[Chem. Pharm. Bull. 25(9)2195—2202(1977)]

UDC 615.357.37.011.5.033 : 546.11.02.3.08

Tritium Labeling of Insulin and Its Application to the Double Isotope Derivative Dilution Analysis[1]

Ushiho Matsumoto,[2a] Kenji Shimada, Mitsuo Ikeda,[2b] Ikuto Hayakawa,[2c] and Yūzō Nagase[2a]

Tokyo College of Pharmacy,[2a] Niigata College of Pharmacy[2b] and Showa Yakuhin Kako Co., Ltd.[2c]

(Received November 2, 1976)

A method of tritiation and application to chemical determination of insulin are presented. Tritium labeling of insulin is performed by a catalytic exchange method with tritiated phosphoric acid-boron trifluoride complex, which is prepared by the use of tritiated water (100 mCi/ml). Bovine crystalline insulin was tritiated with this reagent for 12 hr at room temperature, and the product was confirmed to be identical with unlabeled intact insulin by several chromatographic techniques. Specific radioactivity of ^{3}H-insulin was 6.2—6.5 mCi/mmol.

The most effective coupling condition for ^{3}H-insulin with phenyl isothiocyanate[^{35}S] was at 40° and pH 9.0 in N,N-dimethylallylamine–TFA buffer for 4 hr, which gave a single radioactive peak of ^{35}S-bis(phenylthiocarbamyl)-^{3}H-insulin on radio-paper scannogram. On the double isotope derivative dilution analysis, the radioactive portion was eluted from the paper and the radioactivities of ^{3}H and ^{35}S were measured by a liquid scintillation spectrometer. The loss of insulin through the analytical procedures can be corrected by the use of ^{3}H-insulin added as an internal indicator. The recovery of authentic insulin was 101.5±1.4 to 104.1±3.4%. When ^{3}H-insulin of the present specific radioactivity is used, the determination limit of the method for insulin is 100 μg (0.017 μmol). This isotope derivative method will be available for corrections of the overestimated values obtained by the routine radioimmunoassay as total immunoreactive insulin levels.

Keywords——bovine insulin; acid-catalyzed tritiation of insulin; phenyl isothiocyanate sulfur-35; sulfur-35 phenylthiocarbamylation of tritium insulin; double isotope derivative dilution analysis; tritium and sulfur-35 radioactivity; microdetermination

The quantitative determination of insulin in blood has been for many years a very helpful procedure in the clinical diagnosis for diabetes. Radioimmunoassay and bioassay technique have been widely applied to measure the quantity of insulin. Among them, radioimmunoassay method is recently utilized as the microassay for an extremely small amount of insulin existing in blood. However, the incorporation of radioactive iodine, such as ^{125}I or ^{131}I, into insulin molecule brings in many inherent drawbacks. Of greatest concern was the difficulty of retaining biological activity in iodine-labeled insulin.[3,4] It is known that great a care must be taken to avoid iodination of more than an average of one tyrosine per molecule.[5] It has even been suggested that the biological activity of such preparations may be primarily due to the presence of unlabeled insulin.[4]

In contrast, labeling of protein preparations with tritium has a certain advantage. Tritium-labeled insulin has been prepared in the past either by exposure to tritium gas (Wilzbach) method[6] or by a microwave discharge activation of tritium gas,[7] or by tritium-iodide exchange

1) A part of this work was presented at the 96th Annul Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1976.
2) Location: *a) 1432–1, Horinouchi, Hachioji, Tokyo, 192–03, Japan; b) 5827, Kamishinei, Niigata, Niigata, 950–21, Japan; c) 860, Shimonoge, Takatsu-ku, Kawasaki, Kanagawa, 213, Japan.*
3) J.S. Glover, D.N. Salter, and B.P. Shepherd, *Biochem. J.*, **103**, 120 (1967).
4) E.R. Arquilla, H. Ooms, and K. Mercola, *J. Clin. Invest.*, **47**, 474 (1968).
5) J.L. Izzo, W.F. Bale, M.J. Izzo, and A. Roncone, *J. Biol. Chem.*, **239**, 3743 (1964).
6) C.H. Holt, I. Voelker, and L.V. Holt, *Biochem. Biophys. Acta*, **38**, 88 (1960).
7) W.C. Hembree, R.E. Ehrenkaufer, S. Lieberman, and A.P. Wolf, *J. Biol. Chem.*, **248**, 5532 (1973).

reduction of tyrosine-iodinated insulin.[8] In the Wilzbach method, the major portion of tritium in the tagged products is frequently incorporated into by-products formed by β-radiolysis damage and, furthermore, isolation from purely tagged compound is considerably difficult. Recently, semisynthetic tritiated insulin was prepared,[9] although this method is very attractive, it is rather complicated and requires special techniques. Chemical modification of insulin with tritium-containing substituents is not useful for the present purpose, because the modification changes the primary structure of insulin molecule.

In this paper, we report the tritiation of bovine crystalline insulin and its chemical determination by a double isotope derivative dilution method. For the preparation of tritiated insulin, a catalytic exchange method is applied by use of tritiated phosphoric acid-boron trifluoride complex, which was first reported by Yavorsky and Golin[10] to be a useful labeling method for aromatic compounds of low molecular weight. Fundamental conditions for the isotope derivative method were examined and determination of insulin was carried out by the use of phenyl isothiocyanate[^{35}S] as the derivation reagent for insulin and the tritiated insulin described above.

1. Tritiation of Bovine Crystalline Insulin

Though numerous forms of acid catalysis are known for promoting tritium exchange labeling, evaluation of the powerful acid complex of tritiated phosphoric acid-boron trifluoride ($H_3PO_4{}^3H\cdot BF_3{}^+H_2PO_4{}^-$, $^3HH_2PO_4\cdot BF_3$) for this purpose has revealed significant advantages.[10] 1) Labeling is effected by simply contacting with the tritiating reagent at ambient temperature; 2) preferential tritium labeling takes place in aromatic hydrogen, considerably less for hydrogen on tertiary carbon atoms and essentially none for non-tertiary alkane hydrogen; 3) radiochemically pure tracers are often produced, obviating drastic post-labeling purification that is always required after the Wilzbach method; 4) it is simple, requiring no special equipment. This tritiating reagent has been widely applied to the labeling of different types of organics, but, there is conventionally no previous application of it to biopolymers. Gosztonyi *et al.*[11] tritiated β-lipoprotein by the use of a tritiated acetic acid-boron trifluoride ($CH_3COOH^3H\cdot BF_3{}^+CH_3COO^-$) reagent in radiochemically pure form, without any change in its natural constitution. The labile tritium is washed out with distilled water by repeated lyophilization. These methods of the acid catalyzed tritiation are rather rapid, and may take place under mild conditions permitting the successful labeling even of sensitive biopolymers.

Bovine crystalline insulin was tritiated by using $^3HH_2PO_4\cdot BF_3$, which was freshly prepared by passing boron trifluoride gas into tritiated phosphoric acid ($^3HH_2PO_4$). In the preparation of $^3HH_2PO_4\cdot BF_3$ for the tritium labeling reagent, Yavorsky and Golin[10] prepared $^3HH_2PO_4$ by adding tritiated water to excess of phosphorus pentoxide. In the present experiment, the preparation of $^3HH_2PO_4$ was modified so that the tritiated water (100 mCi/ml) taken was in considerable excess of that theoretically required. The tritiation procedure, therefore, was carried out under the condition in which tritiated water and $^3HH_2PO_4\cdot BF_3$ coexist. The catalytic exchange period for tritiation of insulin with the reagent required 12 hr at room temperature under stirring, since the tritiation with $^3HH_2PO_4$ proceeds much more slowly than with the corresponding BF_3 complex.[10]

Removal of labile tritium in the labeled insulin was examined by several procedures. The most satisfactory result was observed by dialysis against 10^{-3} N hydrochloric acid. Fig. 1 shows the mode of removal of labile tritium in the ^{3}H-insulin molecule. After dialysis against 10^{-3} N hydrochloric acid for 1 week after catalytic tritiation, paper chromatography, cellulose

8) J.F. Dinoman, W.W. Meyers, Y. Aaishi, and A.P. Wysocki, *Fed. Proc.*, **22**, 386 (1963).
9) P.A. Halban and R.E. Offord, *Biochem. J.*, **151**, 219 (1975).
10) P.M. Yavorsky and E. Golin, *J. Am. Chem. Soc.*, **84**, 1071 (1962); P.M. Yavorsky and E. Golin, NYO-10177, 1st Quarterly Technical Status Report, Consolidation Coal Company, Contact No. AT(30-1)-2976, July 1, 1962.

acetate electrophoresis, and disc electrophoresis in 15% polyacrylamide gel are utilized to demonstrate chemical and radiochemical purity of the ^{3}H-insulin. The *Rf* values of ^{3}H-insulin by paper chromatography with three kinds of developing solvents are shown in Table I.

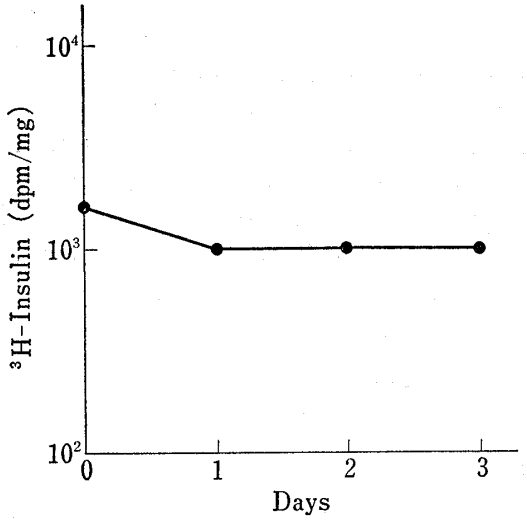

Fig. 1. Removal of Labile Tritium in ^{3}H-Insulin

●—●: dialyzed against 0.001 N HCl (pH 3.0).

Table I. Paper Chromatography of ^{3}H-Insulin

	Solvent system	^{3}H-insulin *Rf* value[a)]
A	BuOH–pyridine–AcOH–H_2O (15 : 10 : 3 : 12, v/v)	0.47
B	*sec*-BuOH–1% AcOH (1 : 1, v/v)	0.25
C	BuOH–AcOH–H_2O (3 : 1 : 4, v/v)	0.53

a) On Whatman No. 1 paper. The *Rf* values are in agreement with those of unlabeled authentic bovine insulin.

When chromatographed with each of the solvent systems A, B, and C, the ^{3}H-insulin shows a single peak on radio-paper scannograms and is identified by comparing its single spot which corresponds to the intact insulin by coloration with *o*-tolidine after chlorination of the amide groups on paper. As seen from Figs. 2 and 3, electrophoresis of the ^{3}H-insulin on cellulose acetate membrane and polyacrylamide gel disc also prove its homogeneity and identity with the intact insulin. The specific radioactivity of the tritiated insulin, which has well confirmed the homogeneity, was 6.2—6.5 mCi/mmol and is similar to those obtained by means

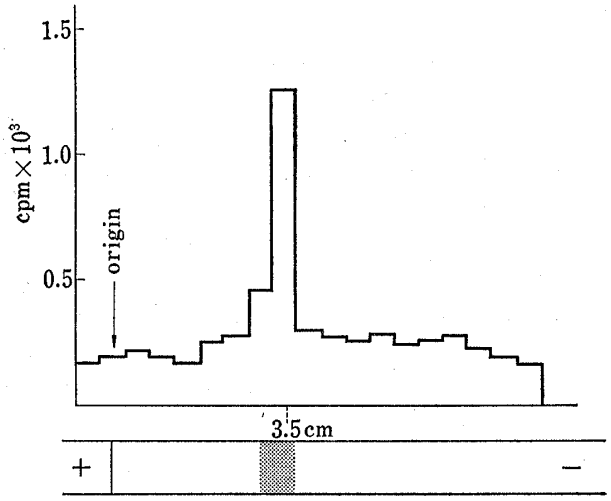

Fig. 2. Electrophoretic Pattern of ^{3}H-Insulin and Its Radioactivity Profile on Cellulose Acetate Membrane

▒: Ponceau S stained zone.
Membrane size: 2.5×18 cm, 40 min at constant voltage, 9 V/cm, in 3 M HCOOH containing 4 M urea.

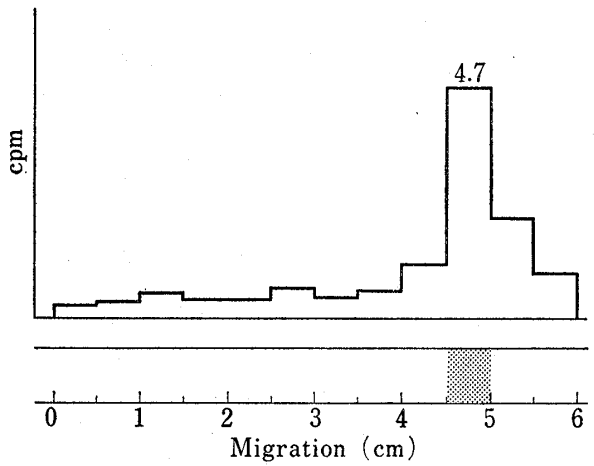

Fig. 3. Disc Electrophoretic Pattern of ^{3}H-Insulin and Its Radioactivity Profile on 15% Polyacrylamide Gel (pH 9.4)

▒: Coomassie Brilliant Blue stained zone.
Gel tube size: 0.5×7 cm, 1.5 hr at constant current, 3 mA/tube.

11) T. Gosztonyi, J. Márton, and A. Kovács, *Nature* (London), **208**, 381 (1965).

of the catalytic exchange method of tritiation in several repeated labeling, independently. Furthermore, the tritiated product of zinc-free insulin, which was prepared from bovine crystalline insulin by the method of Carpenter,[12] was found to have similar chemical and radiochemical behavior and specific radioactivity to that of the crystalline zinc insulin.

2. Double Isotope Derivative Dilution Analysis of Insulin

2.1 Coupling Conditions for Phenyl Isothiocyanate——The double isotope derivative dilution analysis of insulin requires the introduction of a substituent containing β-emitting isotopes other than tritium into insulin molecule. In the present work, phenyl isothiocyanate [^{35}S] (^{35}S–PTC) is taken as the reagent for derivative labeling of insulin by using the first stage of Edman's reaction.[13] The coupling reaction of ^{35}S–PTC with N-terminal amino acid residues in insulin molecule is shown in Chart 1. Africa and Carpenter[14] reported the phenyl-

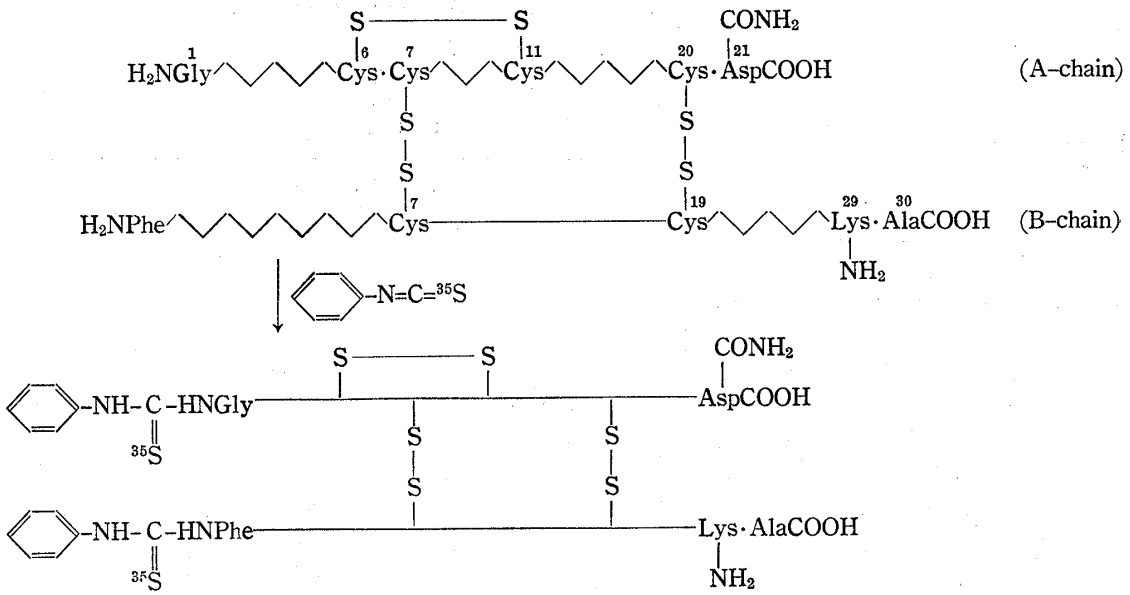

Chart 1

thiocarbamylation of insulin by the use of ^{14}C–PTC at 40°, the reaction medium was maintained at a given constant pH with a pH-Stat. From their experiments, they pointed out that the PTC coupling reaction depends highly on pH value of the medium. Because of this reason, the optimum coupling conditions for insulin with ^{35}S–PTC were previously examined in 5% N,N-dimethylallylamine(DMAA)–trifluoroacetic acid (TFA) buffer solution for various pH values. In 1 ml each of alkaline DMAA–TFA buffer with five kinds of different pH values (pH 8.0, 9.0, 9.5, 10.0, and 11.7), 2 μl of ^{35}S–PTC (20.5 μCi/mmol) was allowed to react with ^{3}H-insulin (2×10^{5} dpm/100 μg) for 1- and 4-hr periods. After the coupling, aliquots of the reaction solutions were extracted repeatedly with benzene, the aliquots of the aqueous layer were concentrated *in vacuo*, and the coupling products were submitted to radio-paper chromatography. The radio-paper scannograms are shown in Fig. 4 (a) and (b). Similar scannograms are observed for each corresponding values, but by a 4-hr coupling in pH 9.0 solution, single and intense radioactive peak was recognized compared with 1-hr coupling at the same pH. These findings show that the optimum coupling condition for insulin with PTC is a 4-hr coupling period in pH 9.0 DMAA buffer solution. Through the above experimental procedures, benzene extraction of unreacted ^{35}S–PTC and DMAA from the coupling

12) F.H. Carpenter, *Arch. Biochem. Biophys.*, **78**, 539 (1958).
13) P. Edman, *Arch. Biochem.*, **22**, 475 (1949); *idem*, *Acta Chem. Scan.*, **4**, 283 (1950).
14) B. Africa and F.H. Carpenter, *Biochemistry*, **9**, 1962 (1970).

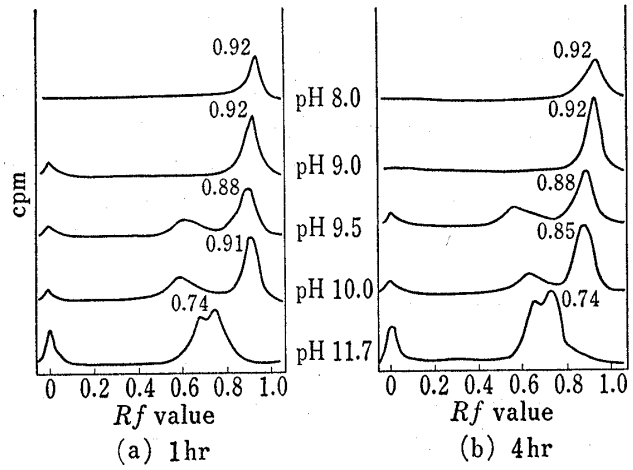

Fig. 4. Radio-scannograms based on pH Values for the Coupling (1 and 4 hours) of ^{3}H-Insulin with Phenyl Isothiocyanate[^{35}S]

Developing solvent: BuOH–pyridine–AcOH–H_2O (15:10:3:12, v/v).

Table II. Recovery of Authentic Insulin by Double Isotope Derivative Dilution Method

Insulin taken (μg)	Insulin found[a] μg	%
522.0	543.1±17.4	104.1±3.4
250.0	256.4± 3.7	102.6±1.5
100.0	101.5± 1.4	101.5±1.4

a) Relative standard deviation, $n=4$.

reaction mixture followed by ethyl acetate, extraction shows no difference on the radio-paper scannograms.

The two chains of bovine insulin contain three primary amino groups, the N-terminal α-amino groups of glycine and phenylalanine residues and the ε-amino group of a lysine residue. The two α-amino groups should react more rapidly at pH 8.5—9.0 than the latter owing to the difference in their pK_a's (7.5 and 9.6, respectively[15]). Consequently, one would expect the product of phenylthiocarbamylation at pH 9.0 to be a disubstituted derivative, substitution having occurred only on the α-amino groups of the insulin molecule. On the basis of these findings, the single radioactive peak appearing at pH 8.0 (Fig. 4a) or 9.0 (Fig. 4b) is presumed to be the terminal amino groups in both A and B chains of insulin reacting with ^{35}S–PTC to produce ^{35}S-bis(phenylthiocarbamyl)-^{3}H-insulin (Chart 1). These coupling conditions were applied to the double isotope derivative dilution analysis of insulin.

2.2 Analytical Procedure——Chemical determination of insulin was carried out to establish the double isotope derivative dilution method for authentic insulin samples. Sample and standard solution of insulin were prepared as follows: For the sample solution, bovine crystalline insulin was dissolved in 5% DMAA–TFA coupling buffer (pH 9.0) at the final concentration of 522, 250, or 100 μg/ml, while 500 μg/ml insulin solution was prepared for the standard solution.

In a 10-ml ground glass stoppered test tube, 1 ml of ^{3}H-insulin (2.41×10^{4} dpm/10 μg/ml) dissolved in 5% DMAA–TFA coupling buffer (pH 9.0) was added to both the sample and standard solutions in order to correct the loss of insulin sample during the analytical procedure. To these solutions, 2 μl of ^{35}S–PTC (40.5 μCi/mmol) was added with a Hamilton microsyringe and nitrogen gas was passed for 25 sec to expel oxygen dissolved in the solution. Then the test tubes were quickly stoppered tightly and incubated in a water bath of 40° with shaking for 4 hr.

The reaction solution was extracted five times with 2 ml each of freshly purified benzene. In each extraction step, layer separation was completed by centrifugation at 3000 rpm for 10 min. The aqueous solution left after the benzene extraction was concentrated to *ca.* 0.2 ml of the original in a desiccator over phosphorus pentoxide under a reduced pressure. The concentrate was paper chromatographed by the ascending technique on Whatman 3MM paper with butanol–pyridine–acetic acid–water (15:10:3:12, v/v) as the developing solvent.

15) C. Tanford and J. Epstein, *J. Am. Chem. Soc.*, **76**, 2163 (1954).

After development the paper was dried in air and the radio-chromatogram was taken on a radio-paper chromatoscanner. The small area of the paper containing ^{35}S-bis(phenylthiocarbamyl)-^{3}H-insulin was cut off and eluted with one 0.3-ml and four 0.2-ml portions of 0.01 N ammonia solution. The eluate was transfered to a counting vial. To the vial 10 ml of the liquid scintillation cocktail was added and radioactivities of ^{3}H and ^{35}S were measured by a liquid scintillation spectrometer. The amount of insulin in the sample was calculated from the following equation:

$$M_p = \left(\frac{S_p}{H_p} \times \frac{H_{st}}{S_{st}} \times \frac{I_p}{I_{st}} \times M_{st}\right) - M_i$$

where M_p is the amount (in μg) of insulin in the sample, M_{st} is the total amount (in μg) of insulin in the standard sample, S_p and H_p are the counts (in dpm) of ^{35}S and ^{3}H in the sample after the analytical procedures, S_{st} and H_{st} are the counts (in dpm) of ^{35}S and ^{3}H in the standard sample after the analytical procedures, I_p and I_{st} are the counts (in dpm) of ^{3}H-insulin added to the sample and the standard sample, and M_i is the amount (in μg) of ^{3}H-insulin added to the sample.

2.3 Results and Discussion——Table II shows the analytical results of known amounts of authentic insulin by the double isotope derivative dilution method by the above procedure. These results show that the quantities of insulin ranging from about 100 to 500 μg (0.017—0.085 μmol) are well determined by the isotope derivative method, when ^{3}H-insulin and ^{35}S-PTC having the specific radioactivity of approximately 6 mCi and 40 μCi per mmol, respectively, were used.

The specific radioactivity of ^{3}H-insulin labeled with $^{3}HH_2PO_4 \cdot BF_3$ complex is retained in mCi/mmol level due to the specific radioactivity of tritiated water employed for the preparation of tritiated phosphoric acid. Therefore, it requires higher specific radioactivity of the tritiated compound to enable to determine nanogram order of insulin, such as radioimmunoassay method. On the other hand, there are certain dangers in radioimmunoassay based on insulin antigenicity to estimate the total "immunoreactive insulin". Proinsulin in blood and urine has a similar immunological activity to insulin, but extremely low biological potency. When blood or urine insulin is assayed by radioimmunoassay, proinsulin also cross-reacts with antisera to insulin to cause overestimation for the quantities of insulin.[16] Furthermore, the "abnormal insulin" or insulin-like substances which may appear in blood in certain disease and have insulin-like immunoreactivity are also bound to insulin antibodies.[17]

Our proposed isotope derivative method should not be interfered by the materials which have insulin-like immunoreactivity such as proinsulin. Moreover, the present method has some great advantages; loss of insulin through the extraction and isolation process can be preliminarily corrected by addition of tritium-labeled insulin as an internal radioactive indicator for corrections of losses during the analytical procedures. The quantities of insulin can be calculated from the above-described formula without requiring the standard curves merely by counting the radioactivities of the diluted derivative, ^{3}H and ^{35}S-double labeled bis-(phenylthiocarbamyl) insulin isolated.

Theoretical basis for the double isotope derivative assays was developed with the use of the tritium tracer and the ^{14}C-labeled acetate derivatives for the assay of acetylatable steroids.[18] In this double isotope derivative dilution method, which is distinct from the direct isotope dilution analysis or a single isotope derivative dilution method, the quantities of the desired constituent in a sample can be determined on the basis of only a portion of the diluted

16) A.H. Rubenstein, S. Cho, and D.F. Steiner, *Lancet*, **1968**, 1353.
17) R.B. Elliot, D. O'Brien, and C.C. Roy, *Diabetes*, **14**, 780 (1965); D. O'Brien, D. Shapcott, and C.C. Roy, *ibid.*, **16**, 572 (1967).
18) P. Avivi, S.A. Simpson, J.F. Tait, and J.K. Whitehead, Proc. 2nd Radioisotope Conf., Vol. 1, Oxford, England, July, 1954, p. 313; B. Kliman and R.E. Peterson, *Fed. Proc.*, **17**, 255 (1958).

derivative isolated. In addition, because of unnecessity for another chemical determination for the quantities of a diluted derivative, the principal advantage of the present method is not restricted to the sensitivity of chemical determination methods.

By further raising the specific activity of the tritiated insulin, determination limit of the present method may be improved by the use of a more concentrated tritiated water. This isotope derivative method will be available for corrections of the overestimated values and determination for the true quantities of insulin, because routine analytical results obtained by the radioimmunoassay calculate antibody-bound values as the total "immunoreactive insulin" levels as described previously. Further investigation is necessary for the application of the present method to practical samples such as blood insulin.

Experimental

1. Reagents and Materials——Bovine pancreas crystalline insulin (Sigma Chemical Co., U.S.A.), N,N-dimethylallylamine (DMAA, Tokyo Kasei Kogyo Co., Tokyo), and trifluoroacetic acid (TFA, Tokyo Kasei Kogyo Co., Tokyo) were commercial products and used without further purification. Phenyl isothiocyanate (Seikagaku Kogyo Co., Tokyo) was purified by distillation under a reduced pressure before use. DMAA–TFA buffer solution: 5% of DMAA in 50% pyridine solution (pH 11.7) was adjusted to pH 8.0, 9.0, 9.5, or 10.0 with TFA. Pyridine was refluxed over BaO for 4 to 6 hr, and distilled in a fractionating column. The middle fraction boiling at 115° was collected.

Radioactive Materials: Tritiated water (Radiochemical Centre, England, specific activity, 5 Ci/ml) and phenyl isothiocyanate[^{35}S] (^{35}S–PTC, Radiochemical Centre, England, specific activity, 0.78 mCi/mmol) were diluted with the unlabeled compounds and used.

Cellulose acetate membrane "Cellogel" (Chemetron Co., Italy, 2.5×18 cm), and Visking tube (seamless cellulose tubing 30/32 type, Visking Co., U.S.A.) were used for electrophoresis and dialysis, respectively.

2. Instruments——Aloka PCS-4 paper chromatoscanner and Aloka LSC-502 liquid scintillation spectrometer (Nihon Musen Medicophysical Laboratory, Tokyo) were used for radioactivity measurements.

3. Counting of ^{3}H- and ^{35}S-Labeled Samples——All samples were counted for 10 min. The counting efficiency of the liquid scintillation spectrometer was 26—27% for ^{3}H and 58—60% for ^{35}S. Suitable corrections were made for background, crossover, and efficiencies of the two labeles.

Liquid scintillation cocktail was prepared as follows from 4.0 g of 2,5-diphenyloxazole (PPO), 0.4 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene(dimethyl-POPOP) and 100 g of naphthalene dissolved in 1000 ml of toluene–dioxane–methyl cellosolve (15: 3: 2, v/v).

4.1 Tritiation Method for Bovine Insulin——In an Erlenmeyer flask, 7.75 g of P_2O_5 was placed and 3 ml of diluted $^{3}H_2O$ (300 mCi/3 ml) was gradually added and stirred under ice-cooling. To $^{3}HH_2PO_4$ thus obtained, BF_3 gas was bubbled to prepare the $^{3}HH_2PO_4 \cdot BF_3$ under similar ice-cooling. An increase in weight of the tritiated material was 7.5 g by passing the BF_3 gas. To the tritiated complex thus prepared, 30 mg of bovine crystalline insulin was added and stirred for 12 hr at room temperature. The reaction mixture was submitted to dialysis in a Visking tube against 10^{-3} N HCl for 1 week. The outer diluted acid phase was replaced with fresh 10^{-3} N HCl two or three times a day. The inner phase treated by dialysis was freeze-dried in order to obtain ^{3}H-insulin, the specific radioactivity of which was 6.2—6.5 mCi/mmol.

^{3}H-insulin thus prepared was identical chemically and radiochemically with intact insulin by the radio-paper chromatography, cellulose acetate electrophoresis, and disc electrophoresis. On paper chromatography with appropriate solvent systems only a single radioactive peak was observed by means of radio-paper chromatoscanner and its *Rf* value agreed with that of the authentic insulin.

4.2 Removal of Labile Tritium in ^{3}H-Insulin——One mg of ^{3}H-insulin prepared freshly by acid catalytic tritiation was dissolved in 5 ml of 10^{-3} N HCl and placed in the Visking tube. Dialysis was carried out for 3 days against 1 l of 10^{-3} N HCl. At 24-hr intervals, 1 ml of the inner phase was taken into a counting vial and its radioactivity measured by a liquid scintillation spectrometer.

4.3 Electrophoresis on Cellulose Acetate Membrane——Electrophoresis was conducted on Cellogel membrane (2.5×18 cm) in 3 M HCOOH containing 4 M urea. The method of Jentsch[19] was slightly modified by keeping the constant voltage at 166 V (9 V/cm). After a 40-min run, ^{3}H-insulin portion was detected by staining with Ponceau S, and its behavior was compared with intact insulin run under similar electrophoretic conditions. The stained Cellogel membrane was cut into 5-mm segments and each segment extracted with 0.01 N HCl. Radioactivities for the extracts were counted by a liquid scintillation spectrometer.

19) J. Jentsch, *J. Chromatogr.*, **76**, 167 (1973).

4.4 Polyacrylamide Disc Electrophoresis——Fifteen percent of polyacrylamide gels (pH 9.4) for disc electrophoresis were prepared by the procedure of Ornstein[20] and Davis.[21] Gel disc electrophoresis (Mitsumi Scientific Industry Co., Tokyo, gel tube size: 0.5×7 cm) was started at a constant current of 3 mA/tube. After 1.5 hr, the run was stopped and the gels removed by rimming with a hypodermic needle. Then the gels were sliced into 5-mm segments with a razor blade. The gel slices were each homogenized and eluted with 0.2 ml of Tris-HCl buffer solution (pH 7.5). Radioactivity of each extract was counted by a liquid scintillation spectrometer. Other gels similarly run was stained with Coomassie Brilliant Blue.

20) L. Ornstein, *Ann. N.Y. Acad. Sci.*, **121**, 321 (1964).
21) B.J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).