The Preparation and Immunological Properties of ^{131}I -Labelled Adrenocorticotrophin

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1. A procedure is described for preparing ^{131}I -labelled adrenocorticotrophin suitable for use in radioimmunoassay. 2. Adsorption of labelled and unlabelled adrenocorticotrophin at low concentrations occurs to various surfaces despite the presence of diluent protein. Adsorption and desorption errors are minimized by low pH and by the use of polystyrene vials. 3. Preparations with low initial damage are obtained if the radioiodination is performed rapidly and the separation of ^{131}I -labelled adrenocorticotrophin from unchanged [^{131}I]iodide is carried out on cellulose columns by using dilute acid. 4. The immunological activity of ^{131}I -labelled α^{1-24} -adrenocorticotrophin, but not of ^{131}I -labelled porcine adrenocorticotrophin, decreases with increasing specific radioactivity. The involvement of tyrosine residues in the immunological specificity of the α^{1-24} -adrenocorticotrophin only is suggested to explain this finding.

The radioimmuno-electrophoretic technique, first developed for the assay of plasma insulin (Yalow & Berson, 1960), requires the routine preparation of labelled peptide hormones of high specific radioactivity. The radioiodination method developed for human growth hormone (Hunter & Greenwood, 1962; Greenwood, Hunter & Glover, 1963) has been applied to a number of peptide hormones. Experiences with this method have been summarized and reviewed (McFarlane & Greenwood, 1966; Greenwood, 1967); it seems clear that both the amount of radioactive iodine transferred to the peptide (yield), and hence the specific radioactivity of the product, and the degree of damage sustained by the peptide varies between different batches of radioactive iodine and between different peptides labelled with the same batch. The quality of the radioactive iodine (isotope abundance) and of the peptide samples (electrophoretic heterogeneity) have been studied as two factors that can lead to low specific radioactivity and high damage (Yalow & Berson, 1966). In practice the specific radioactivity of the product is rarely limiting. However, the degree of damage sustained by a peptide during its radioiodination is critical; thus the precision, sensitivity and specificity of the radioimmunoassay are impaired and this may be compounded by further damage to the labelled peptide during storage and during exposure to plasma. Radioimmunoassay requires only a small fraction (1–2%) of the amount of labelled peptide that is usually

prepared and therefore purification procedures can be used that remove much undamaged labelled peptide provided that they remove all, or the great majority, of damaged labelled hormone.

ACTH* is a straight-chain polypeptide consisting of 39 amino acids. The immunological activity and species specificity resides in positions 25–32 (Li, 1961). Its biological activity, in the first 24 *N*-terminal amino acids, is common to all species studied. Thus biological and immunological measurements of activity *in vitro* and *in vivo* may differ (Imura, Sparks, Grodsky & Forsham, 1965; Fleischer, Givens, Abe, Nicholson & Liddle, 1966). A radioimmunoassay method for the first 24 *N*-terminal amino acids (α^{1-24} -ACTH; Synacthen) was developed to avoid this discrepancy in studying hormone production by bronchial neoplasms (Landon, Crewe & Greenwood, 1967). The method was based on that reported for plasma ACTH (Yalow, Glick, Roth & Berson, 1964). Difficulties were encountered in the preparation of ^{131}I -labelled ACTH by the method of Greenwood *et al.* (1963). The present paper reports the modifications introduced, based upon experience shared with Dr R. S. Yalow and Dr S. A. Berson, for the preparation of ^{131}I -labelled ACTH suitable for radioimmunoassay. For this purpose a preparation is required that contains about 50 ng. of labelled hormone having a specific radioactivity from $100 \mu\text{Ci}/\mu\text{g.}$ upwards, that is initially less than 5% damaged and that

* Abbreviation: ACTH, adrenocorticotrophin.

does not contain more than 10% of damaged labelled peptide after 2 weeks of storage.

MATERIALS

Buffers. All buffers were sodium salts. Unless otherwise specified the solvent used to dilute ^{131}I -labelled ACTH was 0.05 M-phosphate buffer, pH 7.5, containing 5 mg. of human serum albumin/ml. and 0.5% mercaptoethanol (Yalow *et al.* 1964). This is referred to as diluent buffer.

Adrenocorticotrophin. The classification for the various types of ACTH used in this study is that due to Li (1959). The types of ACTH studied were chromatographically purified α_p - and α_h -ACTH (porcine and bovine ACTH respectively, supplied by Organon Laboratories), whose biological activities were 100–120 i.u./mg., α_h -ACTH (human ACTH prepared by the method of de Jager, Homan & de Wied, 1963) with a biological activity 40–50 i.u./mg., and synthetic α_p -ACTH and synthetic α^{1-24} -ACTH (Synacthen), both supplied by Ciba Laboratories Ltd., Basle, Switzerland.

^{131}I - and ^{125}I -Iodide. Samples were obtained from The Radiochemical Centre, Amersham, Bucks., or from Isoerve, Cambridge Nuclear Corp., Boston, Mass., U.S.A.

Albumin. Human serum albumin was a gift from the Blood Products Laboratory, Lister Institute of Preventive Medicine, London, S.W. 1.

METHODS

Preparation of ^{131}I -labelled ACTH

The reaction was carried out in a polystyrene vial to reduce losses of ACTH by adsorption. To the sample of radioactive iodide (0.8–1.0 mc) was added ACTH (2 μg .) and chloramine-T (100 μg .), each in 10 μl . of 0.05 M-phosphate buffer, pH 7.5. After brief mixing, sodium metabisulphite (200 μg . in 10 μl . of 0.05 M-phosphate buffer, pH 7.5) was added to stop the reaction by converting any residual ^{131}I iodine into ^{131}I iodide. Then 50 μl . of 0.1 N-HCl was added by a hooked pipette (50 μl . capacity, E-MIL Gold Line) and mixed, and 50 μl . of the mixture was withdrawn and transferred immediately, with the same pipette, to a dry cellulose column (0.5 g. of Whatman ashless cellulose powder). A larger proportion of the reaction mixture was unnecessary and may require an increased weight of cellulose and hence a slower procedure. The column was eluted discontinuously with two additions of diluent buffer (each of 2 ml.) and one of 2 ml. of 0.1 N-HCl. Compressed air was applied to speed filtration. The two diluent buffer eluates were each collected in vials containing 3 ml. of diluent buffer (W1 and W2). The HCl eluate containing ^{131}I -labelled ACTH was collected into vial S1 (3 drops), S2 (6 drops) and S3 (remainder). All sample vials were surrounded by ice and contained diluent buffer such that the final volume was 5 ml. Finally the cellulose of the column was dislodged into a vial and any remaining adsorbed ^{131}I -labelled ACTH eluted with 1 N-HCl such that the final volume was 5 ml. (column vial).

Separation of damaged and undamaged ^{131}I -labelled ACTH and of ^{131}I iodide

Separation was carried out by chromatoelectrophoresis on filter paper, which differs in several ways from classical

paper electrophoresis (Berson, Yalow, Baumann, Rothschild & Newerley, 1956). A number of variables influencing optimum separation may be mentioned.

The capacity of filter paper to adsorb small amounts of ^{131}I -labelled protein hormones in the presence of plasma proteins varies between batches and between different hormones. Buffer flow to the centre of the strip and hence rate of separation is dependent on the applied voltage, is decreased at low temperature and is influenced by the ionic strength of the buffer. Separation is not influenced by the position of the strips but faster separations are obtained when the sample is applied nearer the cathodal end of the strip.

All samples to be analysed were in 0.05 M-phosphate buffer, pH 7.5, containing 5 mg. of human serum albumin/ml. The addition of more protein did not improve separation and if more than 25 μl . of plasma was added to the sample before chromatoelectrophoresis some elution of undamaged ^{131}I -labelled ACTH from the line of application occurred.

As a result of studies of these variables, chromatoelectrophoretic separation of undamaged ^{131}I -labelled ACTH, damaged ^{131}I -labelled ACTH and ^{131}I iodide was carried out as follows. Twelve strips of Whatman 3MC paper (2.5 cm. $\times$ 44 cm.), after being moistened with 0.06 M-barbitone buffer, pH 8.6, were laid across a horizontal Perspex frame (38 cm. $\times$ 38 cm.) supported on cross-struts of nylon at 2 cm. intervals. The ends of the strips were placed in electrode tanks (36 cm. $\times$ 6 cm.), each filled with 500 ml. of 0.06 M-barbitone buffer, pH 8.6, and the bridge gap was 40 cm. A portion (10–100 μl .) of the sample was applied along a line 10 cm. from the centre towards the cathode. A constant voltage (500 v) was applied, and separation was carried out at room temperature for 60 min. The strips were air-dried, cut into 1 cm. lengths towards the anode, starting at 2 cm. behind the origin, and radioactivity was determined with a Packard autogamma counter.

Undamaged ^{131}I -labelled ACTH remained adsorbed to the paper at its site of application, damaged ^{131}I -labelled ACTH migrated with the albumin or plasma proteins or both towards, but never beyond, the centre, and free ^{131}I iodide moved beyond the centre of the strip. Fig. 1 illustrates the distribution of the three components after chromatoelectrophoretic separation for 60 min. of a sample of an initial reaction mixture and of one of the column eluates with and without the addition of excess of anti-serum. Separation was reproducible so that the percentage of undamaged ^{131}I -labelled ACTH was always determined by counting a 5 cm. length towards the anode, starting from 2 cm. behind the origin, the percentage of damaged ^{131}I -labelled ACTH by counting the next 7 cm. length towards the anode and the ^{131}I iodide peak by counting a further 7 cm. length towards the anode. In a series of replicate experiments the distribution of radioactivity varied by less than 3% with this technique.

Yield of reaction, specific activity and concentration of ^{131}I -labelled, damaged and undamaged ACTH

The yield of reaction, specific radioactivity and concentration of ^{131}I -labelled ACTH were calculated from the distribution of radioactivity after radioiodination and from

Fig. 1. Behaviour on chromatoelectrophoresis of damaged and undamaged ¹³¹I-labelled α_P -ACTH, and of [¹³¹I]iodide and of undamaged ¹³¹I-labelled α_P -ACTH after its reaction with antibody. Samples (10 μ l.) were applied to paper strips at the point indicated by the broken line near the cathode (—). After chromatoelectrophoresis for 1 hr. the strips were scanned for radioactivity. (a) Scan obtained from the initial radioiodination mixture and showing peaks of undamaged ¹³¹I-labelled ACTH, damaged ¹³¹I-labelled ACTH and [¹³¹I]iodide respectively from the origin; (b) scan obtained after purification with a cellulose column (fraction S2) and showing undamaged ¹³¹I-labelled ACTH alone; (c) scan obtained after incubating undamaged ¹³¹I-labelled ACTH (60 pg.) with anti-(α_P -ACTH) serum (final dilution 1:500).

the separation of damaged and undamaged ¹³¹I-labelled ACTH and of [¹³¹I]iodide.

To count a wide range of radioactivity the counting geometry on a Panax scintillation counter (USC-B) was modified such that 1 mc of [¹³¹I]iodide gave approx. 250 counts/sec. (geometry A) or approx. 3800 counts/sec. (geometry B). The following description of the calculation of yield, etc., is based upon the example given in Table 1, of a radioiodination of synthetic α_P -ACTH.

After the addition of [¹³¹I]iodide the reaction vial gave 307 counts/sec. (geometry A) and was therefore calculated to contain 1208 μ C. After the radioiodination of ACTH (2 μ g.) a proportion of the reaction mixture was transferred to the cellulose column. This proportion can be calculated by difference after measurement of the residual radioactivity in the vial and in the transfer pipette, in this instance 307—(118+15) counts/sec. Neither ¹³¹I-labelled ACTH nor [¹³¹I]iodide was significantly adsorbed to the reaction vial or transfer pipette. Provided that mixing is adequate, the percentage transfer (56.7%) of radioactivity represents a transfer of 1.134 μ g. of ACTH and of 0.685 mc of [¹³¹I]iodide respectively. The relative proportion of the radioactivity present as ¹³¹I-labelled ACTH, both damaged and undamaged, and as [¹³¹I]iodide, was determined by

chromatoelectrophoresis of a portion of the remaining reaction mixture. In this instance 86.5% of the radioactivity was peptide-bound; thus the yield of reaction was 86.5% and the calculated specific radioactivity was 522 μ C/ μ g.

The total radioactivity in each of the eluates from the cellulose column and in the column residue were counted in 5 ml. volumes by geometry B and may be summed (2648 counts/sec.). The amount of radioactivity in each fraction due to ¹³¹I-labelled ACTH was calculated from its total radioactivity and its percentage distribution, determined by chromatoelectrophoresis of a sample. The radioactivity in each fraction due to ¹³¹I-labelled ACTH may be summed (2358 counts/sec.) and expressed as a yield of reaction (2358/2648 $\times$ 100%) and specific radioactivity (534 μ C/ μ g.) as a check on the previous calculated values.

The amount of ¹³¹I-labelled ACTH in each 5 ml. fraction was calculated from the radioactivity due to ¹³¹I-labelled ACTH in the fraction, from the sum of the radioactivity associated with ¹³¹I-labelled ACTH (2358 counts/sec.) and from the amount of ACTH transferred to the column (1.134 μ g.), e.g. fraction S1 contained 0.06 μ g. of ¹³¹I-labelled ACTH in 5 ml. [(1.134 $\times$ 125)/2358]. From the chromatoelectrophoretic data, this fraction contained only a small percentage of damaged ¹³¹I-labelled ACTH (1.2%) and was suitable for a subsequent radioimmunoassay. Since 60 pg. amounts are used in radioimmunoassay the loss of undamaged peptide during purification was not limiting.

It has been assumed that the calculated specific radioactivity of the ¹³¹I-labelled ACTH was uniform. It is more likely that a range of molecules was obtained with increasing amounts of total iodine. The calculated specific radioactivity is therefore a mean figure. The influence of the degree of iodination on the appearance as damaged ¹³¹I-labelled ACTH was not studied and the variation in percentage damage from W1 to S3 may represent some resolution of ACTH with different degrees of iodination.

Separation of ¹³¹I-labelled ACTH from ¹³¹I-labelled ACTH bound to antibody

Chromatoelectrophoresis was used to separate free ¹³¹I-labelled ACTH from labelled hormone bound to antibody in obtaining the antibody titration and standard curves from incubated mixtures of ¹³¹I-labelled ACTH, anti-ACTH serum and ACTH. The antibody-bound hormone fraction moves from the origin as γ -globulin and by this technique is indistinguishable from any damaged ¹³¹I-labelled ACTH that may be present after incubation of ¹³¹I-labelled ACTH. Control incubation mixtures were set up containing labelled hormone and diluent buffer in the absence of added antibody to correct for this type of damage. Results were not considered acceptable if the amount of damaged ¹³¹I-labelled ACTH exceeded 10% at the end of the period of incubation.

Preparation and titration of anti-ACTH serum

Antisera to natural α_P -ACTH and to synthetic α^{1-24} -ACTH were obtained in guinea pigs by weekly subcutaneous injections of the antigen (0.25 mg. in 1 ml. of 0.05 M-phosphate buffer, pH 7.5, with an equal volume of Freund's complete adjuvant; Difco Laboratories). Animals were

Table 1. *Counting schedule in the preparation of ^{131}I -labelled ACTH*

α_p -ACTH (2 $\mu\text{g.}$) was radioiodinated with 1.208 mc of [^{131}I]iodide. Details are given in the text. All samples were counted for 100 sec. and corrected for dead time, background and volume.

Geometry A		Radioactivity (counts/sec.)	
Sodium [¹³¹ I]iodide (1.208 mc in vial)		307	
Reaction vial after transfer		118	
Transfer pipette		15	
Column load (calc.)		174	
% transfer, 56.7 : 1.134 μg. of ACTH; 0.685 mc of [¹³¹ I]iodide			
Geometry B		Radioactivity due to ¹³¹ I-labelled ACTH (%)	Radioactivity due to ¹³¹ I-labelled ACTH (counts/sec.)
Eluate (5 ml.)	Total radioactivity (counts/sec.)		
W1	1577	83.6	1318
W2	269	97.0	261
S1	127	98.4	125
S2	46	96.9	46
S3	48	98.0	47
Column wash	581	96.7	561
Total	2648		2358

Chromatoelectrophoretic analyses

Radioactive peak	% of total radioactivity in each peak						Column wash
	Reaction mixture	W1	W2	S1	S2	S3	
Undamaged ^{131}I - labelled ACTH	66.3	73.4	91.2	97.2	94.1	94.4	82.7
Damaged ^{131}I -labelled ACTH	20.2	10.2	5.8	1.2	2.8	3.6	14.0
[^{131}I]Iodide	13.5	16.4	3.0	1.6	3.1	2.0	3.3

Yield 86.5%. Sp. radioactivity 522 $\mu\text{C}/\mu\text{g.}$, calc. from the analysis of the reaction mixture.

bled by cardiac puncture after a variable number of injections (4–12). Blood (4 ml.) was heparinized and centrifuged and the plasma stored at -20° until used. The immunological activity of the ^{131}I -labelled ACTH preparations and of the guinea-pig antisera were determined by constructing antibody-titration curves. Doubling dilutions of an appropriate antiserum were set up by using diluent buffer with a final volume of 200 $\mu\text{l.}$ in polystyrene assay tubes (6 cm. $\times$ 1 cm.; Lockham Ltd.). A volume (10 $\mu\text{l.}$) of diluent buffer containing a constant amount of ^{131}I -labelled ACTH (usually 60 pg.) was then added to each tube. One tube containing labelled peptide and diluent buffer only was set up with each series to enable a correction to be applied for damage. After incubation for 4 days at 4° , 100 $\mu\text{l.}$ of the contents of each tube was spotted on Whatman 3MC paper, separated by chromatoelectrophoresis and counted as described previously.

Inhibition of the reaction between ^{131}I -labelled ACTH and anti-ACTH serum by unlabelled ACTH

Doubling dilutions of unlabelled ACTH were set up by using diluent buffer with a final volume of 200 $\mu\text{l.}$ in polystyrene assay tubes. To each tube was added 10 $\mu\text{l.}$ of diluent buffer containing a constant amount of ^{131}I -labelled

ACTH (usually 60 pg.) followed by 50 $\mu\text{l.}$ of an appropriate dilution of antiserum in 0.05M-phosphate buffer, pH 7.5, containing 2.5 mg. of human serum albumin/ml. Two further tubes were set up with each series, of which one contained labelled peptide and diluent only, to allow a correction for damage and the other labelled peptide and antibody to determine the percentage of ^{131}I -labelled ACTH bound in the absence of unlabelled hormone. After incubation for 4 days at 4° , 100 $\mu\text{l.}$ of the contents of each tube was separated by using chromatoelectrophoresis and the strips were counted as described previously.

RESULTS

Adsorption of α_p -ACTH and α^{1-24} -ACTH to different surfaces

The loss by adsorption of peptide hormones at low concentrations to glass surfaces is well known and the addition of a higher concentration of another protein is standard practice. However, unlabelled and ^{131}I -labelled α_p -ACTH or α^{1-24} -ACTH (50 pg./ml.) in solutions containing 2.5 mg. or 5.0 mg. of human serum albumin/ml. in 0.05M-phosphate buffer, pH 7.4, were found to become

progressively adsorbed to glass with resultant losses which ranged from 10 to 30% over 1–48 hr. Higher concentrations of added protein or siliconized glass were without significant effect, but losses were shown to be lower in polythene or polystyrene containers (maximum loss $4.4 \pm 0.4\%$). Adsorbed peptide was eluted by dilute acid (0.02N-hydrochloric acid–0.15M-sodium chloride) and storage in this solution minimized losses ($5.6 \pm 2.6\%$). Cumulative losses, without the precautions of acidification, in making and transferring standard solutions of α^{1-24} -ACTH amounted to some 75% in one experiment. A greater loss on storage of ^{131}I -labelled α^{1-24} -ACTH than of ^{131}I -labelled α_{p} -ACTH was observed in the diluent buffer on to glass whereas damaged ^{131}I -labelled ACTH was adsorbed less readily.

Radioiodination of ACTH

The procedure described by Hunter & Greenwood (1962) for growth hormone, which employs a Sephadex column for purifying the reaction mixture, was unsatisfactory for ACTH because 32–62% of the labelled hormone produced was damaged. Improved purification and lower initial damage was obtained with a cellulose column from which the labelled hormone was eluted with fresh human plasma (Yalow & Berson, 1960).

With the procedure described in the Methods section, reaction in a polystyrene vial diminished the initial adsorption of hormone and enabled the amounts of ACTH and ^{131}I iodide to be limited to 1–2 μg . and approx. 1 mc respectively. More than 98% of the radioactivity in a reaction vial was removed by two washes of diluent buffer (2 ml. each). The addition of 0.1N-hydrochloric acid after radioiodination prevented significant losses of labelled peptide to the transfer pipette. Undamaged ^{131}I -labelled ACTH was effectively eluted by 0.1N-hydrochloric acid from the cellulose column and this avoided the requirement for fresh plasma. The main advantage, however, was a marked reduction in the rate of progressive damage of the labelled hormone in the sample eluate as compared with the plasma eluates. This finding suggests that the progressive damage previously noted was probably due to degradation by plasma enzymes rather than radiation damage.

With improved control of adsorption and damage, attempts were made to increase the yield of the reaction and hence the specific radioactivity of the product. Two factors suggested by the experiences of Dr S. A. Berson and Dr R. S. Yalow (personal communication) increased the yield, namely the addition of ACTH to the isotope sample before the addition of chloramine-T, and a reduction in the volume of the reactants. Hence to 1 mc of sodium

^{131}I iodide were added appropriate amounts of ACTH and chloramine-T, each in 10 μl . of 0.1M-phosphate buffer, pH 7.5. Twenty radioiodinations of synthetic α_{p} -ACTH and ten of synthetic α^{1-24} -ACTH were performed. Yields of reaction ranged from 27% to 89% with a mean yield of $63 \pm 16\%$ (S.D.) for α_{p} -ACTH and $69 \pm 14\%$ for α^{1-24} -ACTH. The variation in yield of reaction and hence specific radioactivity is presumably due to unknown variations in the different batches of ^{131}I iodine used. The amounts of damaged ^{131}I -labelled ACTH and of free ^{131}I iodide in the best eluate are low. Thus the mean damage, as percentages, of ^{131}I -labelled α_{p} -ACTH and of ^{131}I -labelled α^{1-24} -ACTH were 2.7 ± 1.1 and 2.2 ± 1.0 respectively.

Radioiodination of natural porcine ACTH (six experiments) and of natural bovine ACTH (three experiments) were carried out with similar results to those obtained with the synthetic peptides. The mean yield for these two hormones was $67 \pm 13\%$ and the mean damage was $2.7 \pm 1.0\%$.

The yield of the reaction for the synthetic and natural peptides was only slightly decreased by increasing the amount of isotope that reacted over the range 130–1300 μc . Consequently the specific radioactivity of the labelled hormone was essentially

Fig. 2. Results of the radioiodination of ACTH. No significant differences between synthetic α_{p} -ACTH, natural α_{p} -ACTH, natural α_{b} -ACTH and synthetic α^{1-24} -ACTH were noted, and the results are pooled. (a) Yield of reaction (percentage of radioactivity transferred to ACTH). (b) Observed specific radioactivity as a function of the theoretical specific radioactivity. The line represents the relationship if the yields of the reactions were 100%.

proportional to the theoretical specific radioactivity, calculated from the amounts of [^{131}I]iodide and ACTH that reacted (see Fig. 2).

Radioiodination of natural α_{h} -ACTH (six experiments) was carried out. The mean yield of $49 \pm 18\%$ was significantly lower ($P=0.05$) and the mean damage ($6.5 \pm 2.5\%$) was significantly higher ($P < 0.001$) than those obtained with the natural porcine and bovine ACTH. These differences were attributable to the relative purity of these hormones. Thus the biological activity of the α_{p} - and α_{b} -ACTH exceeded 100 i.u./mg. whereas that of the α_{h} -ACTH was only 40–50 i.u./mg.

Ten radioiodinations were performed with [^{125}I]iodide and 1–2 μg . of synthetic α_{p} -ACTH, of synthetic α^1 - 24 -ACTH and of natural α_{b} -ACTH. Equivalent results were obtained to those found by using [^{131}I]iodide with yields ranging from 52% to 78%. After storage for 4 weeks damage was less than 10.0%.

Immunological properties of ^{131}I -labelled ACTH

Preparation and storage damage to the labelled peptide, as defined by behaviour on chromatoelectrophoresis, was limited to less than 5% by using the above procedure. Since the immunological rather than chromatoelectrophoretic properties of the labelled peptide are utilized in radioimmunoassay the preparation damage was also estimated immunologically, by determining the percentage of

the ^{131}I -labelled ACTH capable of binding to different dilutions of antiserum. Samples of ^{131}I -labelled α_{p} -ACTH and α^1 - 24 -ACTH were prepared at increasing specific radioactivities and shown to contain less than 5% of damaged material on chromatoelectrophoresis. Fixed amounts of the

Fig. 4. Effect of increasing the specific radioactivity of ^{131}I -labelled α^1 - 24 -ACTH. Experimental details are as given in Fig. 3. The specific radioactivities were 162 (●), 610 (○) and 942 (×) $\mu\text{C}/\mu\text{g}$. A progressive decrease in binding of ^{131}I -labelled α^1 - 24 -ACTH to anti-(α^1 - 24 -ACTH) serum with increasing specific radioactivity is shown.

Fig. 3. Effect of increasing the specific radioactivity of ^{131}I -labelled α_{p} -ACTH on binding to antiserum. Samples were prepared at specific radioactivities 140 (●), 540 (○) and 838 (×) $\mu\text{C}/\mu\text{g}$. and contained less than 5% of damaged material by chromatoelectrophoresis. Fixed amounts (60 pg.) were caused to react with antiserum to α_{p} -ACTH at the final dilutions shown. Increasing specific radioactivity and hence increasing the amount of total iodine substituted had no effect on the binding to antiserum.

Fig. 5. Specificities of an antiserum to α^1 - 24 -ACTH. The binding of equal amounts (60 pg.) of ^{131}I -labelled α_{p} -ACTH (●), α_{b} -ACTH (○) and α^1 - 24 -ACTH (×) of similar specific radioactivities (200–250 $\mu\text{C}/\mu\text{g}$.) by increasing dilutions of antiserum to α^1 - 24 -ACTH. The antiserum is shown not to distinguish between the α_{p} - and α_{b} -ACTH. The weight of α^1 - 24 -ACTH used is not corrected for the molecular-weight difference between this peptide and α_{p} -ACTH and α_{b} -ACTH.

Fig. 6. Specificities of an antiserum to α_p -ACTH. Experimental details are as given in Fig. 5. The antiserum to α_p -ACTH used distinguishes between ^{131}I -labelled α_p -ACTH (●), ^{131}I -labelled α_b -ACTH (○) and ^{131}I -labelled α^{1-24} -ACTH (×).

samples were then allowed to react with increasing dilutions of their respective antisera. There was no impairment of binding and therefore by implication no immunological damage of the ^{131}I -labelled α_p -ACTH over the range of specific radioactivities studied, 140–838 $\mu\text{C}/\mu\text{g.}$, as shown in Fig. 3. In contrast, the binding of ^{131}I -labelled α^{1-24} -ACTH was progressively impaired as its specific activity was increased from 162 $\mu\text{C}/\text{mg.}$ to 942 $\mu\text{C}/\mu\text{g.}$ (Fig. 4). Thus there was a fall in the percentage bound at high concentrations of antiserum and an alteration in the slope with dilution of antiserum. Since there was no increase in 'chromatoelectrophoretic

damage' with increasing specific radioactivity this probably represents a reduction in immunological activity, which may be related to the increasing substitution of iodine as ^{131}I and ^{127}I into the molecule. The isotope abundance of the 'carrier-free' [^{131}I]iodine used is not known and hence the total amount of iodine introduced could not be calculated. The same batch of [^{131}I]iodine was used, however, and the specific radioactivities may be taken as a measure of the mean amount of total iodine substituted.

Data obtained on the specificities of the two antisera used suggest an explanation of the greater loss of immunological activity with increasing iodination shown by α^{1-24} -ACTH as compared with α_p -ACTH. α_p - and α_b -ACTH, each of 39 amino acid residues, have their first 24 residues in common with α^{1-24} -ACTH, and 60pg. amounts are bound with equal facility by the anti-(α^{1-24} -ACTH) serum (Fig. 5). It may be noted that the lower binding of the same weight of α^{1-24} -ACTH is uncorrected for the lower molecular weight of this peptide, approximately two-thirds of that of α_p - and α_b -ACTH. Species differences between α_p - and α_b -ACTH in the sequence 25–39 are irrelevant when antibody is used that is directed to the 1–24 sequence of α^{1-24} -ACTH. The antiserum to α_p -ACTH, however, binds α_b -ACTH less readily than α_p -ACTH, and α^{1-24} -ACTH is essentially not bound by this antiserum (Fig. 6). Antibody formation is thus preferentially directed towards the C-terminal sequence of α_p -ACTH and the 1–24 sequence is not involved. Since the two tyrosine residues involved in iodination are located at positions 2 and 23 the substitution of more iodine involves sequences that are significant in the binding with an anti-(α^{1-24} -ACTH) serum but are not involved in the binding between

Table 2. Inhibition by unlabelled analogues of ACTH in the reaction between ^{131}I -labelled ACTH and antiserum

Inhibition with	Amount of ^{131}I -labelled α^{1-24} -ACTH + anti-(α^{1-24} -ACTH) serum (ng.)*	Amount of ^{131}I -labelled α_p -ACTH + anti-(α_p -ACTH) serum (ng.)*
α^{4-10} -ACTH	~2000	> 4000
α -Melanocyte-stimulating hormone	~1000	> 4000
α^{1-24} -ACTH	1	~500
α^{1-24} -ACTH†	5	—
β -Melanocyte-stimulating hormone	~2000	—
α_p^{17-39} -ACTH	~8	0.5
α^{11-24} -ACTH	0.5	—
α_p^{25-39} -ACTH	> 4000	0.5
α_p -ACTH	1.2–1.4	1
α_h -ACTH	2–4	6–12
α_{hi} -ACTH‡	2–32	12–128

* The amount of peptide that must be added to produce an equivalent inhibition of the reaction between labelled peptide and antibody to that produced by 1ng. of the unlabelled homologous peptide.

† α^{1-24} -ACTH in which the two arginines in positions 17 and 18 have been replaced by two ornithine residues.

‡ ACTH extracted from human bronchial neoplasms secreting ACTH.

an anti-(α_p -ACTH) serum and ^{131}I -labelled α_p -ACTH.

Inhibition studies with a number of peptides with sequences in common with ACTH help to substantiate this view (Table 2). Synthetic α -melanocyte-stimulating hormone is a tridecapeptide with its 13 amino acids in common with the *N*-terminal sequence of ACTH, but with an acetyl group on the *N*-terminal serine; synthetic human β -melanocyte-stimulating hormone contains 22 amino acids and its sequence 4-10 is shared by ACTH. Peptides containing amino acids in common with the *N*-terminal sequence of ACTH (α^{4-10} -ACTH, α^{1-24} -ACTH and α -melanocyte-stimulating hormone) were relatively ineffective in inhibiting the reaction between ^{131}I -labelled α_p -ACTH and anti-(α_p -ACTH) serum, whereas those containing the *C*-terminal sequence of ACTH (α_p^{17-39} - and α_p^{25-39} -ACTH) were more effective, on a weight basis, than unlabelled α_p -ACTH itself. As expected, α_p^{25-39} -ACTH did not compete in the reaction between ^{131}I -labelled α^{1-24} -ACTH and anti-(α^{1-24} -ACTH) serum, nor did α^{4-10} -ACTH and α - or β -melanocyte-stimulating hormones. The substitution of two ornithine residues for the arginines present in positions 17 and 18 of α^{1-24} -ACTH resulted in a fivefold loss of immunological activity. It was also found that the α_p^{17-39} -ACTH was partially effective and that α^{11-24} -ACTH was more effective, on a weight basis, compared with α^{1-24} -ACTH. These studies indicated that binding to the particular anti-(α^{1-24} -ACTH) serum used involves the *C*-terminal end of synthetic α^{1-24} -ACTH.

The studies with α_n -ACTH obtained from pituitary or from 'ACTH'-secreting bronchial neoplastic tissue (Table 2) emphasizes the value of an assay based on labelled α^{1-24} -ACTH and an antiserum to this peptide. The samples were not highly purified, but were measured with greater sensitivity and specificity with anti-(α^{1-24} -ACTH) serum than with anti-(α_p -ACTH) serum.

DISCUSSION

The sensitivity of a radioimmunoassay depends, in part, on the amount of labelled peptide used as a tracer. Basal plasma ACTH concentrations lie in the pg./ml. range (Yalow *et al.* 1964) and maximum sensitivity requires a labelled peptide of high specific radioactivity if excessively long counting times are to be avoided. In our hands the sensitivity of the radioimmunoassay for plasma ACTH is limited by factors other than the specific radioactivity of the labelled peptide.

The radiation sustained by peptides during radioiodination may cause alterations, termed damage, such that non-specific binding to plasma proteins

takes place, as described for insulin by Berson *et al.* (1956). The radioiodination procedure used for human growth hormone was initially reported to produce undamaged labelled hormone (Greenwood *et al.* 1963) but subsequent experiences, including studies with ACTH, have shown that the presence of damaged material frequently constitutes a problem (McFarlane & Greenwood, 1966; Yalow & Berson, 1966). Routine analysis for damaged material must be regarded as essential since precision, sensitivity and finally specificity of a radioimmunoassay is lost by using increasingly damaged material (Greenwood, 1967). In our hands the chromatoelectrophoretic analysis of Yalow & Berson (1960) is the most useful technique presently available. Of the two most widely used methods for purifying undamaged ^{131}I -labelled peptides, namely the use of Sephadex or cellulose columns, the latter proved more suitable for the present studies. The use of dilute hydrochloric acid proved advantageous as compared with the use of plasma for elution of labelled ACTH from the cellulose because this mitigated against the risk of storage damage.

The adsorption of ^{131}I -labelled ACTH from neutral solutions to glass presents problems in making radioiodination and radioimmunoassay quantitative. Adsorption was found to be minimal, however, to polystyrene at neutral pH and to glass at low pH. The adsorption of ACTH to glass and its desorption at low pH has been reported previously by Stouffer & Lipscomb (1963).

If a labelled peptide is used in a subsequent radioimmunoassay, then the larger the percentage of damaged material in the sample, as determined by chromatoelectrophoresis, the greater the effect in the immunological assay. For routine purposes chromatoelectrophoresis damage may be used to forecast the effects of gross immunological damage. In the absence of damage a further immunological effect is still possible, however, namely a progressive loss of ability to bind to antiserum with increasing substitution of iodine. This effect was demonstrated for α^{1-24} -ACTH but not for α_p -ACTH in a series of studies in which the specific radioactivity of the preparation was used as an index of the total iodine substituted. Like ^{131}I -labelled growth hormone, the quality of a preparation of ^{131}I -labelled α^{1-24} -ACTH will thus be more dependent upon variations in the total iodine content of a [^{131}I]iodine sample than in ^{131}I -labelled α_p -ACTH.

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