York) :

Hepatic interstitial albumin — A possible regulator of albumin synthesis.

Hypoalbuminemia per se does not stimulate albumin production and dextran and gamma globulin infusions depress albumin synthesis suggesting a colloid osmotic regulatory mechanism controlling albumin production. Since the plasma colloid osmotic pressure was not reduced, this mechanism was postulated to reside in hepatic extravascular fluid. This report describes the measurement of hepatic extracellular space (^{14}C-sucrose), plasma albumin (^{125}I-albumin), and total exchangeable albumin (^{131}I-albumin) in control rabbits, rabbits treated with dextran, and dextran and cortisone. Following distribution equilibrium of ^{131}I-albumin, ^{125}I-albumin and ^{14}C-sucrose were injected, livers and lung removed and tissue radioactivity compared with that in the plasma. Hepatic interstitial volume averaged 12 % in all groups. During dextran administration total exchangeable albumin fell 20 %, albumin degradation fell from 273 to 217 mg/kg/dav, hepatic interstitial albumin rose from

27 to 52 mg/100 g liver weight. Cortisone reversed these findinds. Exchangeable albumin remained stable and albumin degradation rose from 245 to 320 mg/kg/day. Hepatic interstitial albumin decreased to 11 mg/100 g wet liver weight. The available hepatic interstitial space for albumin was limited to 10 % of the sucrose space. In the lung the pulmonary albumin space approximated 50 % of that for sucrose. Albumin is excluded from most of the hepatic interstitial fluid and this volume appears to provide a sensitive system for monitoring changes in colloid concentration. These results support the concept that albumin synthesis may be regulated by interstitial albumin or colloid within the liver.

F. FEDERIGHI, R. BIANCHI, F. VITEK (Clinica Medica, Pisa):

Kinetics of iodinated human serum albumin following expansion of the albumin pool in man.

The kinetics of human serum albumin was studied in 5 subjects (3 normal and 2 patients suffering from nephrotic syndrome) using ^{131}I labelled HSA and analog computer analysis. The relevant parameters were evaluated by means of transfer functions obtained from the computer under general conditions avoiding assumptions concerning the compartmental composition of the extravascular space. After an initial period of 4 days during which the values of the parameters were determined by means of injection of ^{131}I labelled HSA, an infusion of 40-50 grams of non-labelled HSA was given in a 12 hours interval. The changes of the parameters after infusion were studied by means of a new injection of ^{125}I labelled HSA.

K. HØEDT-RASMUSSEN, E. HASCH, S. JARNUM (Medical Department Bispebjerg Hospital, Copenhagen) :

Extravascular degradation of ^{131}I labelled serum albumin.

A comparison of the catabolic rate of albumin after intravenous and subcutaneous administration.

The commonly accepted site of albumin degradation is the intravascular pool. The methods for calculating the catabolic rate assuming intravascular degradation are based upon the activity in urine/activity in plasma ratio. The same result can be obtained by analysis of the plasma curve. Theoretically, these calculations would give the same results after both intravenous and subcutaneous administration of iodized albumin.

In 10 cases, ^{131}I-albumin was injected subcutaneously and the radioactivity was measured in urine and in blood samples for three weeks. In 6 of the cases, ^{125}I-albumin was simultaneously injected intravenously.

The urine excretion of activity was almost identical after subcutaneous and intravenous administration of albumin, while the plasma activity after subcutaneous injection first reached its maximum value after about 5 days. Calculation of the intravascular degradation of albumin in accordance with various commonly accepted principles leads to the conclusion that 10-20 % of the subcutaneously injected albumin is catabolized extravascularly in normal subjects. In patients suffering from protein-losing diseases, there was found to be a smaller percentage of extravascular albumin catabolism than in normal subjects.

The finding is discussed with particular reference to protein denaturation and lymphatic flow.

G. G. PINTER (Department of Physiology, University of Maryland, School of Medicine, Baltimore, Maryland) :

Quantity and role of extravascular plasma albumin in the kidney.

Confirming the results of previous experiments (Proc. Symp. Preparation and Bio-Medical Application of Labeled Molecules. EURATOM, 1964. Brussels, p. 287) it was demontrated that a fraction of the renal albumin pool is extravascular. The quantity of extravascular albumin, ranging from seven to sixty-five per cent of the total, is largest in the outer cortex and smallest in the papilla.

Dynamic hematocrit values calculated on the basis of the intravascular compartment were smaller than the simultaneous arterial hematocrits. Thus, it appears likely that a relative separation of red blood cells from plasma takes place in the renal blood vessels, the extent of this process, however, is not as great as it was previously assumed.

G. MILHAUD (Laboratoire des Isotopes, Institut Pasteur, Paris) :

^{131}I-serum albumin metabolism in the rat.

Rat serumalbumin was prepared and labelled with ^{131}I. After I. V. injection, the changes of the specific radioactivity, R_s, with time can be expressed by the relation :

$$R_s = A_1 e^{-a1t} + A_2 e^{-a2t}$$

Serumalbumin metabolism can be represented by a two compartment model. In the normal rat as well as in the rat with tied ductus lymphaticus we have measured : the masses of the compartments, the rate of exchange between the compartments, rate of total way out from the pool, the foecal excretion of serumalbumin.

N. RICCIONI, M. F. BECCHINI, F. VITEK (Clinica Medica, Pisa) :

Analogue computer study of the kinetics of extravascular distribution of ^{131}I labelled plasma proteins in normal and tumoral tissues.

An attempt has been made to interpret the time course of radioactivity in extravascular space following injection of human serum albumin (RIHSA) and fibrinogen (RIHF), both labelled with ^{131}I, in normal tissues, inflammatory lesions and tumors.

An analogue computer has been used to simulate the observed behaviour, on the basis of compartment models. Models based on first order reactions adequately describe the curves of extravascular activity observed in any case with RIHSA and in normal and inflammatory tissues with RIHF. On the contrary the course of extravascular activity curves observed with RIHF in tumors cannot be accounted for by such a model. Attempts are made to interpret this special pattern, and the relative implications are discussed.

G. B. GERBER, A. REUTER, F. KENNES, J. REMY-DEFRAIGNE (Euratom and C. E. N., Dept. Radiobiology, Mol) :

Preparation of ^{3}H serumproteins and their use in catabolic studies.

The concentration of several serumprotein fractions decreases after wholebody irradiation, an effect which could be due to a decreased synthesis or an increased intra or extrahepatic catabolism.

Studies on incorporation of phenylalanine and tryptophan demonstrated that synthesis of the fractions involved increases rather than decreases. The aromatic aminoacids were chosen since they occur in a relatively large concentration in the fractions most affected after irradiation.

Hepatic catabolism was then investigated. To this end, serumproteins labeled with ^{3}H-phenylalanine were biosynthetically prepared by a rat liver perfusion. Labeled serumproteins were then added to livers perfused at different time periods after irradiation and the decrease of total activity and specific activity were followed over periods of perfusion up to 12 hours. Formation of volatile radioactivity due to degradation of liberated phenylalanine was also measured. These data do not suggest a difference in hepatic catabolism after irradiation. However, the differences in activity, even during a 12 hour period of perfusion, were rather small. Next, a mixture of serumproteins labeled with ^{131}I and of serumproteins biosynthetically prepared with ^{3}H phenylalanine was injected into rats and the ^{131}I and ^{3}H radioactivity in serumproteins, as well as in feces and in urine, was followed in normal and X-irradiated rats. These data suggest that extrahepatic loss of proteins is responsible for the changes in concentration of serumproteins after irradiation.

Symposium on : **Labelled proteic hormones for metabolic studies.**

Chairman : E. F. PFEIFFER, Endokrinologische Abteilung, Medizinische Universitätsklinik, Frankfurt/Main.

R. S. YALOW, A. S. BERSON (Veterans Administration Hospital, Bronx, New York):

Considerations in the labeling of protein hormones.

This presentation will deal principally with the preparation and purification of ^{131}I-labeled hormones at specific activities of 500-1200 mC/mg. Consideration will be given to the specific activity requirements for the ^{131}I-labeled hormones used as tracers in the radioimmunoassay of hormones in plasma at very low concentration as well as to the theoretical and practical limitations in achieving the optimum specific activity for these labeled tracers. The potential usefulness and limitations of other radioactive labels for hormones in the radioimmunoassay technique will be evaluated. The significance of iodinating at more than 1 atom per molecule protein in affecting the suitability of the tracer and the methods for evaluating the extent of iodination of some labeled protein hormones will be examined. Procedures will be described for minimizing damage to the labeled hormone during its preparation and purification and during incubation with plasma and for maximizing the sensitivity of the radioimmunoassay technique.

U. ROSA, A. MASSAGLIA, G. F. PENNISI (Sorin, Saluggia); C. A. ROSSI, I. COZZANI (Istituto di Chimica Biologica, Pisa) :

Correlation of chemical changes due to iodination with insulin biological activity.

The effect of progressive iodination on the biological activity of labelled insulin has been investigated. Iodination up to 8 iodine atoms per insulin molecule has been performed under standardized and controlled conditions. Preparations were tested for their biological activity using the epidimal fat method in rats.

The distribution of iodine among the four tyrosine residues has been measured and tentatively correlated with the drop of biological activity occurring above a certain level of iodination, on one side, and the reduction in SS bridges reactivity towards sulphite on the other.

H. A. OOMS, E. R. ARQUILLA (Laboratoire de Médecine Expérimentale Université de Bruxelles and Department of Pathology, University of California, Los Angeles) :

Influence of iodination on the immunological properties of insulin.

It is generally accepted that varying degrees of iodination have little effect on the binding of radioactive insulin to the anti-insulin antibodies, and this, up to the high level of 5 at. of I per mol. of insulin.

In the radioimmunoassay, cristallin insulin and radioactive iodinated insulin are in competition with the anti-insulin antibodies. We have studied this competition in two different experimental designs :

1° deducting the amount of insulin, not bound to the antibody, from the repartition of the radioactivity (radioimmunoassay) and measuring it directly by the immune hemolysis assay.

2° measuring the insulin, not bound to the antibody, from a first immuno-assay (using ^{125}I insulin as a tracer) in a second radioimmunoassay using ^{131}I insulin as a tracer.

The results indicate that, when in competition with unlabeled insulin, iodinated insulin demonstrates a significant impairment to combine with insulin antibodies. This impairment increases with the degree of iodination.

Moreover, the analysis of the results suggests that iodinated insulin does not react with all the antigenic sites available in an antiserum.

These results will be discussed in the interpretation of the radioimmunoassay.

L. G. HEDING (Novo Research Institute, Copenhagen) :

Sources of error in radioimmunoassay.

Errors may stem from faults and irregularities in the analytical performance of the assay or they may be of a more specific nature related to the use of immunochemical reactions and labeled proteins. Some sources of error of the first kind are : adsorption of proteins to glassware, contamination of reagents with the compound to be assayed for, and lack of control with reaction times, temperature, pH, and salt concentration.

Sources of error in the second category include : (1) *Species difference.* The standard antigen can be from another species than the antigen assayed, and then it should be carefully checked whether the reaction of the antigens from the two species towards the antibody is identical. (2) *Structural difference.* The structure and thereby the specific immunological activity of the antigen may be changed in the extraction process used for the preparation. (3) *Incomplete precipitation.* One major problem is the separation of free and antibody-bound antigen, and several different methods have been developed for this purpose. In the double antibody method, which includes the use of an anti-guinea pig γ-globulin rabbit serum, errors may occur due to cross reactions between human γ-globulin and the second antibody. (4) *Presence of antibody in the sample.* Falsely high or low results, depending on the method of separation chosen, may occur if the samples (e.g. serum) already contain antibodies capable of reacting with the labeled antigen. (5) *Inactivation of immunological activity.* Degradation of antigen or antibody, e.g. by enzymes, may give rise to falsely high values.

All the antigen in a sample may not be detectable in the assay, but may be present in an immunologically inactive state. Thus still another kind of error is possible, which may be important in the biological research.

E. SAMOLS (Department of Medicine, The Royal Free Hospital, London) :

Report on insulin radioimmunoassay.

Endogenous and exogenous insulin levels in blood may be measured with great sensitivity and precision by various radioimmunoassay techniques, permitting

studies of the relationships between plasma insulin, blood glucose, and other metabolic parameters in man, in health and disease.

Immunologically reactive insulin has been measured in the fasting state, and after provocative tests (including oral and intravenous glucose loads, and the administration of tolbutamide, l-leucine and glucagon) in different hypoglycaemic conditions, diabetes mellitus, and in non-diabetic obese subjects. Such insulin assays have proved of great value in the diagnosis of islet cell tumours and in the differential diagnosis of hypoglycaemia, and may be helpful in the early diagnosis of diabetes mellitus. Radioimmunoassay of insulin has also contributed to the understanding of the physiological mechanism involved in these provocative tests. The insulin response to provocative tests in diabetes is frequently much greater than the glucose response would suggest. The diabetic insulin response, which is also observed in non-diabetic obese subjects, indicates that there is peripheral tissue resistance to the action of insulin in many diabetics, although it is possible that an abnormal insulin may be produced in some diabetics.

By means of immunological insulin assays it has been demonstrated that glucagon directly stimulates insulin secretion in normal subjects and in some diabetics. The effect of glucagon on insulin secretion introduces new concepts of the role of glucagon in glucose homeostasis.

Session on : **Labelled proteic hormones for metabolic studies.**

Chairman : C. A. Rossi, Instituto di Chimica Biologica, Università di Pisa, Pisa.

P. G. Crosignani (Clinica Ostetrica e Ginecologica, Università di Milano); F. Polvani (Centro di Endocrinologia Ginecologica, C. N. R., Milano) :

Radioimmunoassay of HCG.

The availability of pure preparations of protein hormones and of more versatile and sensitive immunochemical techniques has permitted the production of specific antibodies and their use in identifying and evaluating these hormones.

Radio-immunological methods were recently employed in the immunoassay, those methods seem to be much more sensitive and precise in connection with the classical techniques.

Antisera against HCG have been obtained. They have been found capable of neutralizing the biological effect of hormone, of agglutining HCG-coated red cells and of reacting with ^{131}I-labelled HCG. The complex formed during the latter reaction is soluble; furthermore antibodies carefully precipitated (coagulated) with organic solvents are capable of binding ^{125}I-HCG, and bound activity can be separated by centrifugation.

When ^{125}I-HCG and unlabelled hormones are mixed the specific activity of the former is decreased. An aliquot of the mixture may be removed using a given amount of coagulated anti-HCG serum, and the radioactivity of the precipitate will be directly related to the specific activity of the mixture.

A preparation of HCG is iodinated using electrolysis according to the method described in this conference. The results obtained in this procedure will be discussed.

Labelled HCG and rabbit coagulated antibodies to HCG are used in a radio-immunological assay of human chorionic gonadotrophin.

K. JØRGENSEN (Novo Research Institute, Copenhagen) :

^{125}I-insulin as a tracer of insulin in different chemical processes.

Ten-times-crystallized pork insulin was iodinated in acid solution with carrier-free $^{125}I^- + {}^{127}IO_3^-$ (the latest in excess) to give a preparation containing about 5 atoms of $^{125}I + {}^{127}I$ per 100 insulin monomers corresponding to about 10 mC/mg of insulin. Precipitation with $ZnAc_2$, filtration and washing were used for purification.

With fresh preparations or with preparations stored for less than two months at —25 °C (1 μg insulin and 1 mg albumin/ml), it was found that the radioactivity (95-100 %) completely follows added pork insulin through at least four crystallizations. The radioactivity could also be completely bound to antibodies against insulin.

However, radioactivity did not follow carrier insulin in certain electrophoresis experiments (while it did follow in other experiments of electrophoresis). Furthermore, in a certain reversed-phase partition chromatography about half of the radioactivity followed the single peak of carrier insulin while the other half appeared as « trailing ».

The results demonstrate the need for comparing — if possible — the behaviour of radioiodinated insulin with that of insulin in the specific process for which the preparation is intended as a tracer of insulin.

L. HEDING (Novo Research Institute, Copenhagen) :

A simplified insulin radioimmunoassay method.

Insulin-antibody-complex is separated from free dissolved insulin by precipitation with 80 % ethanol. With this technique, the sources of error found with the double-antibody reaction are avoided and the procedure is rapid and easy. With the simplified method, it was found that any of the ions Cl^-, NO_3^-, $HCOO^-$, CH_3COO^-, and I^- increases the insulin-antibody reaction rate and the amount of antibody-bound insulin. The following ions were found inactive : Na^+, K^+, NH_4^+, SO_4^{--}, HPO_4^{--}, BO_3^{---}, citrate, and oxalate.

The insulin content of serum and plasma from normal persons has been determined and the influence on the insulin content of different anticoagulants used for the preparation of the plasma investigated.

R. H. UNGER, M. S. MCCALL (University of Texas Southwestern Medical School and Veterans Administration Hospital, Dallas, Texas) :

Demonstration in man of a relationship between ^{131}I-insulin binding and action in peripheral tissues.

In vitro studies by Stadie *et al*, indicated that insulin binding by rat diaphragm is prerequisite for its action. Experiments were, therefore, designed to determine 1) if ^{131}I-insulin binding is demonstrable *in vivo* in man, and 2) if so, if it is correlated with insulin effect.

Twenty normal adults were given 0.5 U of ^{131}I-insulin by femoral artery; external radioactivity was recorded simultaneously over each calf. First circulation intracellular ^{131}I-insulin binding to tissue would be reflected by higher radioactivity in the injected extremity, ^{131}I-albumin (intravascular distribution) or Na-^{131}I (extracellular distribution). The latter substances gave one-hour intercalf radioactivity ratios (ICRR) (injected calf/opposite calf) of 1.1 and 1.3 respectively, whereas ICRR of ^{131}I-insulin was 3.2, with at least 4 hours required for equalization of intercalf radioactivity. Arterial pre-injection of unlabeled insulin reduced ICRR to 1.6; in diabetics with circulating insulin antibodies, ICRR fell to 1.3. A ratio of summated arteriovenous differences across each leg (injected leg/opposite leg) was 2.3, indicating

that greater insulin binding on the injected side was accompanied by greater insulin action on that side.

Conclusions. — 1) Intercalf radioactivity patterns of ^{131}I-insulin suggest first circulation intracellular binding to tissues of the human leg; 2) they correlate qualitatively with indices of glucose uptake; 3) the binding and action of labeled and unlabeled insulin appear to be qualitatively similar.

W. MALAISSE, J. R. M. FRANCKSON (Laboratoire de Médecine Expérimentale, Université de Bruxelles) :

Binding of radioiodinated insulins with the rat diaphragm in vitro.

In vitro studies of the rat diaphragm show that the fixation of different radioiodinated insulins can be divided into two stages : a rapid and reversible binding occurring in the early minutes of incubation and a slow process easily isolated from the 10th minute onwards. The results of numerous experiments are compatible with the hypothesis that the first phenomenon is an adsorption process, poorly specific.

Incubation or pretreatment of insulin with guinea pig anti-insulin antibodies inhibits the tissue fixation of the labeled compounds, revealing an identical immunological behaviour of cristalline insulin and of the radioactive fraction which binds to the muscle.

On the contrary, cristalline insulin, whatever the concentration or the incubation conditions, does not reduce the fixation of labeled material on the diaphragm. This lack of competition between the hormone and the radioisotopes is discussed.

Y. ARNOULD, C. DELCROIX, E. RASIO, H. A. OOMS, J. M. R. FRANCKSON (Laboratoire de Médecine Expérimentale et Laboratoire de Médecine Nucléaire, Université de Bruxelles) :

Analysis of plasma disappearance curves of cristalline and radioiodinated insulins.

Different amounts of cristalline insulin (0.2 to 100 U/kg) or of radioiodinated insulins (0.3 to 1 mC) have been rapidly injected by the venous route to normal anesthetized dogs, maintained in normoglycaemia by glucose compensation. Blood samples were collected from the 2nd minute to the 6th hour following injection. Plasma insulin was estimated by radio-immunoassay and by bioassays; plasma insulinic radioactivity, by paper chromatography and immunochromatography. The blood disappearance curves have been submitted to mathematical analysis (digital computer).

The disappearance curves of cristalline insulin (radioimmunoassay) can be expressed by a sum of exponentials, variable according to the injected amounts. The shape of the radio-insulin curves are different, they tend to an assymptote differing from zero and can only be expressed by a sum of exponentials and of one constant.

Simultaneous injections of a same tracer of radioinsulin wih graded loads of cristalline insulin, only produces a small change in the disappearance rate of the labelled compound. Several hypothesis can be forwarded to explain the poor competition between both types of insulin.

E. E. GABBE, H. C. HEINRICH (Institute of Physiological Chemistry, University of Hamburg) :

Whole-body turnover of ^{131}I-labelled insulin in man.

Crystalline bovine and human insulin were ^{131}I-labelled by a modification of the iodine-monochloride technique. The labelled products which contained one atom iodine per molecule of insulin were purified and identified by dextrangel filtration (Sephadex-G 50) and ion-exchange chromatography (DEAE-Sephadex A 50). The

homogeneous ^{131}I-insulin preparation was injected intravenously in normal diabetic patients, under complete iodide blockade (99.84 % ^{131}I$^-$ decorporation effect) during the entire period of investigation.

The ^{131}I-label of the injected ^{131}I-insulin was used as a metabolic tracer and measured in the whole human body with a large-volume radioactivity detector with organic liquid scintillator. The biological half-life and turnover of the ^{131}I-insulin in the human body was calculated from the measured whole-body ^{131}I-insulin retention.

R. BIANCHI, L. DONATO, G. FEDERIGHI, F. VITEK (Clinica Medica, Pisa) :

In vivo measurement of the rate of degradation of ^{131}I-insulin in humans.

Using an original method which permits to measure in man the rate at which labeling iodine is liberated from rapidly degraded iodinated molecules, the rate of deiodination of ^{131}I labeled insulin has been measured in humans.

The method demands the simultaneous injection of ^{131}I labeled insulin and Na ^{125}I, and the continuous recording of thyroid uptake or urinary excretion of the two radioisotopes for two hours.

The results obtained with preparations at different degree of iodination and the possibility of use of the technique for physiological investigation are discussed.

Round table on : **Applications of tracer theory to protein turnover studies.**

Participants :

M. BERMAN (Office of Mathematical Res., N. I. H., Bethesda, Md); L. DONATO (Clinica Medica, Pisa); C. M. E. MATTHEWS (M. R. C. Cyclotron Unit, Hammersmith Hospital, London); B. NOSSLIN (Malmö Allmanna Sjukhuset, Malmö); G. SEGRE (Istituto di Farmacologia, Camerino); F. VITEK (Institute of Biophysics, Prague).

Subjects :

1. Validity and limitations of multiexponential analysis, in relation to the physical model assumed, and to the significance of the rate constants obtained.
2. Possibility of measuring the parameters of the system under unsteady state conditions.
3. Mathematical interpretation of experiments with rapidly degraded proteins, like proteic hormones.

Round table on : **Prospects in radioimmunoassay of human growth hormone and parathormone.**

Participants :

J. P. FELBER (Clinique médicale universitaire, Lausanne); P. FRANCHIMONT (Institut de Médecine, Liège); F. C. GREENWOOD (Imperial

Cancer Research Fund, London); R. MICHEL (Collège de France, Paris); G. MILHAUD (Institut Pasteur, Paris); J. T. POTTS (National Health Institute, Bethesda); H. J. QUABBE (Medizinischen Universitätskliniken Berlin); V. STEWART (Universitätsklinik, Frankfurt/Main); H. VAN CAUWENBERGE (Institut de Médecine, Liège).

Subjects :

1. Availability of highly purified human growth hormone and parathormone.
2. Preparation of ^{131}I-human growth hormone and ^{131}I-parathormone.
3. Preparation of antisera.
4. Procedures of immunoassay.