The Preparation and Evaluation of Tritiated Polyalanyl Insulin Derivatives[1]

ROBERT E. CANFIELD,[2] GORDON I. KAYE,[2] AND SUSAN B. WEST

From the Department of Medicine and the F.H. Cabot Laboratory of the Division of Surgical Pathology, Departments of Surgery and Pathology, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032

ABSTRACT. A series of polyalanyl insulin derivatives (PAI) have been synthesized and characterized. Their content of added alanine ranged from 0.49 to 16.6 moles per mole of insulin. PAI derivatives with an average of up to 3.75 moles of added alanine per mole of insulin retained full biological activity as measured by the epididymal fat pad assay. Data from phenylisothiocyanate degradation indicate that 87% of the molecules in such a derivative probably possess at least one added alanine, so it is concluded that insulin with minimal addition of alanine is biologically active.

A preparation of high specific activity tritiated polyalanyl insulin has also been synthesized and shown to be fully biologically active by the epididymal fat pad assay. Preliminary autoradiographic studies using this insulin derivative demonstrate that the hormone is bound to the sarcolemmal membranes of both striated and cardiac muscle. ^{3}H-polyalanyl insulin was also concentrated in the proximal tubules of the kidney, but barely detectable in the liver at seven minutes after intravenous injection. (*Endocrinology* **90**: 112, 1972)

THE present study was undertaken to determine whether polyalanyl derivatives of protein hormones might be prepared with preservation of their biological function. If this can be accomplished, protein hormones with added radioactive polyalanyl side chains could be used in tracer studies to locate sites of hormone binding at the target cell.

The reaction of proteins with N-carboxy-D,L-alanine anhydride has been used to synthesize derivatives with multiple polyalanyl side chains attached to the alpha amino group and to the epsilon amino groups of proteins (1,2). One notable feature of these derivatives is that the addition of large numbers of alanine residues is accompanied by little or no interference with the biological action of a particular protein. For example, it has been demonstrated that up to an average of 700 poly-D,L-alanine residues may be added to gamma globulin without altering the capacity of the antibody to bind antigen (3). The addition to ribonuclease of up to 50 alanine residues, in multiple polyalanyl side chains, actually appears to enhance enzymic function on certain substrates and does not interfere with the normal reoxidation of the reduced protein (4). Polyalanyl derivatives of trypsin with more than 100 added alanines have enhanced stability and no loss of enzyme function. Similarly polyalanyl derivatives of chymotrypsin have been shown to retain their enzymic activity (5). Finally, a preparation of poly-D,L-alanyl rabbit myosin has been shown to retain most of its Ca ATPase activity (6).

The present report describes the synthesis and characterization of a series of polyalanyl insulin derivatives as well as preliminary autoradiographic studies with a biologically active tritiated polyalanyl insulin. Studies with polyalanyl human growth hormone have been described elsewhere (7).

Materials and Methods

Crystalline bovine insulin was obtained from the Boots Pure Drug Co., Ltd. (Batch 2189, 23 IU/mg). This particular lot of insulin was prepared at the request of The Commission on Proteins, IUPAC, and is accepted as standardized material. Phosphocellulose (P-70) was obtained from H. Reeve Angel. Leuch's anhydride (N-carboxy-D,L-alanine) was purchased from the Pilot Chemical Co. and Dowex (AG 50 × 4)

Received May 17, 1971.

[1] Supported by USPHS grants AM 09579 and AM 12396.

[2] Career Investigators of the Health Research Council of the City of New York.

was purchased from Calbiochem. Tritiated D,L alanine was prepared as a custom synthesis by the method of Humphrey *et al.* (8) by the New England Nuclear Corporation and D-glucose-1-^{14}C was also purchased from the New England Nuclear Corporation.

Preparation of Polyalanyl Insulin Derivatives (PAI). Crystalline insulin (3.0 g) was dissolved in 200 ml of 0.02N HCl. Following the addition of 800 ml of 0.1M NaH_2PO_4, the mixture was adjusted to pH 7.0 with concentrated NaOH. The solution, which was quite turbid following addition of the phosphate, became clear after adjustment of the pH. Nine 100 ml aliquots of this solution were chilled on ice. Two grams of N-carboxy-D,L-alanine were dissolved in 50 ml of anhydrous dioxane and a slight amount of residual insoluble material was eliminated by centrifugation. Eight different volumes of the dioxane solution (0.25 ml, 0.5 ml, 0.75 ml, 1.0 ml, 2 ml, 5 ml, 10 ml, and 25 ml) were added with vigorous stirring to the chilled 100 ml insulin-containing aliquots. The polyalanyl insulin products obtained from these eight mixtures will be referred to as PAI 1 through 8, sequentially. A ninth sample (PAI 9) received no N-carboxyl-D,L-alanine, but was carried through all the other procedures as a control. Following one hour of stirring, sufficient 1N HCl was added to each sample to adjust the pH to 3.0. The material was then frozen and stored at —20° C.

Chromatography of Polyalanyl Insulin (PAI) Derivatives. The frozen insulin samples were warmed to room temperature and applied to a 2.5 × 10 cm column of phosphocellulose that had been equilibrated with 1N acetic acid adjusted to pH 3.5 with NH_4OH. The column was eluted at room temperature with ammonium acetate buffers mixed in a three chamber varigrad. The first chamber contained 1N acetic acid adjusted to a pH of 3.5 with NH_4OH, the second chamber contained 2N acetic acid adjusted to a pH of 3.8 with NH_4OH, and the third contained 4N acetic acid adjusted to a pH of 4.5 with NH_4OH. The volume of each chamber was 250 ml; the fraction size was 5 ml. These conditions were chosen to permit elution of insulin without precipitation of the hormone. The fractions containing the insulin were pooled and passed through a 5 × 80 cm column of Sephadex G-25, which previously had been equilibrated with 0.1N acetic acid. The same solution was used to elute the column after the passage of the PAI fractions. The insulin-containing eluate, free of ammonium ions, was lyophilized and stored at —20° C.

Chemical Characterization of PAI Derivatives. Portions of each PAI sample (1 mg) were hydrolyzed in 6N HCl in evacuated tubes for 24 hours at 110° C and, following evaporation of HCl, subjected to amino acid analysis on a Spinco Model 120 B Amino Acid Analyzer. The number of added alanines per mole of insulin is tabulated in the second column of Table 1.

An additional portion of each sample (30 mg) was subjected to performic acid oxidation by the method of Hirs (9). The resulting mixture of oxidized A and B insulin chains was dissolved in 15% aqueous formic acid at 70° C and applied to a jacketed, 0.9 × 47 cm column of Dowex AG 50 × 4 maintained at 70° C. The column was developed with 15% formic acid until a sharp peak of optical density absorbing at 280 nm emerged. The eluting solution was then changed to 10% NH_4OH and a second UV absorbing peak emerged from the column coincident with the change in effluent pH. The columns were operated at elevated temperatures to avoid precipitation of the oxidized insulin chains. The composition of material from the first peak was identical to the composition of the insulin A chain; the composition of material from the second peak was identical to that of the insulin B chain. This same technique was then applied to the isolation of the oxidized A and B chains from each of the PAI derivatives. The four cysteic acid residues in the oxidized A chain made it sufficiently acid to prevent adsorption on Dowex under the conditions of application, whereas the histidine and the lysine residues in the oxidized B chain apparently provided sufficient positive charges to permit binding. The number of alanines in each insulin chain could then be determined by amino acid analysis. These results are listed in the third and fourth columns of Table 1. The calculated sum of these values for the individual oxidized chains (fifth column) is in excellent agreement with the results of the amino acid analyses of the unoxidized PAI samples (second column).

Two to three milligram samples of each iso-

lated A and B chain preparation were subjected to the Edman degradation by a procedure described previously (10). The residues removed were calculated by difference from amino acid analyses of samples taken before and after one complete Edman cycle. The degree of alanylation of epsilon amino groups of the lysine residue in position 29 of the B chain was determined by deamination of the unreacted lysine side chains with nitrous acid followed by acid hydrolysis and amino acid analysis (4). These results are expressed as the fraction of substituted lysines (Table 2).

Epididymal Fat Pad Assay. Samples of each of the polyalanyl insulin derivatives were assayed for biological activity by the epididymal fat pad assay procedure (11,12). As noted by these authors, the metabolic activity of portions of an individual fat pad may be variable. Therefore, we rotated the tissue sections in the experimental procedure to compensate for these differences. In the assays reported here, each fat pad was sectioned into three approximately equal pieces and these were placed in separate flasks. One flask contained 500 uU/ml of the standard insulin, the second contained an equal amount of the PAI insulin derivative to be tested, and the third flask contained no insulin and served as a blank. Each unknown was tested with at least 20 sets of triple flasks, and the inner, middle and outer sections of the fat pads were rotated to minimize the effect of the anatomical differences. The biological activity listed in Table 1 is expressed as the ratio of counts liberated from D-glucose-1-^{14}C by the polyalanyl insulin derivative to the counts liberated by an identical quantity of standard insulin after each set of counts had been corrected for the counts in the blank. A ratio of 1.0 was taken to indicate full biological activity in the derivative being tested. The level of insulin assayed was chosen because it produced a tenfold increase in radioactivity over the blank value. When the standard Boots insulin was compared with the control sample (PAI 9) in this assay system, the ratio of activity was 1.01 with a standard error of 0.06. It should be noted that each polyalanyl insulin test solution was prepared on a weight basis assuming 23 IU/mg of insulin. Therefore, samples PAI 6, 7, and 8, with heavy alanine enrichment, would have had higher biological activity ratios had the comparisons been made on a molar basis. No assay was performed on samples PAI 1 and 3.

Preparation of Tritiated D,L Alanyl Insulin. N-acetyldehydroalanine was subjected to catalytic reduction in tritium and a portion of the product (7 Ci, 20 Ci/mM) was converted to Leuch's anhydride according to a modification of the procedure of Humphrey *et al.* (8). The labeled alanine was suspended in dioxane at 50° C and phosgene was continuously bubbled through the suspension with continuous stirring for two hours. The dioxane was then evaporated by passing anhydrous nitrogen (Mathe-

TABLE 1. Relationship between alanine addition and biological activity of polyalanyl-insulin derivatives (PAI)*

Insulin derivative	Moles of added alanine/mole protein				Biological activity	
	Insulin	A-chain	B-chain	Calc. total	Ratio	Standard error
PAI 1	0.49	0.29	0.21	0.50	**	—
2	0.95	0.56	0.38	0.94	1.03	0.06
3	1.54	0.82	0.60	1.42	**	—
4	1.96	1.11	0.79	1.90	1.09	0.06
5	3.75	2.17	1.67	3.84	0.98	0.05
6	7.99	4.58	3.46	8.04	0.81	0.05
7	12.25	6.21	5.76	11.97	0.71	0.05
8	16.60	8.81	7.63	16.44	0.46	0.06
9 (control)	0.01	−0.01	−0.02	−0.03	1.01	0.06

* Biological activity of the PAI derivatives was measured by epididymal fat pad assay and a tabulation of data for PAI 4, illustrating the details of one particular assay, is given in Table 3. All of the other assays employed the same procedure.

** Epididymal fat pad assays were not performed on these two derivatives.

son) through the reaction vessel. The oily residue of ^{3}H-alanine Leuch's anhydride (N-carboxy-D,L-alanine anhydride) remaining after evaporation of the dioxane was redissolved in 5 ml of anhydrous dioxane and immediately added to a chilled aqueous solution of insulin (20 mg) prepared as described earlier. After 30 minutes the product was acidified (pH 3.0) and purified by the techniques for the isolation of PAI derivatives described above using a 5 × 1 cm column of Sephadex G-25. The purified ^{3}H-PAI was then lyophilized and redissolved in 0.001N HCl and its concentration determined by measuring absorption at 280 nm. Within 24 hours of the reaction of insulin with the ^{3}H-N-carboxy-D,L-alanine anhydride, the derivative was purified and assayed for biological activity with the epididymal fat pad system. At the same time that the assay flasks were incubating, 100 μg of the radioactive insulin preparation were injected into the tail vein of an anesthetized 300 g male albino rat. Seven minutes after the injection of the labeled insulin, the rat was sacrificed and samples of skeletal and cardiac muscle, kidney and liver were removed, fixed in Bouin's solution for 24–48 hours, dehydrated in ethanol and embedded in paraffin.

Preparation of Autoradiographs. Sections cut at 5 μ and mounted on glass slides were subsequently deparaffinized in toluene and rehydrated through a series of ethanol solutions of decreasing concentration.

Kodak NTB-2 nuclear track emulsion was melted at 45° C and then maintained at 37° C in a continuous flow water bath. In total darkness, racks of slides were dipped for 30 seconds in the emulsion, drained for five minutes, and placed for two hours in a desiccator containing a saturated atmosphere of hydrogen peroxide in order to oxidize artifactually reduced silver in the emulsion. The slides were dried for three hours in a light-proof box through which a stream of cool air was passed. The emulsion-coated slides were stored in lightproof boxes at −10° C and allowed to expose for 11, 18 and 20 days. Two dippings were made, one of three slides of each tissue, and one of two slides of each tissue, using a fresh lot of emulsion for each dipping. Only slides from the same dipping were compared in evaluating uptake of ^{3}H-insulin.

Following exposure, slides were warmed to 20° C, developed for three minutes in Kodak D-19, rinsed in running water for 30–60 seconds, and fixed in Ansco Rapafix (1:3 dilution) for seven minutes, all in total darkness. The developed slides were washed for 20–30 minutes, placed in 4% formalin for 20 minutes to harden the emulsion, and then washed for an additional ten minutes. The sections were then stained either with Harris' hematoxylin and eosin Y (H&E) or with hematoxylin, phloxine and safran (HPS) (13), dehydrated, cleared in toluene and mounted.

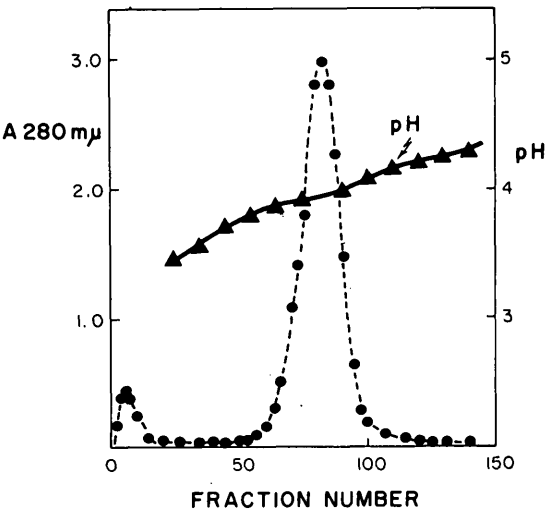


FIG. 1. Elution of a 300 mg sample of PAI-1 from phosphocellulose P-70 under conditions described in the text. The closed circles indicate absorbance at 280 nm and the closed triangles indicate the pH of selected effluent fractions.

Results

Fig. 1 depicts the results of chromatography of the insulin derivative, PAI 1, on phosphocellulose. Phosphocellulose is a useful ion-exchange resin for peptide chromatography (14) and was chosen in this instance because most of the monophosphate groups are fully ionized at pH 3.5. At this pH insulin is bound to the phosphocellulose, but alanine and polyalanine, by-products of the reaction with N-carboxy-D,L-alanine anhydride, pass through the column. It was observed that when the column was developed with high concentrations of ammonium acetate at room temperature, the insulin and polyalanyl insulin derivatives were eluted at

sufficiently low pH values to prevent precipitation of the protein.

Insulin samples with larger amounts of added alanine (*i.e.*, PAI 5–8) were eluted from phosphocellulose at an earlier time than the control insulin samples. Amino acid analyses of material from the mid-portion of the ascending edge of the insulin peak of these PAI derivatives contained approximately 30% more added alanine than material from the mid-portion of the descending edge of the same peak. The content of alanine in the fraction with the highest insulin concentration, as well as that from the pooled material, was approximately the average of these other two values. These findings reflect a moderate degree of heterogeneity among the substituted insulin molecules.

The number of added alanines, calculated from amino acid analyses of each PAI sample, is listed in the second column of Table 1. The third and fourth columns of the Table show the number of added alanines found by analysis of the isolated A and B chains. The results suggest that the alpha amino group of the A chain is slightly more reactive with the N-carboxyanhydride than that of the B chain, which had a portion of its added alanines attached to the lysine residue at position 29. Those lysine residues that had alanines linked to their epsilon amino groups were protected from deamination. Their estimated values are given in Table 2.

TABLE 2. Substitution of alpha and epsilon-amino groups measured in isolated A and B chains of polyalanyl-insulin derivatives (PAI)

Insulin derivative	Percent of NH_2-terminal amino acid substituted with alanine*		Percent of lysine substituted with alanine**
	A-chain	B-chain	
PAI 1	7	8	7
2	26	10	10
3	44	36	13
4	47	25	16
5	74	55	22
6	69	65	34
7	100	76	51
8	93	97	52
9 (control)	0	0	0

* In the control sample (PAI 9) 68% of the available glycine and 91% of the available phenylalanine were removed by a single Edman degradation on the isolated A and B chains. The percentage of NH_2-terminal amino acids substituted with alanine in derivatives PAI 1–8 was calculated from the observed reduction in the release of glycine and phenylalanine following subtractive Edman degradation performed under identical conditions on the isolated A and B chains of the derivatives (10).

** The percent of epsilon amino groups substituted with alanine was determined by amino acid analysis of isolated B chains of the PAI derivatives following deamination with nitrous acid and acid hydrolysis (4).

The results of the Edman degradation performed on A and B chains isolated from the polyalanyl-insulin derivatives are also summarized in Table 2. Glycine and phenylalanine are the NH_2-terminal residues of the A and B chains respectively. In the control sample 0.68 moles/mole of the glycine and 0.91 moles/mole of the phenylalanine were removed despite the insolubility of the oxidized peptide chains. The percent of normal NH_2-terminal groups accessible for removal by the Edman degradation decreased progressively with increasing alanylation. It is apparent that virtually all of the alpha amino groups were substituted in PAI 7 and 8, yet one-half of the lysines remained unreactive. The lesser reaction of the epsilon amino groups may be attributed to their lowered reactivity at pH 7.0.

The results of the epididymal fat pad bioassays (Table 1) indicate that through PAI 5 the insulin derivatives were as active as the standard Boots insulin. The details of data for a representative epididymal fat pad bioassay of a PAI sample in comparison with standard Boots insulin are presented in Table 3. Selected polyalanyl insulin samples were also assayed for their glucose lowering ability in alloxan-treated rats. These samples were found to be biologically active in this experimental system as well as in the fat pad assay. Since the epididymal fat pad assay provided more quantitative data, this method was used for the systematic comparison of the biological activity of the insulin derivatives. The assay system was designed to as-

certain which of the PAI samples, if any, were fully active. Therefore, the data are expressed as the percent of the $^{14}CO_2$ released by an equal amount of standard insulin. When this ratio is less than one (PAI 6–8), it only indicates that the insulin derivative was less active than the control. Precise estimates of the specific biological activity of these less active PAI derivatives would require more extensive testing in view of the logarithmic dose-response relationship of this assay (11,12).

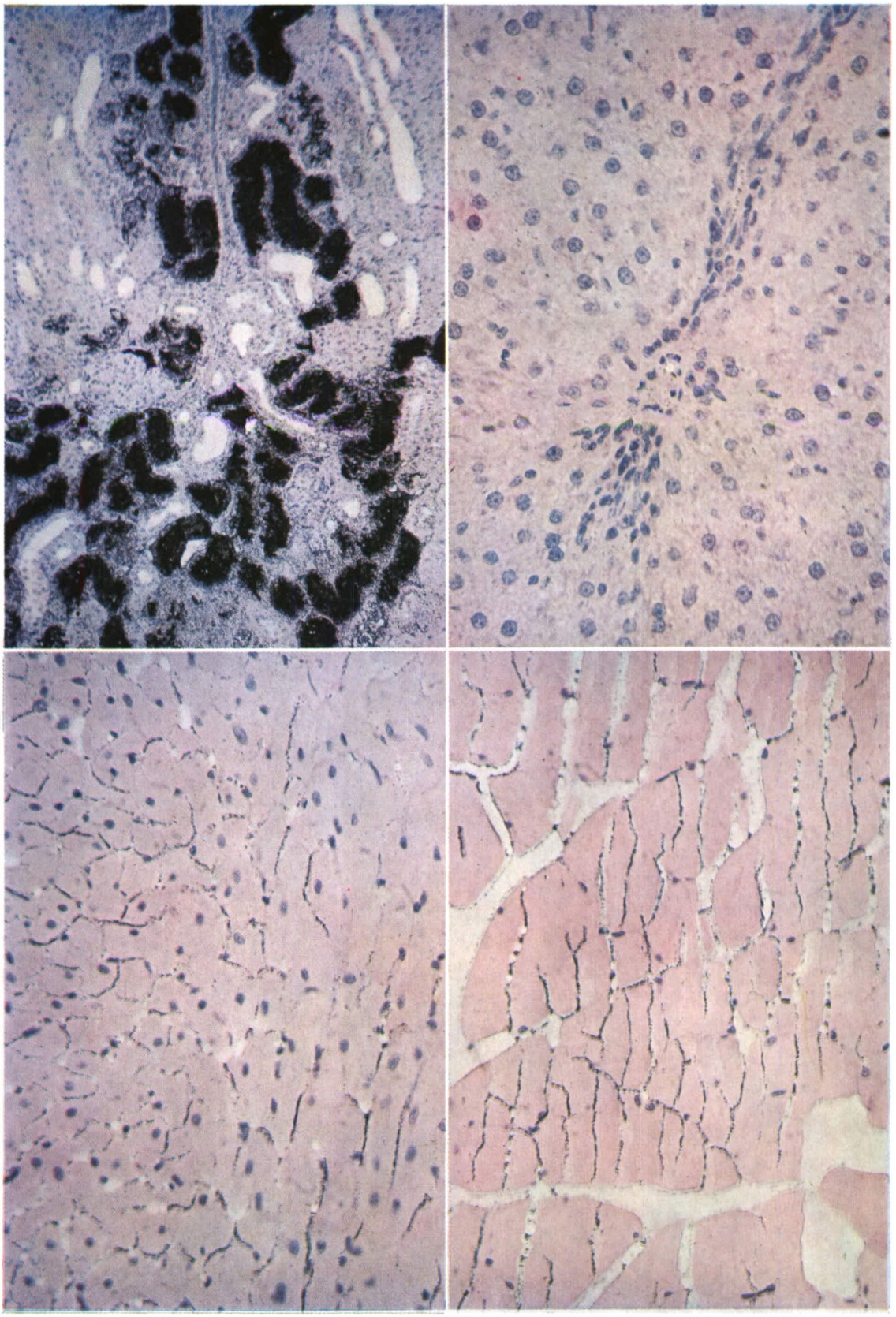
The conditions for the synthesis of tritiated polyalanyl insulin were chosen to result in the addition of two or less moles of alanine per mole of insulin in order to preserve full biological activity. The high specific activity of the tritiated polyalanyl insulin product made it impossible to perform a standard amino acid analysis, but estimates of the specific radioactivity of the tritiated polyalanyl insulin indicated that it was approximately 20 Ci/mM, or the equivalent of one added alanine per mole. Thus the tritiated sample was approximately the equivalent of PAI 2, which would have been expected to be biologically active according to the data shown in Table 1.

TABLE 3. Epididymal fat pad bioassay data for PAI 4

Assay	Preparation*	Fat pad section (mg)	CPM (mg)	PAI 4** STD	Assay	Preparation*	Fat pad section (mg)	CPM (mg)	PAI 4** STD
	Standard	42.9	286			Standard	74.1	248	
1	PAI 4	82.3	266	0.92	11	PAI 4	66.2	232	0.93
	Blank	60.1	35			Blank	92.6	24	
	PAI 4	51.1	256			PAI 4	62.1	251	
2	Blank	67.2	38	1.29	12	Blank	65.9	40	1.33
	Standard	91.9	207			Standard	86.3	198	
	Blank	51.0	55			Blank	92.7	16	
3	Standard	45.7	289	0.81	13	Standard	77.3	112	0.92
	PAI 4	47.4	244			PAI 4	65.8	104	
	Standard	45.6	313			Standard	100.7	117	
4	PAI 4	42.4	305	0.97	14	PAI 4	96.9	110	0.93
	Blank	56.1	43			Blank	103.9	20	
	PAI 4	49.8	238			PAI 4	39.9	197	
5	Blank	65.1	35	1.24	15	Blank	45.5	24	0.88
	Standard	81.5	199			Standard	69.8	220	
	Blank	62.7	42			Blank	55.7	22	
6	Standard	91.4	213	0.88	16	Standard	64.6	196	0.83
	PAI 4	113.5	193			PAI 4	63.0	166	
	Standard	81.6	141			Standard	40.6	133	
7	PAI 4	64.8	151	1.08	17	PAI 4	51.9	166	1.29
	Blank	87.4	22			Blank	34.3	20	
	PAI 4	74.0	192			PAI 4	54.4	167	
8	Blank	97.3	22	1.68	18	Blank	62.1	18	1.62
	Standard	93.0	123			Standard	68.9	110	
	Blank	53.8	34			Blank	69.6	16	
9	Standard	45.1	258	1.29	19	Standard	67.3	165	0.77
	PAI 4	46.6	322			PAI 4	59.5	131	
	Standard	48.1	342			Standard	72.0	146	
10	PAI 4	59.6	317	0.92	20	PAI 4	67.8	173	1.20
	Blank	60.7	32			Blank	98.3	14	

* The sequence of preparations was varied in the assays so that anatomical differences in the epididymal fat pads would be averaged (see Methods).

** The biological activity ratio of the PAI derivative is expressed as the ratio of counts liberated from D-glucose-1-^{14}C by the polyalanyl insulin derivative to the counts liberated from this substrate by an equal weight of the standard insulin after subtraction from each measurement of the counts liberated in a blank containing no insulin.



A & B

C & D

The epididymal fat pad assay data confirmed that the tritiated polyalanyl insulin derivative was biologically active. The value for the ratio of $^{14}CO_2$ released by the ^{3}H-PAI when compared to an equal quantity of standard insulin was 1.04 with a standard error of 0.05. Ten sets of three flasks were used in this assay. The entire experiment was conducted within 24 hours to reduce the possibility that the high degree of specific radioactivity would destroy the biological activity of the insulin.

The autoradiographic preparations were intended to determine the feasibility of using preparations of tritiated polyalanyl insulin with this high level of specific activity to localize the hormone in various tissues. The results indicate that the method is a useful one.

Fig. 2 shows the extent and localization of labeling in four tissues, kidney, liver, cardiac muscle and skeletal muscle, fixed seven minutes after intravenous injection of the tritiated insulin.

In the renal cortex (Fig. 2A) the major portion of the labeled material is found to have cleared the glomeruli and localized in the first portions of the proximal convoluted tubules. The loop of Henle and the distal convoluted tubules are unlabeled. The extent of clearance of the ^{3}H-insulin by the kidney in the seven minute period is quite evident.

The liver, on the other hand (Fig. 2B), shows only slight labeling. This labeling is restricted to the region immediately surrounding the portal vein and to a few sinusoids radiating from it. The hepatocytes, hepatic venules and canalicular regions are unlabeled.

Both heart muscle (Fig. 2C) and skeletal muscle (Fig. 2D) show extremely clear the apparently specific localization of the label at the level of the sarcolemma. In both cross-sections and longitudinal-sections the individual muscle fibers are outlined by the silver grains of the autoradiograph. The absence of silver grains over the blood vessels in the muscle sections emphasizes the efficiency of clearance by the glomeruli (Fig. 2A). In addition, despite the high specific activity of the ^{3}H-PAI, there is very low background in the autoradiographs.

Discussion

In order for a polypeptide hormone to begin its action, the hormone's three-dimensional structure must be recognized by a specific receptor site in the target cell. If the hormone is bound at cellular sites of action for a period of several minutes (in contrast to the more rapid turnover in enzyme-substrate interactions), the location of these receptor sites may be determined by use of radioactive polypeptide hormones. Such attempts at localization would require that the labeled hormone have full biological

←

FIGS. 2A–D. Autoradiographs prepared from representative sections of kidney, liver, cardiac muscle and skeletal muscle of a rat sacrificed seven minutes after intravenous administration of 100 μg of ^{3}H-poly alanyl insulin.

(**A** top, left) The heaviest labeling is found in the kidney. In the renal cortex the major portion of the labeled material is shown to have cleared the glomeruli and to be localized in the proximal tubules. The distal tubules and the ascending limb of the loop of Henle are unlabeled. ×100.

(**B** top, right) In the liver there is very little labeling. This labeling is restricted to the region immediately surrounding the portal vein and to a few sinusoids radiating from it. Hepatocytes, hepatic venules and canaliculi are unlabeled. ×250.

(**C** bottom, left) Cardiac muscle and (**D** bottom, right) skeletal muscle show extremely clear and apparently specific localization of the ^{3}H-PAI at the sarcolemma. The individual muscle fibers are outlined by the silver grains. The absence of silver grains over the blood vessels in these muscle sections emphasizes the efficiency of renal clearance indicated by the absence of label over the glomeruli in Fig 2A. There is also extremely low background and no diffuse labeling despite the very high specific activity of the ^{3}H-PAI. ×250.

activity as well as sufficient specific radioactivity to permit experiments with reasonable pharmacologic doses.

Because there is considerable knowledge concerning the chemistry and mode of action of insulin (15), many studies with radioactive insulin have been attempted (16–21). Most of these studies, however, have used iodinated insulin. There is doubt whether such labeled molecules retain full biological activity, so there is reason to evaluate other methods of preparing labeled protein hormones. Arquilla *et al.* have reviewed the evidence concerning the effect of iodination and report that purified iodoinsulin derivatives have a markedly attenuated biological activity (22). A recent report suggests that the technique of preparing iodinated insulin may account for the loss of biological activity (23), and iodinated insulin preparations have been successfully employed in studies of membrane binding in fat cells and liver (23–25).

As noted earlier, the addition of polyalanyl side chains to the amino groups of antibodies and enzymes frequently produces little, if any, alteration in the biological action of these molecules. Presumably, the hydrophilic polyalanyl side chains which are added to amino groups at the surface of the protein serve to complement the existing hydrophilic exterior shell of the molecule (26). In contrast, the tyrosine residues are frequently a part of the hydrophobic interior of the protein where there is no free space, so that the addition of a bulky iodine atom might be disruptive to the architecture of the protein. Thus, the addition of radioactive atoms by reaction with the N-carboxy-D,L-alanine anhydride offers some theoretical appeal (7). In addition to the insulin studies reported here, biologically active preparations of polyalanyl human growth hormone (7) and of polyalanyl human chorionic gonadotropin (27) have been prepared. However, the studies with polyalanyl enzymes and antibodies involved molecules in which the biological activity was measured against soluble substrates or antigens. It is possible that the polyalanyl side chains on hormones could inhibit their "fit" into a receptor site at the surface of the cell, leaving open the possibility that there may be a limit to the total number of alanine residues that can be added.

In 1953 Fraenkel-Conrat showed that insulin would react with the N-carboxyanhydride of leucine and, while no specific bioassay was reported, he noted that the insulin derivative possessed some activity (28). The development of techniques to prepare tritiated alanine with a high specific radioactivity by reduction of N-acetyldehydroalanine made it possible to design experiments using this amino acid. The D,L alanine mixture is customarily employed to prevent the formation of side chain alpha helices which might reduce the solubility of the modified protein. Another advantage of the D,L polyalanyl side chain is that circulating exopeptidases are not likely to release the added alanines (29).

The results of the bioassay of unlabeled polyalanyl insulin derivatives indicated that up to 3.75 moles of alanine could be added per mole of insulin (PAI-5) without reduction of the biological activity of the hormone when it was compared to the starting material. The A chains consistently received more alanine than the B chains, despite the fact that the latter possessed two types of reactive sites. The control insulin sample that was subjected to all the procedures including the addition of dioxane did not lose biological activity when compared with the starting material.

The Edman degradation data for the isolated A and B chains indicated that the majority of the NH_2-terminal glycine and phenylalanine residues in PAI-5 were protected from removal by the addition of alanines. It is concluded that at least 74% of the A chains of insulin molecules in PAI-5 possessed at least one added alanine residue indicating that insulin molecules with minimal alanine addition are biologically active. Furthermore, since over half of the B chains in PAI-5 were also shown to be substituted

with alanine, it is likely that over one half of those insulin molecules that did not have alanine added to the A chain (*i.e.*, 26%) had at least one added alanine on their B chains. Therefore, the percentage of substituted insulin molecules in PAI-5 is probably close to 87%, and the remaining 13% of unsubstituted molecules are insufficient to account for the finding of full biological activity in the sample (11,12).

Once it was established that biologically active polyalanyl insulin derivatives could be prepared, the second step was to synthesize a tritiated polyalanyl insulin and to evaluate such a derivative in autoradiographic localization studies.

While the unlabeled N-carboxyanhydride of D,L alanine is commonly available, the tritiated reagent is not. However, techniques to prepare tritiated alanine with high specific activity have been developed in studies of labeled antigens. These techniques were duplicated on a reduced scale for the insulin studies described here (8). Several pilot experiments were conducted until conditions were established for the synthesis of a polyalanyl insulin derivative that possessed between one and two moles of labeled alanine per mole of insulin. These conditions were then employed using the high specific activity, tritiated D,L alanine (20 Ci/mM). A determination of the specific radioactivity of the purified product indicated that it had received approximately one added mole of alanine per mole of insulin. This figure must be taken as approximate because it is based on the vendor's estimate of the specific activity of the tritiated alanine and also because multiple dilutions were necessary to obtain satisfactory concentrations to determine radioactivity. However, the estimates of concentrations of the tritiated polyalanyl insulin for the bioassay and tail vein injection were accurate because they were based on the optical density of the ^{3}H-PAI solution measured at 280 nm.

The autoradiographs shown in Fig. 2 indicate that tritiated polyalanyl insulin derivatives can be used effectively for such studies. Because of the low energy of its beta decay, tritium provides excellent resolution for autoradiography (30,31). Equally clear results probably would have been obtained if the animal had received 10 μg of ^{3}H-PAI, *i.e.*, an amount closer to a physiological level.

While the results illustrated in Fig. 2 are primarily intended to demonstrate the feasibility of this method for preparing labeled insulin, they also provide an interesting comparison with earlier studies which employed iodinated insulin.

Several investigators have reported that iodinated insulin molecules are concentrated by kidney proximal tubule cells, and have concluded that the labeled insulin is filtered at the glomerulus and reabsorbed in the proximal tubule (16,19,21). Presumably the appearance of the labeled insulin molecules in the glomerular filtrate is a function of molecular size and would occur whether or not the insulin derivative was biologically active. Thus, proximal tubule localization of grains in Fig. 2A is consistent with these earlier autoradiographic studies and also with other studies concerning the role of the kidney in insulin metabolism (32,33). The tritiated insulin, however, gives a much clearer autoradiograph than iodinated insulin even where the ^{3}H-PAI is present in such high concentrations as shown in Fig. 2A.

The almost uniform distribution of grains along the sarcolemmal membranes in the cross-section of the rat's striated muscle and in the section of cardiac muscle provides graphic confirmation of the cell fractionation data of Edelman and Schwartz (20). These authors observed that virtually all of the ^{131}I-insulin in rat striated muscle could be found attached to sarcolemmal tubules or released into a soluble fraction. This pattern of the ^{3}H-PAI labeling contrasts with the diffuse localization obtained by previous workers with ^{131}I- or ^{125}I-insulin (17–19). This diffuse labeling had been interpreted by some workers as evidence for intracellular penetration of the iodinated insulin (18). In addition to the autoradiographic and bio-

chemical data which indicate that insulin is bound and acts at membrane in muscle cells, Cuatrecasas has shown that insulin covalently bound to Sepharose is capable of interacting with superficial membrane structures of isolated fat cells to initiate transport and other metabolic functions (34).

Since insulin normally reaches the liver via the portal circulation and since the labeled insulin was administered in this study by injection into the peripheral circulation, it is difficult to interpret the paucity of grains in the liver section. Diminished liver uptake of insulin following peripheral injection has been reported (35). It should be noted in this regard, that in the experiments reported here all tissues were taken from the same animal at a single time point so that observations of the relative distribution of labeling are valid.

The present study demonstrates, therefore, that a biologically active tritiated polyalanyl insulin (^{3}H-PAI) can be synthesized and used effectively in autoradiographic studies of the cellular localization of this polypeptide hormone.

Acknowledgments

The authors are indebted to Dr. Michael Sela for many useful discussions and to Dr. Israel Schechter for the gift of N-acetyldehydroalanine.

References

1. Stahmann, M. A. (ed.), Polyamino Acids, Polypeptides and Protein, University of Wisconsin Press, Madison, Wisconsin, 1962.
2. Katchalski, E., M. Sela, H. I. Silman, and A. Berger *In* H. Neurath (ed.), The Proteins, Vol. II, Academic Press, Inc., New York, 1964, p. 405.
3. Fuchs, S., and M. Sela, *J Biol Chem*, **240**: 3558, 1965.
4. Anfinsen, C. B., M. Sela, and J. P. Cooke, *J Biol Chem* **237**: 1825, 1962.
5. Epstein, C. J., C. B. Anfinsen, and M. Sela, *J Biol Chem* **237**: 3458, 1962.
6. Edelman, I. S., E. Hoffer, S. Bauminger, and M. Sela, *Arch Biochem Biophys* **123**: 211, 1968.
7. Canfield, R. E., *Ann NY Acad Sci* **148**: 293, 1968.
8. Humphrey, J. H., B. A. Askonas, I. Auzins, I. Schechter, and M. Sela, *Immunology* **13**: 71, 1967.
9. Hirs, C. H. W., *J Biol Chem* **219**: 611, 1956.
10. Canfield, R. E., *J Biol Chem* **238**: 2698, 1963.
11. Renold, A. E., D. B. Martin, Y. M. Dagenais, J. Steinke, R. J. Nickerson, and M. C. Sheps, *J Clin Invest* **39**: 1487, 1960.
12. Sheps, M. C., R. J. Nickerson, Y. M. Dagenais, J. Steinke, D. B. Martin, and A. E. Renold, *J Clin Invest* **39**: 1499, 1960.
13. Bencosme, S. A., *Am J Clin Path* **24**: 1324, 1954.
14. Canfield, R. E., and C. B. Anfinsen, *J Biol Chem* **238**: 2684, 1963.
15. Levine, R., *Am J Med* **40**: 691, 1966.
16. Elgee, N. S., R. H. Williams, and N. D. Lee, *J Clin Invest* **33**: 1252, 1954.
17. Narahara, H. T., N. B. Everett, B. S. Simmons, R. H. Williams, *Am J Physiol* **192**: 227, 1958.
18. Stein, O., and J. Gross, *Endocrinol* **65**: 707, 1959.
19. Worthington, W. C., Jr., D. J. Jones, and M. G. Buse, *Endocrinol* **74**: 914, 1964.
20. Edelman, P. M., and I. L. Schwartz, *Am J Med* **40**: 695, 1966.
21. Beck, L. V., and N. Fedynskyj, *Endocrinol* **81**: 475, 1967.
22. Arquilla, E. R., H. Ooms, and K. Mercola, *J Clin Invest* **47**: 474, 1968.
23. Freychet, P., J. Roth, and D. M. Neville, Jr., *Biochem Biophys Res Commun* **43**: 400, 1971.
24. Cuatrecasas, P., Abstracts *Am Soc Clin Invest* 1971, p. 22a.
25. House, P. D. R., and M. J. Weidemann, *Biochem Biophys Res Commun* **41**: 541, 1970.
26. Dickerson, R. E., and I. Geis, The Structure and Action of Proteins, Harper & Row, New York, 1969.
27. Eshkol, A., *In* Karolinska Symposia on Research Methods in Reproductive Endocrinology: First Symposium: Immunoassay of Gonadotropins, E. Diczfalusy (ed.), p. 145, Stockholm, 1969.
28. Fraenkel-Conrat, H., *Biochim Biophys Acta* **10**: 180, 1953.
29. Schechter, I., and A. Berger, *Biochemistry* **5**: 3371, 1966.
30. Taylor, J. H., P. S. Woods, and W. L. Hughes, *Proc Nat Acad Sci* **43**: 122, 1957.
31. Stumpf, W. E., *J Histochem Cytochem* **18**: 21, 1970.
32. Rubenstein, A. H., and I. Spitz, *Diabetes* **17**: 161, 1968.
33. Rabkin, R., N. M. Simon, S. Steiner, and J. A. Colwell, *New Eng J Med* **282**: 182, 1970.
34. Cuatrecasas, P., *Proc Nat Acad Sci* **63**: 450, 1969.
35. Madison, L. L., B. Combes, R. H. Unger, and N. Kaplan, *Ann NY Acad Sci* **74**: 548, 1959.