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Studies on the Xanthurenic Acid-Insulin Complex

III. Distribution of Xanthurenic Acid and Formation of Xanthurenic Acid-Insulin Complex in Serum

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The distribution of added xanthurenic acid (XA) in the serum was studied by *in vitro* experiments with unlabeled and tritium labeled XA, both with healthy and diabetic serum, with and without added insulin. From this experiment it was elucidated that XA binds to both albumin and insulin fractions. The amounts of XA bound to the serum protein are larger in a normal serum than a diabetic serum. The distribution of ^{131}I-insulin in the serum was also studied. To elucidate the binding of XA to insulin, tritium labeled XA was injected to the rat and the sera were subjected to chromatography on the Sephadex column after it was treated with anti-insulin serum. The distribution of ^{3}H-XA was checked in eluates. The result shows that injected ^{3}H-XA binds to the circulating insulin and the resulting complex combines to the anti-insulin serum. Thus, we can confirm the binding of XA with insulin in the serum.

In a previous paper (*1*) it was suggested from *in vitro* experiments that XA might combine with insulin, and in a successive report, it was suggested that the insulin-XA complex might be responsible for diabetes (*2*) from the results of the blood glucose measurement and the uptake of glucose by rat diaphragm and adipose tissue. In the present paper, the unlabeled and tritium labeled XA distribution was studied as a preliminary experiment and then the binding of XA and insulin in the serum was studied both *in vitro* and *in vivo* experiments.

MATERIALS AND METHODS

1. Human Serum—Serum was obtained from the blood of normal healthy subjects and patients in the National Hospital of Nagoya, and we indebted to Dr. Arakawa for his kindness for the sampling of the diabetic serum.

2. Column Chromatography—A column of Sephadex G-150 (15 mm×1,200 mm) was previously equilibriated with Krebs Ringer Phosphate Buffer (K.R.P.), pH 7.4. A mixture of 2 ml of serum and 2 ml of K.R.P. containing 2.5 mg of XA and/or 20 mg of insulin was applied to the column. The column was eluted with the same buffer, and the fractions of 3 ml of eluates were collected. Their optical densities at 280 mμ and XA contents were measured.

3. Determination of XA—A simple and rapid method was developed to estimate the

amount of XA in the chromatographic experiments. This was based on the method (*3*) for the estimation of the alkaline phosphatase [EC 3.1.3.1].

4. Labeled Materials and Other Reagents—^{131}I-insulin was purchased from the Abott Laboratories Co., and ^{3}H-XA of crystalline type and of ammoniacal solution were both kindly gifted from Meiji Seika Co., and insulin antibody (insulin binding protein) was purchased from Wellcome Research Laboratories.

RESULTS AND DISCUSSIONS

1. Determination of XA with 4-Aminoantipirine (A.A.P.)—Since Musajo (*4*) discovered XA in the urine, the quantitative determination of XA has been attempted by many investigators. Glazer *et al.* (*5*) estimated XA in the urine colorimetrically as its ferric salt, but this method was not sufficiently sensitive for our purposes. Fluorometric procedures were reported by Sato and Price (*6*) and Tsuzi and Hasegawa (*7*) but these methods are very complicated and time consuming. Accordingly, we devised the following method. The principle of the color reaction is shown in Fig. 1. The resulting quinone dye has the absorption spectrum shown in Fig. 2. The spectrum of the colored compound formed under the same conditions by 3-hydroxykynurenine (3-OH-Kyn) is also shown. Figure 2 shows that optical density of 3-OH-Kyn at 510 mμ is about one third of that of XA. Price *et al.* (*8*) reported that 0 to 6.0 mg/day of 3-OH-Kyn is normally excreted in the urine. This is about one tenth of the amount of urinary XA normally excreted (*9*), and in diabetes the amount of urinary 3-OH-Kyn increases markedly. Therefore, this method is not suitable for the determination of XA in the urine containing various compounds related to XA. However, the concentration of these quinoline compounds in sera are low (Table I), so that they are negligible in the presence of a large amount of added XA. The values in Table I, measured by the A.A.P. method, show that the amount of quinoline compounds, expressed as XA in the serum in the case of diabetes, is about 11 μg/ml (s.e. 3.1 μg/ml), and no XA or related compounds to XA were detected under the same conditions in 8 samples of the normal serum.

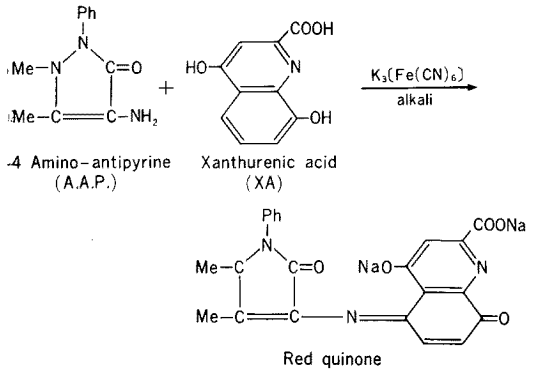

Fig. 1.

The details of the A.A.P. method are as follows: 0.5 ml of a sample was added to 3 ml of a mixture of 0.1 M sodium bicarbonate and 0.1 M sodium carbonate solutions (13 : 7, v/v). Then, 1 ml of 2.4% potassium ferricyanide was added. The resulting color was measured at 510 mμ. The quinoline dye is stable between 20 and 60 min after the color was developed. The color is also stable at pH 10± 0.1. Figure 3 shows the standard curves for XA and 3-OH-Kyn.

2. Distribution of XA in the Serum—i) Distribution of unlabeled XA in the serum:

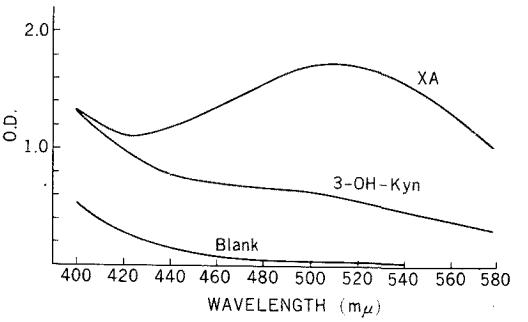

Fig. 2. The spectra of colored compounds formed with 4-aminoantipirine. 0.5 ml of XA solution and 1 ml of bicarbonate buffer (0.1 M, pH 10±0.1) were mixed. Then 1 ml each of 4-aminoantipirine solution and 2.4% potassium ferricyanate solution was added in this order. Spectra were measured 30 to 60 min after addition of all the reagents.

TABLE I. Contents of XA related Compounds colored by A.A.P. Method in the Sera of Diabetic Patients.

Number of patients	Volume of serum tested (ml)	XA and XA related compounds (μg/ml)
1	0.25	8
2	0.25	8
3	0.20	0
4	0.20	5
5	0.25	12
6	0.20	5
7	0.20	5
8	0.15	0
9	0.20	25
10	0.20	0
11	0.20	0
12	0.40	15
13	0.20	37.5
14	0.40	10
15	0.40	20
16	0.40	22.5
		mean : 10.8
		s.e. : 3.1

To investigate the binding of XA with insulin in the serum, insulin and XA were added to the serum and the mixture was applied to the column as described in the "MATERIALS AND

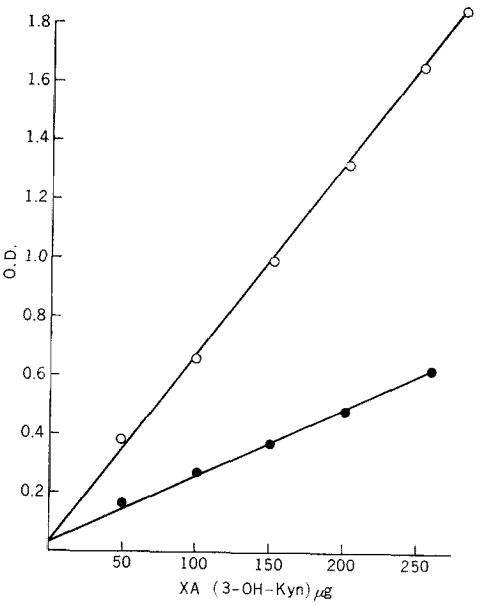

Fig. 3. Standard curves for XA and 3-hydroxykynurenine. ○, XA; ●, 3-hydroxykynurenine.

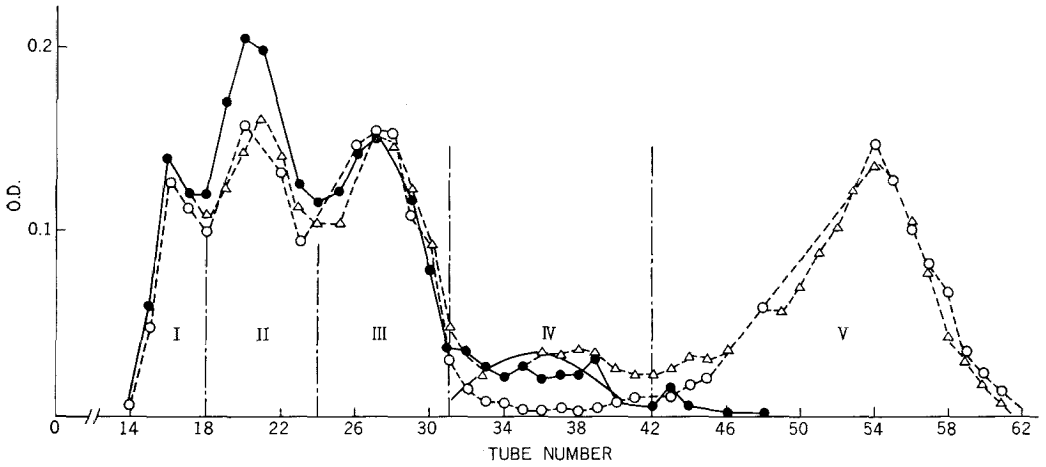

Fig. 4. Elution patterns of human sera from Sephadex G-150. A mixture of 2 ml of serum and 2 ml of K.R.P., pH 7.4 was applied to a Sephadex G-150 column (15 mm × 1,200 mm). 20 mg of insulin and/or 2.5 mg of XA were added to the K.R.P. as indicated. The column was eluted with K.R.P. and fractions of 3 ml of effluents were collected. A 0.2 ml aliquot of each fraction was taken for measurement of the optical density at 280 mμ after dilution with 4 ml of water. ●, serum+insulin; ○, serum+XA; △, serum+insulin+XA; ——, insulin only.

METHODS." The column was eluted with K.R.P. solution (pH 7.4), and 3 ml each fraction were collected. A 0.2 ml aliquot of each fraction was diluted with 4.0 ml of water and the optical density at 280 mμ was measured. As a reference, XA and/or insulin was added to the serum and it was treated in the same way. The results are shown in Fig. 4. The contents of protein and XA are expressed as the optical density at 280 mμ, since XA has maxima at both 280 and 330 mμ.

As shown in this figure, free insulin appeared in tubes No. 30 to 44 and free XA appeared in tubes No. 45 to 60. As indicated in Fig. 4 the fractions of eluates were classified into five groups (Frs. I to V). As described above, insulin mainly appeared in Fr. IV, while free XA appeared in Fr. V. Frs. I to IV were respectively dialyzed, and then lyophilyzed. XA contents in each fraction were determined by D.H.T. method (*1*, *10*). Table II shows the yields in each fractions, and the figures showed the average of the six experiments. As shown in this table the yields of Fr. IV are very low, and for the D.H.T. method more than 15 mg of a sample is required (*1*), so that in this experiment the XA content was measured in a mixture of Frs. III and IV. The XA contents of each fraction were summarized in Table III which were determined by the D.H.T. method. These results showed that the XA contents of Frs. I and II were zero. However with added insulin and XA, XA contents in Fr. III plus Fr. IV increased to 13.7 and 31.5 μg XA per 10 mg protein in normal and diabetic patients, respectively.

ii) The Distribution of ^{3}H-XA in the serum: In the previous experiment, distribution of XA was studied using unlabeled XA, whereas in this experiment tritium labeled XA was used. 0.3 ml (0.3 mCi) of ^{3}H-XA was added to 2 ml of serum and diluted with 75 mM sodium diphosphate (pH 8.4) to 4 ml and this mixture was applied to the column of Sephadex G-150 previously equilibriated with the same solution, and 3 ml each of eluate was collected. The 0.2 ml of aliquots were taken from each fraction and diluted with 4 ml of distilled water. The radioactivities were measured by using 0.2 ml of this diluted solution with the liquid scintillation counter. The measurements are kindly helped by Dr. Hagino, Dr. Kobayashi, and Dr. Kato of the Medical School of

TABLE II. Yield of serum protein fractions for details of the experiments, see the text. Sample A : 2 ml serum, 20 mg insulin, and 2 ml K.R.P. B : 2 ml serum, 2.5 mg XA, and 2 ml K.R.P. C : 2 ml serum, 20 mg insulin, 2.5 mg XA, and 2 ml K.R.P. D : 20 mg insulin and 4 ml K.R.P. Values are yields of each fraction after lyophilization in mg.

Sample	Fractions			
	I	II	III	IV
A	18.0	30.5	62.3	7.8
B	17.7	31.5	65.5	4.7
C	18.3	30.5	58.0	9.7
D				5.4

TABLE III. Distribution of XA in serum protein determined by D.H.T. method. About 15 to 20 mg of protein are required in this method. Since the yield of Fr. IV was always very low, the content of XA was determined in the mixed sample of Frs. III and IV.

Serum	Added reagents to the serum	Number of experiment	XA (μg/10 mg protein)		
			Fr. I	Fr. II	Fr. III+IV
Normal	None	1	0	0	0
	Insulin	2	0	0	5.2
	Insulin+XA	2	0	0	13.7
Diabetic	None	3	0	0	18.1
	Insulin	3	0	0	24.8
	Insulin+XA	3	0	0	31.5

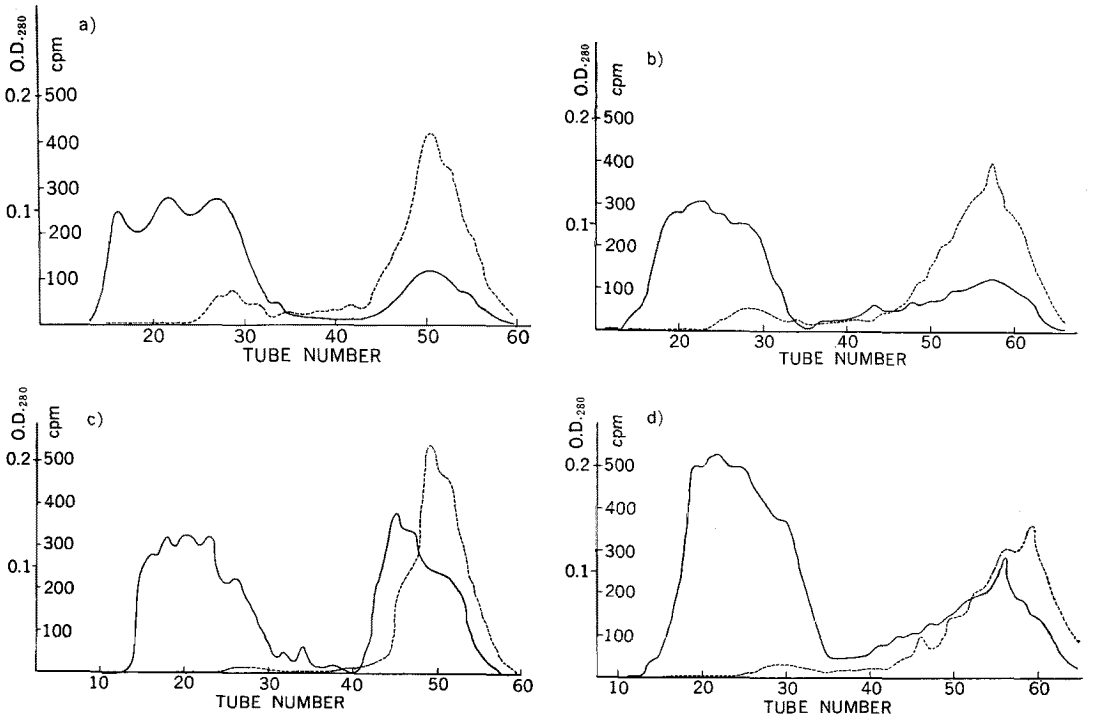

Fig. 5. The distribution of ^{3}H-XA in the serum. a) healthy serum+^{3}H-XA, serum 2 ml, 0.3 mCi ^{3}H-XA; b) healthy serum+^{3}H-XA+insulin; c) diabetic serum+^{3}H-XA; d) diabetic serum+^{3}H-XA+insulin, serum 2 ml, insulin 18 mg, 0.3 mCi ^{3}H-XA. ——, O.D. $_{280}$; ----, cpm.

Nagoya University. The absorbance at 280 mμ was also measured.

Figure 5 (a to d) shows the distribution of radioactivity and absorbance at 280 mμ with healthy and diabetic serum with and without added crystalline insulin. The elution patterns were divided into four groups: tube No. 13 to 23 (Fr. A mainly includes globulin), tube No. 24 to 35 (Fr. B mainly includes albumin), tube No. 36 to 45 in the case of Fig. 5a and c (Fr. C mainly includes added insulin, reference to Fig. 4), tube No. 36 to 48 in the case of Fig. 5b and d and finally tube No. 46 to 60 and/or 49 to 65 (Fr. D mainly includes free XA). From these figures following results may be derived. 1) The distribution of ^{3}H-XA in globulin region is almost negligible. 2) ^{3}H-XA combines to albumin fraction. 3) ^{3}H-XA combines also to added insulin. Table IV shows the percentage of distribution of ^{3}H-XA calculated from cpm. This results show that

TABLE IV. Percentage of distribution of radioactivity in the serum.

Serum	Added reagents to the serum	Fractions			
		A	B	C	D
Health	^{3}H-XA	0.7	11.1	6.3	81.9
	^{3}H-XA+insulin	1.0	8.5	11.1	79.3
Diabetic	^{3}H-XA	0.9	2.7	3.8	92.6
	^{3}H-XA+insulin	0.9	5.0	3.2	90.9

the distribution of ^{3}H-XA in globulin fraction is as low as below 1% in all cases, and that the amount of bound ^{3}H-XA to albumin fraction in a diabetic serum is smaller than in a healthy serum. The exact reason of this result is not clear, but following phenomena may account for one of the reasons; from the result of Table I, in the case of diabetic serum, XA or XA related compounds are already

present in the serum, so that externally added ^{3}H-XA may be inhibited their binding. The table also shows that ^{3}H-XA binds to added insulin, and these results confirm the result already reported (*1*).

In order to have further information about the distribution of XA in the serum, following experiments were carried out. To 2 ml of healthy or diabetic serum were added 2 ml of K.R.P. containing 20 mg of unlabeled XA, 0.2 ml (0.2 mCi) of ^{3}H-XA solution and 4 mg of crystalline insulin. The mixture was then applied to the Sephadex G-150 column and treated as described before. In this case XA contents were determined by A.A.P. method, in which we can determine the contents of XA in Fr. III and Fr. IV separately. The radioactivity was also determined for each fraction after dialysis and lyophilyzation. The results were shown in Table V. This table showed that radioactivity in Fr. I is the lowest both in healthy serum and diabetic serum, and the amount of ^{3}H-XA in Fr. IV is larger in healthy than in diabetic serum. These results are in good agreement with those in Table IV in the previous experiments.

3. The Distribution of ^{131}I-Insulin in the Serum—The distribution of insulin and XA was studied using ^{131}I-insulin which was diluted with crystalline insulin. The experiment was carried out as follows: 0.1 ml of ^{131}I-insulin (*ca.* 62 μCi/0.1 ml, 2.25 μg), 18 mg of crystalline insulin and 20 mg of unlabeled XA (dissolved in 1.9 ml of K.R.P.) were added to 2 ml of serum and the mixture was applied to the column, and treated as before. Figure 6 shows the distribution of radioactivity and absorbance at 280 and 510 mμ (correspond to the content of XA determined by A.A.P. method). Table VI showed the mean values of each three determinations for the distribution of ^{131}I-insulin in both healthy and diabetic serum. These results show that in the case of diabetic serum more insulin invaded to Fr. V (mainly containing free XA). Although this reason is not clear now, this phenomenon suggests the possibility that XA-insulin complex may be consisted of two components, one of which has ordinary molecular weight (*11*) and the other has smaller molecular weight.

4. The Binding XA-Insulin to Insulin Binding Protein— i) Preparation of ^{3}H-XA-insulin complex: As a preliminary experiment

TABLE V. The distribution of XA in serum protein assayed by the A.A.P. method and radioactivity. The sample was assayed directly without deproteinization, since the color development was negligible in the experimental conditions. Values in parenthesis indicate the number of experiments.

Fraction	Normal		Diabetic	
	XA (μg/10 mg protein)	cpm	XA (μg/10 mg protein)	cpm
I	0.8(2)	520	0 (8)	222
II	0 (2)	695	0 (8)	408
III	5.9(2)	7600	10.1(8)	9400
IV	5.2(2)	139000	13.2(8)	55000

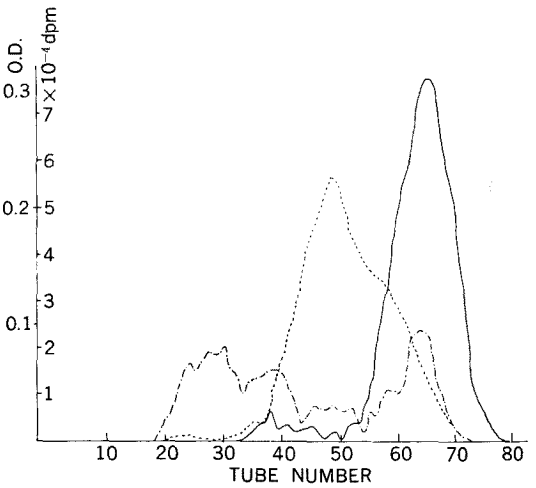

Fig. 6. Distribution of ^{131}I-insulin and XA in the serum. XA was measured by A.A.P. method. ——, O.D.$_{280}$; ----, cpm; -·-·-, XA (510 mμ).

TABLE VI. The distribution of ^{131}I-insulin in the serum. The ditails of the experiments are described in the text. Figures are expressed in percentage.

Serum	Fractions				
	I	II	III	IV	V
Health	1.0	0.3	2.0	93.7	2.9
Diabetic	1.0	0.2	2.3	90.5	5.9

for the binding of XA and insulin in the rat serum, an effect of XA-insulin complex to insulin binding protein was examined. For this purpose, ^{3}H-XA-insulin complex was prepared. 150 mg of crystalline insulin was dissolved in 2 ml of 75 mM disodium phosphate solution and 2.1 ml of XA solution, which was prepared from a mixture of 10 mg of unlabeled XA dissolved in 2 ml of 75 mM disodium phosphate solution and 0.1 ml of ^{3}H-XA solution (0.1 mCi/0.1 ml) was added to it. The mixture was applied to the column of Sephadex G-150. In this case Sephadex Column K-25/45 Jacketed (Pharamacia Uppsala) was used. The column was cooled by Coolnics, CTR-18 (Komatsu-Yamato) at 15°C. Figure 7 showed the distribution of radioactivity. The ratioactivity appeared in regions A, B, and C. The radioactivity in regions A and B shows that XA binds to insulin molecule. After dialysis of the regions A and B, both fractions were lyophilyzed, the yield of the resulting sample are 120 and 10 mg, respectively. The later sample was used for the following experiment. The region A sample has radioactivity of 27.45 cpm/mg and the B sample has a 22.31 cpm/mg, respectively. Since total cpm was 261,876 corresponding 20.5 mg XA, above A sample contains about 5 moles of XA per insulin (the molecular weight of insulin being assumed as 48,000) and one mole of XA per insulin dimer in the case of B sample.

ii) The binding ability of insulin-binding protein with insulin: In order to check the binding ability of insulin-binding protein with insulin, following experiment was performed. A mixture of 2 ml of insulin-binding protein (the powdered sample was dissolved to 8 ml in a vial), 40 μl of ^{131}I-insulin (about 2.5 μCi, 0.9 μg of insulin) and 2.0 ml of 75 mM disodium phosphate solution were applied to the Sephadex G-150 column (15 mm × 1,200 mm) after the mixture was incubated at 4°C for four days. The elution pattern was shown in Fig. 8. The figure showed that radioactivity and absorbance at 280 mμ appeared in separate regions, the former is in a high molecular weight region and the latter in a low molecular weight region. These data showed that ^{131}I-insulin binds to insulin-binding protein. Moreover, the control experiment with the same conditions using ^{131}I-insulin solution (without added insulin-binding protein) showed no radioactivity and absorbance appeared in a high molecular weight region.

iii) Binding of ^{3}H-XA-insulin complex with insulin-binding protein: Two milliliters of ^{3}H-XA-insulin complex prepared in section 4-i) were dissolved in 2 ml of 1.5 M disodium phosphate solution and was mixed with 2 ml of insulin-binding protein solution (as described

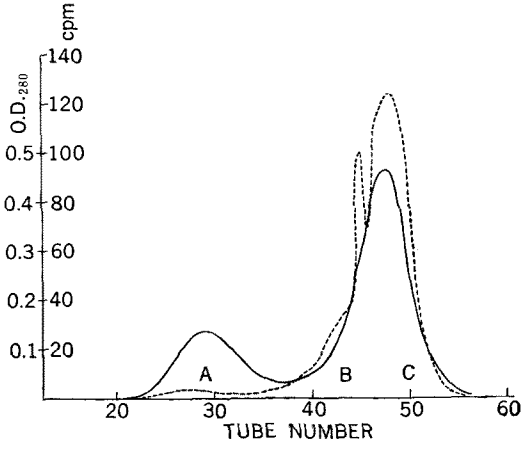

Fig. 7. The column chromatography of ^{3}H-XA added insulin solution. For the conditions of the experiment, see the text. ——, O.D. $_{280}$: ----, cpm.

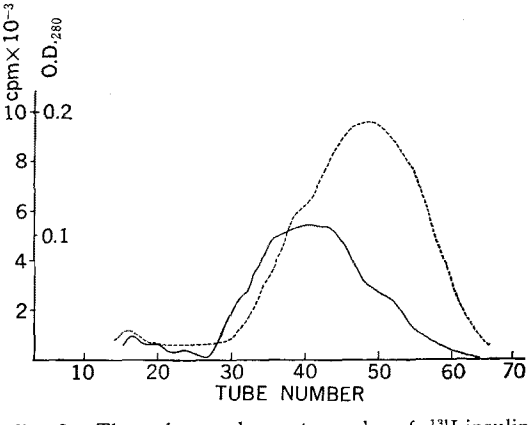

Fig. 8. The column chromatography of ^{131}I-insulin with insulin-binding protein. For the conditions of the experiment, see the text. 2.5 μCi ^{131}I-insulin, 2 ml insulin-binding protein. ——, O.D. $_{280}$; ----, cpm.

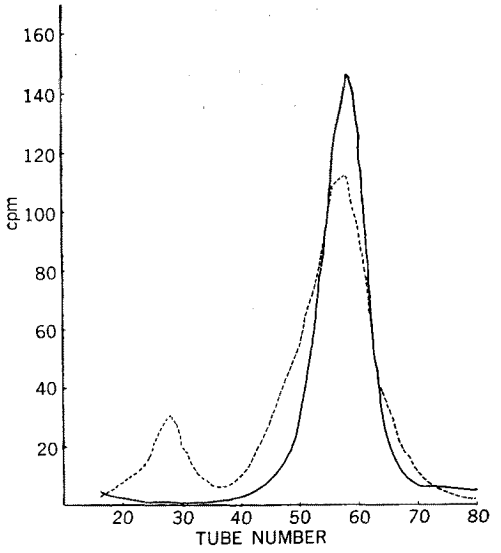

Fig. 9. The column chromatography of ^{3}H-XA-insulin complex with insulin binding protein. For the conditions of the experiment, see the text. 2 ml insulin-binding protein, 2 mg ^{3}H-XA-insulin complex. ——, cpm of ^{3}H-XA-insulin complex only; ----, cpm of ^{3}H-XA-insulin complex after incubated with insulin-binding protein at 4°C for four days.

in section 4-ii)). The mixture was applied to the column after being kept at 4°C for four days. Figure 9 showed the data with and without added insulin-binding protein to the ^{3}H-XA-insulin complex. The data suggested clearly that ^{3}H-XA-insulin complex bound to insulin-binding protein.

5. Binding of ^{3}H-XA to Circulating Insulin— i) The distribution of injected ^{3}H-XA in the serum: A male rat of the Wister strain, weighing 215 g, was starved for 24 hr. After starvation 1.5 g of glucose was administrated intragastrically. After 90 min, the rat was injected intravenously with 4 mg of ^{3}H-XA (910 μCi/mg) dissolved in 0.3 ml of 0.1 M phosphate buffer, pH 7.6. Five minutes after the injection, the blood was taken with the addition of heparin. The serum was obtained by centrifugation. 2.5 ml of this serum and the same volume of K.R.P. solution were mixed and applied to a Sephadex G-150 column as described before, and the column was eluted with the same solution as described above. Three milliliters each fraction were collected,

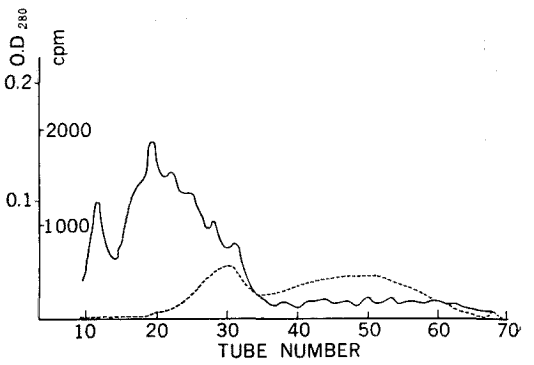

Fig. 10. The distribution of injected ^{3}H-XA in rat serum. For the details of the experiment, see the text. ^{3}H-XA, 970 μCi. ——, O.D.$_{280}$; ----, cpm.

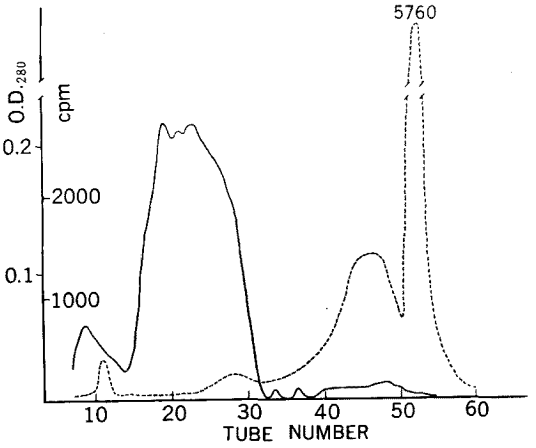

Fig. 11. The distribution of injected ^{3}H-XA in the rat serum after treated with insulin-binding protein. For the details of the experiment, see the text. ^{3}H-XA, 17 m Ci. ——, O.D.$_{280}$; ----, cpm.

and 0.2 ml aliquot from each fraction was diluted to 4 ml and absorbance at 280 mμ and radioactivity were measured. The data were shown in Fig. 10. This figure shows that ^{3}H-XA combined with serum protein (mainly albumin fraction) and with insulin fraction. In a large molecular weight region no appreciable amount of ^{3}H-XA was detected.

ii) Formation of ^{3}H-XA-insulin complex in rat serum in vivo experiment: In order to have valid evidence for the binding of ^{3}H-XA with circulating insulin, an antigen-antibody reaction between insulin-binding protein and ^{3}H-XA-insulin complex was examined. The

experiment was carried out as described in section 5-i), and a male rat weighing 210 g was used. After administration of the same amount of glucose and injection of ^{3}H-XA (4 ml of solution containing 1.7 mCi/7.48 mg of ^{3}H-XA), the blood was taken as described before, and the serum of 2 ml was mixed with 2 ml of insulin-binding protein solution and kept for 4°C for four days. The mixed serum was applied to the same column as described in a preceeding section and treated as before. The elution pattern was shown in Fig. 11.

As shown in this figure, ^{3}H-XA appeared in a large molecular weight region, which probably correspond to γ-globulin fraction, and as shown in Fig. 10 we cannot observe any radioactivity in this region, when insulin-binding protein was not added. These facts suggested that ^{3}H-XA-insulin complex was formed in rat serum *in vivo* and this complex may in turn react with insulin-binding protein as the preliminary experiment shown in Fig. 9 indicated.

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