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The corticotrophic activity of tetracosactide in the adrenal ascorbic acid depletion test

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Using the subcutaneous adrenal ascorbic acid depletion test in hypophysectomized rats it was found that tetracosactide (synthetic 1–24 corticotrophin) could not be assayed against the International Working Standard (IWS) for natural corticotrophin because of a difference in the slopes of the dose-response curves of the preparations. Valid potency estimates were obtained when tetracosactide was assayed against a standard preparation closely similar to the 2nd International Standard for natural corticotrophin. By comparing this standard with the IWS, the best estimate of the potency of tetracosactide in terms of the IWS was 99 (92–106) units/mg. Five different batches of tetracosactide have yielded identical biological potencies. Since tetracosactide can be adequately standardized by physical and chemical-analytical criteria, labelling and dosage on a mg base instead of in units is recommended.

Peptides with amino-acid sequences corresponding to the N terminal 20 to 24 amino-acids of corticotrophin possess strong corticotrophic activity (Schwyzer, 1964). The member of this group of peptides investigated most extensively is undoubtedly tetracosactide.* In a publication on the pharmacological behaviour of tetracosactide a potency of 100 U/mg has been reported (Schuler, Schär & Desaulles, 1963). This value was established in the adrenal ascorbic acid depletion test, using subcutaneous administration. In these assays a standard preparation of the same type as the International Working Standard was used. No indication of a deviation of parallelism between the log dose response curves was found. However other studies indicate that the shorter peptide shows a pharmacokinetic behaviour different from that of the longer chain of corticotrophin (Rerup, 1966).

This observation is in agreement with data obtained on peptides derived from the natural hormone by pepsin digestion (Hays & White, 1954). Although the connection between ascorbic acid depletion and steroidogenesis is far from being elucidated, the ascorbic acid depletion test is widely employed for assaying biological potencies of corticotrophin. This test provides the basis on which clinical potency of natural corticotrophin preparations can be predicted. Therefore an exact evaluation of tetracosactide in terms of the existing International Working Standard was found desirable. In this paper it will be shown that the slope of the log dose response curve of tetracosactide in the subcutaneous adrenal ascorbic acid depletion test differs from that of the International Working Standard so markedly that in many instances valid potency estimates cannot be obtained. A crude corticotrophin preparation resembling the abandoned 2nd International Standard showed a log dose response curve much more like that of the synthetic tetracosactide, making calculation of relative potencies possible.

* Tetracosactide is the proposed international non-proprietary name for the peptide with the amino-acid sequence of the N terminal 24 amino-acids of corticotrophin. This sequence is the same for all species in which it has been studied, including man.

EXPERIMENTAL

Materials and methods

In this work the following preparations were used.

Tetracosactide. Different production batches of 1–24 corticotrophin were prepared according to Kappeler & Schwyzer (1961). These batches were characterized and standardized *inter alia* by amino-acid analysis, ultraviolet spectrum, specific rotation, electrophoresis, acetic acid, water- and peptide content.

Crude porcine corticotrophin, batch NH 160, having a potency of about 3 IU/mg was prepared from hog pituitaries by the acid-acetone extraction technique of Lyons (1937). Of this preparation a laboratory standard was made, consisting of vials each containing exactly the same amount (about 7 mg) of material which was assayed in the subcutaneous adrenal ascorbic acid depletion test against the 2nd International Standard. Each vial contained 23·4 U with a confidence interval ($P = 0{\cdot}95$) of 20·7 to 26·5.

Carboxymethylcellulose-purified porcine corticotrophin (de Jager, Homan & de Wied, 1963), batch 1094, having a potency of 86 (75–99) U per mg in the subcutaneous test compared to the International Working Standard.

Enzymatically hydrolysed carboxymethylcellulose-purified corticotrophin preparations were made by peptic and carboxypeptidase hydrolysis and purified by column chromatography on carboxymethylcellulose. Preparations with predominantly the 1–31 and 1–26 sequence of porcine corticotrophin had the following potencies in the subcutaneous test using the 2nd International Standard as a reference: Predominantly 1–31 sequence: 106 (89–127) U/mg; 148 (127–172) U/mg. Predominantly 1–26 sequence: 99 (69–177) U/mg; 84 (57–137) U/mg.

Subcutaneous adrenal ascorbic acid depletion tests were made according to the directions given in the U.S. Pharmacopeia XVI (1960) with a few minor modifications. A randomized block design was used and the results were calculated according to the rules given in the USP for such a design. Results of individual assays on the same preparation were tested for homogeneity and combined according to Meier (1953) using the modification of Cochran & Carroll (1953). Usually, the assays were repeated until the fiducial limits of the total estimate, obtained by combination of the individual assays on the preparation, were 87 and 115% of the estimated potency.

RESULTS

Soon after the estimation of corticotrophin activity of tetracosactide, using the subcutaneous ascorbic acid depletion test, was started it became apparent that, when the International Working Standard was used, many results had to be discarded because the tests did not fulfil the criterion of slope parallelism. As it is well known that slope differences exist between crude and carboxymethylcellulose-purified corticotrophin as well as between the latter and hydrolysed purified preparations, the slope of tetracosactide was compared with slopes of various other preparations to classify the behaviour of tetracosactide in this test. In Table 1 the median slopes of different corticotrophin preparations, found in a number of assays, are given. Each line comprises figures obtained in the same assay. The difference between the slopes of the Working Standard and the 2nd International Standard is obvious; so is the fact that the slopes of the latter, and also tetracosactide, crude corticotrophin and the hydrolysed preparations, are similar.

Table 1. *Medians of slopes of different types of corticotrophins in the subcutaneous adrenal ascorbic acid depletion test, using gelatin* (16% *w/v*) *as a diluent*

Number of assays	Standard preparation		Tetracosactide	Crude corticotrophin	Hydrolysed corticotrophin*
	IWS	2nd IS			
38	—225		—130		
36	—207			—135	
8	—221				—115
15		—144			—137
17		—181	—124		

IWS = International Working Standard.

2nd IS = Second International Standard.

* Enzymatically hydrolysed carboxymethylcellulose purified corticotrophin preparation, containing polypeptides with amino-acids 1–26 to 1–31.

Table 2. *Slopes of log dose response curves of tetracosactide and NH* 160

Assay number	Slope		Slope difference	Confidence limits of slope difference ($P = 0{\cdot}95$)	
	Unknown b_u	Standard b_s	$b_s - b_u$		
1	—192·1	—112·4	79·7	—22·6	182·0
2	—108·0	—104·7	3·3	—95·4	102·0
3	—150·9	—104·7	46·2	—52·5	144·9
4	—132·6	—109·0	23·6	—59·8	107·0
5	—122·1	—153·1	—31·0	—114·5	52·5
6	—159·9	—139·9	20·0	—76·0	116·1
7	—120·1	—129·8	—9·7	—133·7	114·3
8	—88·3	—84·7	3·6	—94·4	101·6
9	—103·5	—84·7	18·8	—79·2	116·8
10	—132·6	—46·2	86·4	—29·7	202·5
11	—114·0	—46·2	67·8	—48·3	183·9
12	—134·0	—41·5	92·5	—4·9	189·9
13	—105·2	—41·5	63·7	—33·7	161·1
14	—119·3	—214·3	—95·0	—200·1	10·1
15	—142·3	—214·3	—72·0	—177·1	33·0
16	—102·4	—114·1	—11·7	—106·6	83·2
17	—71·4	—114·1	—42·7	—137·6	52·2
18	—128·7	—98·6	30·1	—87·6	147·8
19	—134·8	—98·6	36·2	—81·5	153·9
20	—113·0	—147·4	—34·4	—120·2	51·4
21	—101·3	—147·4	—46·1	—113·2	21·0
22	—77·7	—169·8	—92·1	—192·8	8·6
23	—64·8	—105·5	—40·7	—175·0	93·6
24	—16·1	—211·5	—195·4	—405·7	14·9
25	—97·2	—211·5	—114·3	—324·6	96·0
26	—144·0	—157·0	—13·0	—99·0	73·0
27	—118·8	—157·0	—38·2	—124·2	47·8
28	—219·8	—160·3	59·5	—62·9	181·9
29	—137·0	—160·3	—23·3	—145·7	99·1
30	—87·8	—124·0	—36·2	—140·0	67·6
31	—83·3	—124·0	—40·7	—144·5	63·1
32	—140·4	—108·5	31·9	—49·9	113·7
33	—158·1	—91·4	66·7	—67·6	201·0
34	—86·0	—91·4	—5·4	—139·7	128·9
35	—106·5	—85·8	20·7	—91·7	131·1
36	—118·8	—85·8	33·0	—78·4	144·4
37	—194·4	—189·6	4·8	—104·5	114·1
38	—128·7	—189·6	—60·9	—170·2	48·4
39	—85·5	—88·9	—3·4	—103·6	96·8
40	—130·4	—88·9	41·5	—58·7	141·7
41	—117·9	—60·6	57·3	—37·1	151·7
42	—122·1	—60·6	61·5	—32·9	155·9
Mean	—119·—	—111·—	—1·37 ± 1·32		

These findings indicated that tetracosactide should be tested against the 2nd International Standard to obtain valid assays. Because of the limited availability of the latter preparation, a laboratory standard (NH 160) was prepared from hog pituitaries. It had about the same purity as the 2nd International Standard against which it was assayed and found to contain 23·4 (20·7–26·5) U/vial. However, it was essential to know the activity of tetracosactide in terms of the present Working Standard since the 2nd International Standard is no longer in use. Although an exact relation between the activities of these two standards cannot be established by means of the subcutaneous adrenal ascorbic acid depletion test, it should be kept in mind that the WHO used this test as a guide for the assignment of the potency of the Working Standard in terms of the 2nd International Standard (Bangham, Musset & Stack-Dunne, 1962). In the same way an evaluation of the potency of the laboratory standard in terms of the Working Standard was made, applying experience obtained in the past, when the activities of many purified preparations had to be compared to that of the 2nd International Standard then generally in use. According to this line 20 U per vial seemed to be the best estimate of the laboratory standard in terms of the Working Standard.

Using this reference preparation, 42 assays of tetracosactide were made. In Table 2 the slopes of the log dose response curves are given.

None of the assays had to be discarded because of slope difference and the mean value of $b_s - b_u$ is close to zero. In Table 3 potencies of different batches of tetracosactide are given. As they correspond closely, a combined estimate of 99 (92–106) U/mg can be regarded as the best estimate of the potency of tetracosactide in terms of the Working Standard. This value is in close agreement with the value of 106 IU/mg reported by Schuler & others (1963).

Table 3. *Assay of 5 different batches of tetracosactide against NH 160*

Batch of tetracosactide	½ L*	Potency† U per mg
A	0·057	104 (92–119)
B	0·065	96 (83–111)
C	0·063	101 (87–117)
D	0·064	92 (80–106)
E	0·070	86 (73–101)
Combined result of 5 batches		99 (92–106)

* ½L is half the length of the confidence interval in logarithms. 87–115% confidence limits correspond to ½ L of 0·061.

† Confidence interval $P = 0{\cdot}95$ between parentheses.

DISCUSSION

The results presented show that owing to a slope difference, tetracosactide cannot be standardized against the International Working Standard of corticotrophin using the subcutaneous adrenal ascorbic acid depletion test. Hence we have to face the fact that pharmacokinetic behaviour of the two preparations in the rat is sufficiently different to render biological assays of this kind fundamentally invalid. Inspection of Table 1 reveals that enzymatic hydrolysis of purified corticotrophin to a mixture of peptides with chain lengths comparable to that of tetracosactide lowered the slope in the adrenal ascorbic acid depletion test to the same level. Obviously, a relation exists between the slope of the dose response curve and the length of the peptide chain.

This does not, however, explain the existing slope difference between crude and purified corticotrophin.

From the similarity of the slopes of tetracosactide and the 2nd International Standard, or a preparation of a similar nature, it was deduced that, if these preparations were to be standardized against one another, valid results would be obtained. This was proved in 42 assays.

Although this eliminated the problem as to which standard was to be used for tetracosactide it did not solve the problem of evaluating tetracosactide in terms of the Working Standard of corticotrophin, now in general use. This could not simply be done by calculation because of the existing difference in slope between the 2nd International Standard and the Working Standard (Bangham & others, 1962). Therefore, the only possibility remaining was to assess the activity of NH 160 in terms of the Working Standard using an equivalence which had presented itself from many similar comparisons of crude and purified corticotrophin preparations. The best estimate seemed to be 20 Units/ampoule. In fact this figure should be considered as an approximation of an entity not attainable in any exact manner.

Using NH 160 as a standard, it was found that all batches of tetracosactide investigated showed the same biological potency in assays, with a high degree of precision. This result is not unexpected since the synthesis of tetracosactide and its intermediates can be adequately controlled by physical and analytical chemical methods. This constancy of quality of the synthetic product, which has now been confirmed by biological testing, renders regular bioassay of batches superfluous and allows dosage of the preparation on a milligram basis instead of in international units. This can be regarded as a distinct advantage over the natural product, i.e. corticotrophin. The latter contains a mixture of biologically active peptides which cannot be characterized sufficiently by chemical means, so that a biological standardization is essential.

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