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BIOLOGICAL ACTIVITY OF CORTICOTROPHIN PEPTIDES
WITH HOMO-ARGININE,
LYSINE OR ORNITHINE SUBSTITUTED
FOR ARGININE IN POSITION 8

By

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ABSTRACT

The steroidogenic and lipolytic activities of corticotrophin-(1-24)-tetracosapeptide and [Lys^{17,18}]corticotrophin-(1-18)-octadecapeptide amide were compared with those of the corresponding analogues variously substituted in position 8 with homo-arginine, ornithine or lysine. Peptides substituted with homo-arginine showed a surprisingly high degree of biological activity; in all the tests performed, the loss of activity due to the substitution did not exceed a factor of 3 to 10, while peptides substituted with ornithine or lysine in position 8 only showed residual biological activity or none at all, depending on the test system used. The results reported demonstrated that the presence of a guanidino group (arginine or homo-arginine side chain) in position 8 of the ACTH peptide is essential for the preservation of a high level of biological activity; the length of the aliphatic chain carrying the guanidino group is of minor importance.

In previous communications (Tesser & Schwyzer 1966; Tesser & Rittel 1969), the effect of the substitution of ornithine (cf. formulae, Table 1) for the arginine residue in positions 8, 17 and 18 of corticotrophin-(1-24)-tetracosapeptide (I)¹ was described.

It was shown that as far as the biological activity of the peptide is concerned the presence of a guanidino group in position 8 is more important than the presence of such a group in positions 17 or 18. The need for arginine⁸ had already been demonstrated earlier by Chung & Li (1967), who found that the biological activity of corticotrophin (1-17) heptadecapeptide amide substituted with lysine⁸ was remarkably weak as compared with that of the peptide containing arginine⁸. In addition, according to Geiger (1971) and Schwyzer *et al.* (1971) the active site of corticotrophin is located between positions 5 and 10; arginine⁸ is thus one of the amino acids composing the active centre.

In the present studies, the effect of structural alterations in position 8 on steroidogenic and lipolytic activity has been further investigated.

Peptides substituted in position 8 with homo-arginine, ornithine or lysine were compared with the unmodified peptide containing arginine.

MATERIAL AND METHODS

Peptides. – The synthesis of the analogues II and VI will be described elsewhere (Tesser *et al.*, in prep.). Peptide I was prepared according to Kappeler & Schwyzer (1961); III was synthesized by Tesser & Rittel (1969). Peptides IV and V were prepared by a route analogous to that used in the synthesis of I (Kamber *et al.*, unpublished results). The structures of the tested peptides are shown in Table 1.

Steroidogenesis. – *In vitro*, adrenal slices were incubated for 2 h according to the method described by Saffran & Schally (1955). The corticosterone content of the incubation medium was measured by acid fluorescence (Guillemin *et al.* 1959).

In vivo, the peptides were injected once intravenously into male rats hypophysectomized 24 h previously. The concentration of corticosterone in the plasma was determined as described by Desaulles & Rittel (1968).

Lipolysis. – In the *in vitro* experiments, epididymal fat pads from rats were incubated essentially as described by Jungas & Ball (1963); 4% bovine albumin was added. After two hours' incubation the unesterified fatty acids (FFA) released into the incubation medium were measured by titration (Dole 1956), and the glycerol content estimated as described by Wieland (1957).

Lipolytic activity *in vivo* was estimated on the basis of the concentration of unesterified fatty acids determined colorimetrically in the plasma (Duncombe 1963), 30 min after the intravenous administration of the peptide.

¹) Nomenclature according to the recommendations of the IUPAC-IUB Commission of Biochemical Nomenclature (1972).

Other abbreviations used: Har = homo-arginine, FFA = unesterified fatty acids, i. e. lysine⁸ = lysine in position 8 of the amino acid sequence.

Table 1.
Formulae of the corticotrophin peptides tested.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----------------|-----|-----|-----|-----|--------|----|
| I: H-Ser | Tyr | Ser | Met | Glu | His | Phe | Arg | Trp | Gly | Lys | Pro | Val | Gly | Lys | Lys | Arg | Arg | Pro | Val | Lys | Val | Tyr | Pro-OH | |
| II: H-Ser | | | | | | | Har | | | | | | | | | Arg | Arg | | | | | | Pro-OH | |
| III: H-Ser | | | | | | | Orn | | | | | | | | | Arg | Arg | | | | | | Pro-OH | |
| IV: H-Ser | | | | | | | Lys | | | | | | | | | Arg | Arg | | | | | | Pro-OH | |
| V: H-Ser | | | | | | | Arg | | | | | | | | | Lys | Lys | NH ₂ | | | | | | |
| VI: H-Ser | | | | | | | Har | | | | | | | | | Lys | Lys | NH ₂ | | | | | | |
| I: corticotrophin-(1-24)-tetracosapeptide | | | | | | | | | | | | | | | | | | | | | | | | |
| II: [Har ⁸]corticotrophin-(1-24)-tetracosapeptide | | | | | | | | | | | | | | | | | | | | | | | | |
| III: [Orn ⁸]corticotrophin-(1-24)-tetracosapeptide | | | | | | | | | | | | | | | | | | | | | | | | |
| IV: [Lys ⁸]corticotrophin-(1-24)-tetracosapeptide | | | | | | | | | | | | | | | | | | | | | | | | |
| V: [Lys ^{17,18}]corticotrophin-(1-18)-octadecapeptide amide | | | | | | | | | | | | | | | | | | | | | | | | |
| VI: [Har ⁸ , Lys ^{17,18}]corticotrophin-(1-18)-octadecapeptide amide | | | | | | | | | | | | | | | | | | | | | | | | |

Animals. – The experiments were performed on male albino rats of the Sprague-Dawley strain (Ivanovas, Kisslegg, Germany) which had been kept on a standard laboratory diet and under controlled lighting conditions. The *in vitro* steroidogenesis assays were carried out with adrenals excised from animals weighing 180–200 g. In the *in vitro* lipolytic assays, epididymal fat from rats weighing 140–160 g was used. Rats weighing 180–200 g were selected for the *in vivo* experiments.

Statistical analysis. – Dose-response curves were constructed from the mean data derived from 6 to 10 experiments. Regression lines within the linear part of the dose-response curves were calculated by the Hewlett-Packard programme Stat-Pac V-6. The relative steroidogenic potencies of the peptides *in vitro* were determined in 4-point assays by means of parallel regression analysis (Finney 1964).

RESULTS

Steroidogenesis in vitro (Fig. 1, Table 2)

The dose-response regression lines of the two standard preparations, I and V, do not differ significantly, though the latter peptide is slightly more active

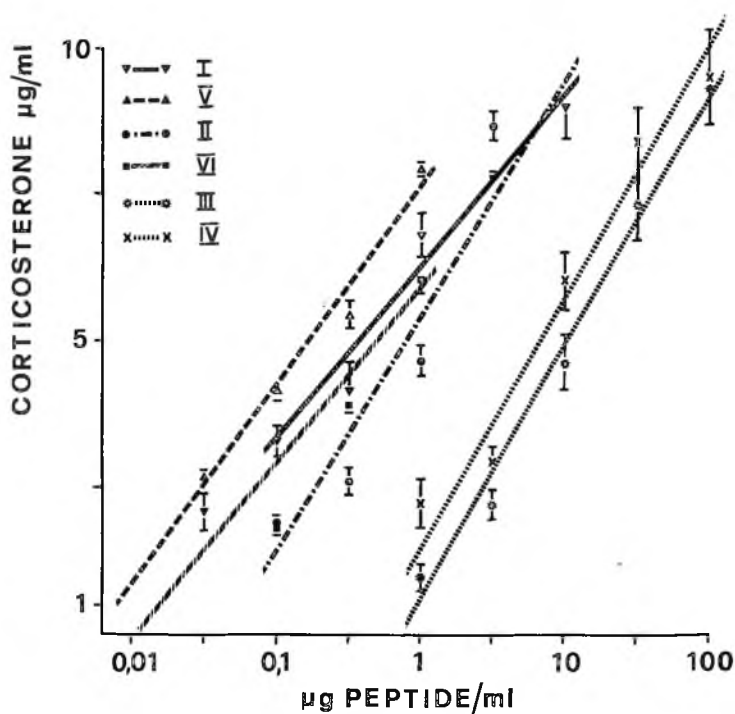


Fig. 1.

In vitro steroidogenesis: Dose-response regression lines of corticosterone released from rat adrenal slices incubated for 2 h at 37°C. Each point constitutes the mean of 6 to 10 incubations \pm SEM.

Table 2.
In vitro steroidogenesis: relative potencies determined in four point assays.
(adrenal slices of rats incubated for 2 hours).

| Standard compound | Test compound | Potency | Confidence limits 95 % |
|-------------------|---------------|------------------|----------------------------|
| I | II | 0.25 | 0.23 -0.28 |
| I | III | 0.036* | 0.029-0.046 |
| I | IV | 0.076* 0.046* | 0.061-0.094 0.024-0.082 |
| V | VI | 0.25 0.24 | 0.19 -0.33 0.20 -0.29 |

* Owing to significant differences in the slopes of the dose-response curves a valid comparison cannot be made.

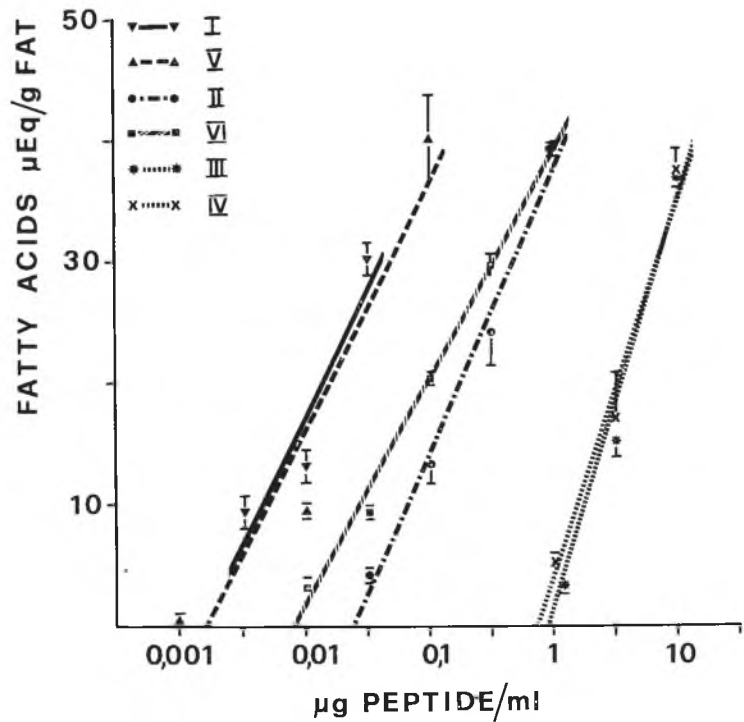


Fig. 2.

In vitro lipolysis: Dose-response regression lines of FFA released from rat epididymal fat pads incubated for 2 h at 37°C. Each point constitutes the mean of 4 to 8 incubations \pm SEM.

Table 3.
In vitro lipolysis: activities determined by glycerol release (epididymal fat pads of rats).

| Test compound | μg peptide per ml incubation medium | | | | | | | |
|---------------|--|---------------|----------------|----------------|----------------|----------------|----------------|----------------|
| | 0.001 | 0.01 | 0.03 | 0.1 | 0.3 | 1.0 | 3.0 | 10 |
| I | $0.9 \pm 0.2^*$ | 8.2 ± 0.6 | 15.9 ± 0.3 | 20.7 ± 0.6 | - | - | - | - |
| II | - | 0.2 ± 0.2 | 2.7 ± 0.7 | 9.1 ± 0.8 | 14.7 ± 1.6 | 21.2 ± 0.7 | - | - |
| III | - | - | - | - | 2.3 ± 0.5 | 3.8 ± 1.2 | 10.0 ± 3.5 | 19.0 ± 1.2 |
| IV | - | - | - | - | 0.5 ± 0.2 | 4.7 ± 1.3 | 9.8 ± 4.0 | 20.3 ± 4.0 |
| V | 1.2 ± 0.5 | 7.9 ± 0.6 | - | 20.0 ± 1.5 | - | - | - | - |
| VI | - | 4.6 ± 1.2 | 7.1 ± 1.2 | 15.5 ± 0.5 | 19.1 ± 0.1 | 20.4 ± 1.3 | - | - |

* μMol glycerol per g fat; each value is the mean of 6-9 incubations.

than the former. The two peptides substituted with Har⁸ (II and VI) yield dose-response regression lines parallel to those of the respective standards; their relative potencies, as determined in 4-point assays, amounts to about 0.25 of that of the standards (= 1). The two analogues III and IV, substituted with Orn⁸ and Lys⁸ respectively, are markedly less active. In the 4-point assays they differ from the standards in the slope of their dose-response regression lines, so that no valid comparison of their potencies can be made. At the semi maximal activity level (middle part of the regression lines), their potency in relation to that of the respective standards is about 0.04.

Lipolysis in vitro (Fig. 2, Table 3)

The mobilization of unesterified fatty acids (FFA, Fig. 2) and the release of glycerol are in good overall agreement with the steroidogenic activity of the peptides. The two standards, I and V, show equal activity. The two Har⁸

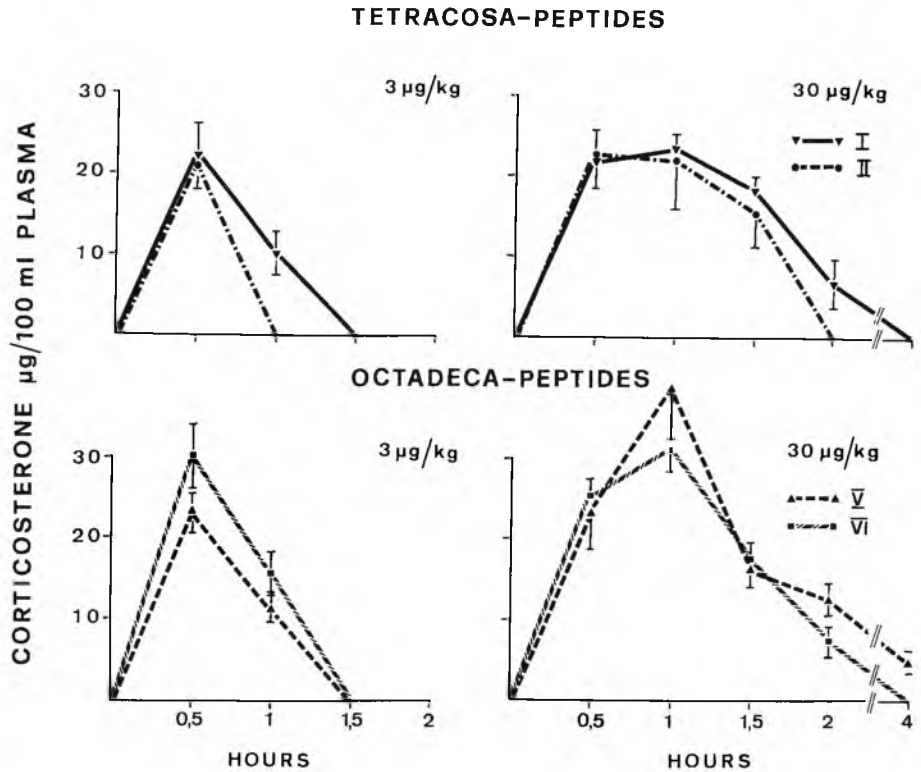


Fig. 3.

In vivo steroidogenesis: Time course of plasma corticosterone concentrations in 24 h hypophysectomized rats after a single intravenous injection of the peptides. Each point represents the mean of 6 to 12 animals \pm SEM.

peptides, II and VI, also exhibit similar activity. Their dose-response regression lines are parallel to those of the standard compounds but a dose 10 times higher is necessary to obtain the same lipolytic effect. The two peptides with Orn⁸ or Lys⁸ (III and IV) yielded somewhat steeper dose-response curves than the other corticotrophin peptides. When compared at the level of medium effect (20 μ Mol/g fat) both peptides proved to be about 300 times less active than the standard compounds. The results obtained by measuring glycerol-release are identical to those found by determining FFA mobilization (Table 3).

Steroidogenesis in vivo (Fig. 3)

Steroidogenic activity *in vivo* was assessed on the basis of the time-response curves obtained after the intravenous administration of single doses of 3 and 30 μ g/kg of each of the peptides.

At these dose levels, the maximum corticosterone concentrations produced by the 4 peptides tested (I, II, V and VI; III and IV were not assayed) do not

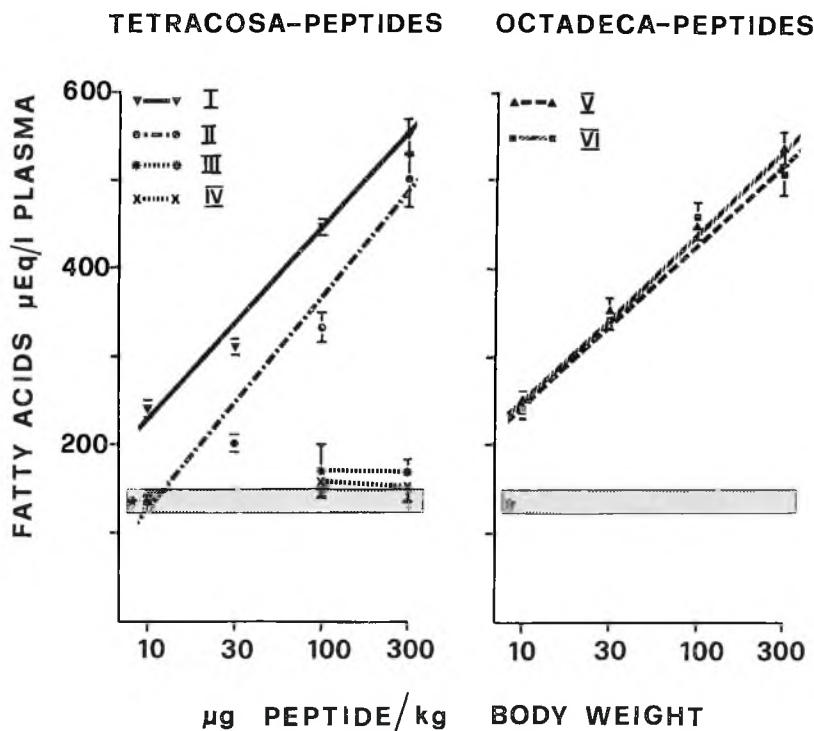


Fig. 4.

In vivo lipolysis: Dose-response regression lines of plasma FFA concentrations in intact rats 30 min after a single intravenous injection of the peptides. Each point represents the mean of 14-21 animals \pm SEM. Basal values (182 ± 12) of 50 rats injected with saline only are shown as shaded area.

differ significantly, but the duration of their steroidogenic effects varies. The two standard peptides have an almost identical duration of action, the 10 fold dose eliciting an almost doubly prolonged effect. The [Har⁸]tetracosapeptide (II) has a shorter duration of effect than the standards, I and V; a dose roughly three times greater than that of the standard peptide I would have to be given to achieve the same duration of effect. The [Har⁸, Lys^{17,18}]octadecapeptide (VI) does not differ significantly from the two standards.

Lipolysis in vivo (Fig. 4)

The slopes of the dose-response regression lines of the four active peptides are parallel. The standard compounds, I and V, and [Har⁸, Lys^{17,18}]octadecapeptide (VI) display identical activity, whereas the [Har⁸]tetracosapeptide (II) is only half as potent. The two tetracosapeptides, III and IV, with Orn⁸ and Lys⁸ show no lipolytic activity up to the highest dose tested (1 mg/kg).

DISCUSSION

The biological importance of arginine in position 8 of corticotrophin peptides has been investigated. Replacement of arginine by homo-arginine (Fig. 5) results in only a slight reduction in the steroidogenic and lipolytic activity of the peptides, while the substitution of ornithine or lysine drastically diminishes these effects.

The four analogues differ as regards not only the pK_a of the basic group in position 8 (guanidino-group pK_a 12.5, amino group pK_a 10.5), but also the length of the aliphatic chain which carries the basic grouping. Our results clearly demonstrate the eminent importance of the guanidino group, while the length of the aliphatic chain is obviously of minor significance, the biological activity of the homo-arginine analogues being only slightly reduced.

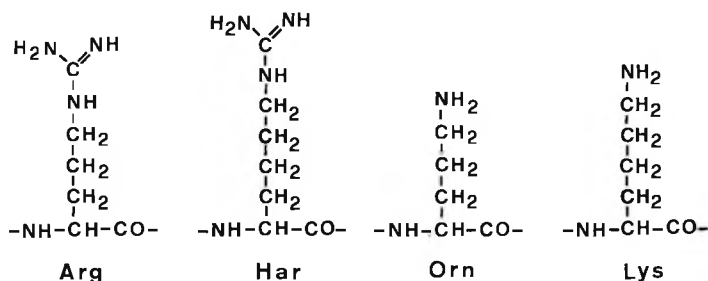


Fig. 5.

Chemical structures of the amino acids arginine (Arg), homoarginine (Har), ornithine (Orn) and lysine (Lys).

These findings confirm and add precision to the results of earlier investigations (Tesser & Schwyzer 1966; Tesser & Rittel 1969) indicating the importance of arginine⁸. It has been shown that the arginyl residues in position 17 and 18 are not indispensable as regards biological activity; they can be replaced by ornithine (Tesser & Schwyzer 1966) or by lysine (Desaulles *et al.* 1969) without substantially altering the effects of the peptides. The 11–24 region of corticotrophin is assumed to be essential to the binding of the peptide to its cell-membrane receptor, but is less important, if at all, for the stimulation of the receptor (Hofmann *et al.* 1970; Schwyzer *et al.* 1971; Finn *et al.* 1972). On the other hand, the 5–10 region is considered to represent the stimulating locus of corticotrophin. This diversity of function might explain the unequal importance of the arginyl residue in position 8 as compared with positions 17 and 18. To stimulate the receptor, a guanidino group is a necessity, while an amino group appears to be inadequate; in the binding region (17/18), however, either of the basic residues suffices. It is tempting to believe that the receptor can discriminate between two groups whose base strengths differ by a factor of 100. Although this difference is most conspicuous, it is reasonable to assume that both residues are completely protonated in aqueous solution under physiological conditions prevailing in the serum or plasma.

It is therefore anticipated, that a protonated guanidino group in position 8 also occurs when the peptide is in intimate contact with the receptor site. The inadequacy of the ammonium group to stimulate the true receptor site might be attributed to the small spatial extent of its positive charge in contradistinc-

Table 4.
Approximate potencies.

| Test compound | Steroidogenesis | | Lipolysis | |
|---------------|-----------------|----------------|-----------------|----------------|
| | <i>in vitro</i> | <i>in vivo</i> | <i>in vitro</i> | <i>in vivo</i> |
| I* | 1 | 1 | 1 | 1 |
| II | 0.25 | 0.3 | 0.1 | 0.5 |
| III | 0.06 | – | 0.003 | ∅** |
| IV | 0.04 | – | 0.003 | ∅** |
| V | 1 | 1 | 1 | 1 |
| VI | 0.25 | 1 | 0.1 | 1 |

* Activity was arbitrarily set at 1.

** Inactive up to a dose of 1 mg/kg iv.

tion to the larger resonance-stabilized guanidinium group. The latter, moreover, affords the possibility of interaction with some bidentate structure in the receptor site by way of a hydrogen bond, additional to its electrostatic bond.

The steroidogenic and the lipolytic potencies of the peptides investigated in this study are indicated in Table 4. It is evident that the relative activities of the tested corticotrophins are highly similar in the two *in vitro* test systems. It may therefore be speculated that the corticotrophin receptors of the adrenal and the fat cell are similar in structure.

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A NOVEL PHOTOCYCLIZATION, STARTING FROM
1,4-DIARYLBUTENYNES

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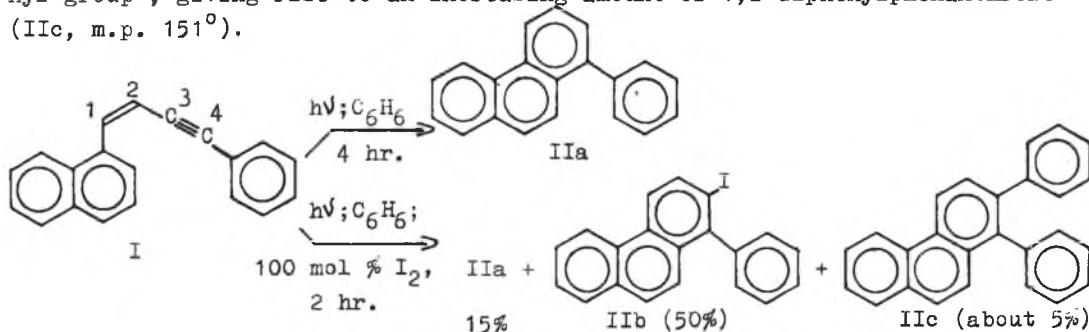
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Cis-trans isomerization is the only known photochemical reaction of arylbutenyynes¹ until now. We found, however, that irradiation under nitrogen of a dry and deaerated solution ($5 \cdot 10^{-4}$ molar) of 1-(α -naphthyl)-4-phenylbut-1-en-3-yn²(I) in benzene with a 360 nm fluorescence lamp (Sylvania blacklite F8T5) during four hours converted the solute into 1-phenylphenanthrene (IIa), 45%.

Although no oxidation step is involved the cyclization is slightly faster in the presence of air; in a solution saturated with oxygen or supplied with a small amount of iodine ($3 \cdot 10^{-5}$ molar) the rate is about twice as fast as in a deaerated and iodine-free solution.

The main irradiation product in a deaerated solution of I containing an equimolar amount of iodine is an iodo derivative, 1-phenyl-2-iodophenanthrene (IIa). On prolonged irradiation of this solution iodine is replaced by a phenyl group³, giving rise to an increasing amount of 1,2-diphenylphenanthrene (IIc, m.p. 151°).



The cyclization is a light-induced reaction. Refluxing a benzene solution of I in the dark or heating of pure I in an evacuated tube at 300° during four hours did not yield any cyclization product.

Irradiation under nitrogen of an iodine-free solution of I ($5 \cdot 10^{-4}$ molar) in hexane in the presence of triplet-sensitizers like 2-methylantraquinone, benzophenone, benzil or acetophenone ($0-1.2 \cdot 10^{-2}$ molar) did not lead to higher yields of IIa. Also the presence of the triplet-quencher azulene did not influence the result of the photoreaction.

The radical scavenger di-tert.butylnitroxide⁴ (10^{-3} - 4.10^{-4} molar) had no influence upon the cyclization of I into IIa. However, on irradiation a solution of I containing iodine as well as this radical scavenger only IIa and not IIb and IIc was formed.

From these results it may be concluded that IIa arises from a singlet excited state of I. It is known that acetylenes in the first excited state have non-linear trans configurations⁵, which should be very favourable for the ring-closure observed. The formation of the new C-C bond and the 1,5-hydrogen shift may be a concerted. In the presence of iodine cyclization product IIb is apparently formed via a radical intermediate, possibly formed by the addition of an iodine atom to the triple bond of I.

The new photocyclization reaction has been investigated on several other diarylbutenyne (Table).

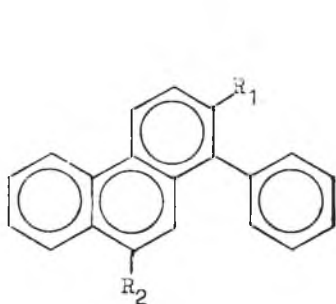
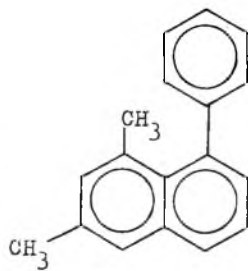
Table

Irradiation products from diarylbutenyne²,
 $Ar^1-CH=CH-C\equiv C-Ar^2$

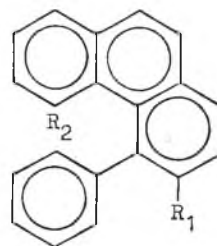
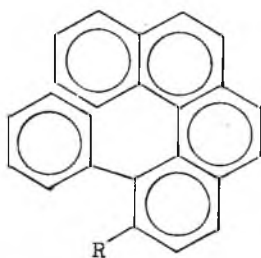
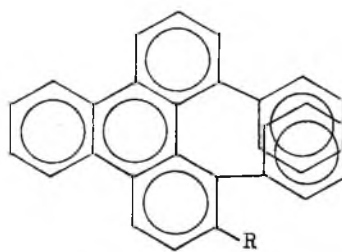
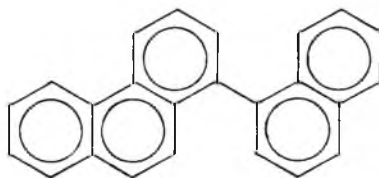
| Ar ¹ | Ar ² | without iodine | | | with iodine | | |
|--------------------------------|--------------------|----------------|------------|--------------------|--------------|------------|--------------------|
| | | pro- duct | yield % | m.p. ^{°C} | pro- duct | yield % | m.p. ^{°C} |
| 1 α -naphthyl | phenyl | IIa | 45 | 74-76 | IIb | 50 | 166-168 |
| 2 phenyl | phenyl | - | | | - | | |
| 3 p. methoxyphenyl | phenyl | - | | | - | | |
| 4 3,5-dimethylphenyl | phenyl | IIIa | 44 | liquid | *) | | |
| 5 β -naphthyl | phenyl | IVa | 55 | 82-84 | IVb | 44 | 160-162 |
| 6 α -(4-methylnaphthyl) | phenyl | IIId | 54 | 89-92 | IIe | 19 | 93-95 |
| 7 β -(8-phenylnaphthyl) | phenyl | IVc | 65 | 169-171 | IVd | 16 | 135-140 |
| 8 3-phenanthryl | phenyl | Va | 50 | 109-113 | Vb | 32 | 131-133 |
| 9 2-benzo [c]phenanthryl | phenyl | -** | | | - | | |
| 10 9-(1-phenylphenanthryl) | phenyl | VIa | 60 | 150-152 | VIb | 44 | 254-260 |
| 11 phenyl | α -naphthyl | - | | | - | | |
| 12 α -naphthyl | α -naphthyl | VIIa | 22 | 116-118 | *) | | |
| 13 β -naphthyl | α -naphthyl | VIIIa | 38 | 150-152 | VIIIb | 38 | 137-140 |

*) Irradiation had to be carried out with a high pressure mercury lamp in hexane; under these circumstances carbon-iodine bonds are broken.

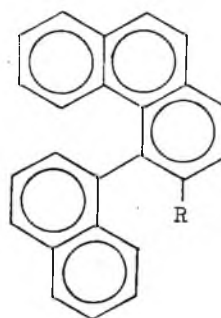
***) Only a mixture of dimers had been formed.

IIa: $R_1 = R_2 = H$ b: $R_1 = I, R_2 = H$ c: $R_1 = C_6H_5, R_2 = H$ d: $R_1 = H, R_2 = CH_3$ e: $R_1 = I, R_2 = CH_3$ 

IIIa

IVa: $R_1 = R_2 = H$ b: $R_1 = I, R_2 = H$ c: $R_1 = H, R_2 = C_6H_5$ d: $R_1 = I, R_2 = C_6H_5$ Va: $R = H$ b: $R = I$ VIa: $R = H$ b: $R = I$ 

VIIa

VIIIa: $R = H$ b: $R = I$

The negative results in the experiments 2, 3 and 11 compared with the cyclization in 4 suggest that some factor related to the electron-distribution in the relevant ring is of decisive importance for the cyclization. The failure of cyclization in experiment 9 may be due to the strong tendency of the starting compound to dimerization, even in dilute solutions. In all other cases only one type of cyclization product was obtained; bond formation occurs always between an acetylenic carbon (C_4 in I) and the aromatic ring at C_1 . In this respect the reaction is clearly different from photocyclizations in diarylbutadienes⁶, which lead to two products if different arylresidues are present in the starting compound⁷. The experiments 7, 8 and 10 reveal that even highly crowded polycyclic aromatics can be obtained quite specifically and in good yields from properly chosen diarylbutenyne.

Full experimental details concerning the syntheses and spectroscopic data and properties of the products will be given elsewhere.

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7. Although Leznoff mentioned high specificity in photocyclizations of 1,4-diarylbutadienes we found in general more than one product. This discrepancy may be due to the very low yields (about 10%) in Leznoff's experiments.

SIMPLE ASSAY OF PLASMA TESTOSTERONE USING A COVALENT PROTEIN-SEPHAROSE COMPLEX

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1. Introduction

In previous communications, we [1] described the use of Sepharose-linked binding proteins for a convenient and precise assay of *cyclo-3',5'*-adenosine monophosphate (*cAMP*) according to the principle of competitive protein binding (CPB) between unlabelled and labelled *cAMP*. The method had the advantage of allowing a simple and rapid separation of "bound" from "free" radioactive *cAMP* by mere filtration of the "immobilized" protein linked to the Sepharose. It avoided all the difficulties arising with soluble *cAMP* binding proteins that had been in use hitherto (for references see [1]).

It occurred to us that similar improvements could possibly be achieved for the CPB assays of plasma testosterone. In a collaborative effort, we succeeded in devising an equally convenient and precise testosterone assay using Sepharose-linked testosterone binding proteins from human late pregnancy serum.

2. Materials and methods

Water was deionized and twice distilled before use. For measuring aqueous solutions, we used Oxford, for solutions in organic solvents, glass pipettes. Buffer I: 0.1 N NaHCO_3 /0.1 N NaCl ; buffer II: 0.05 M Tris-HCl , pH 7.4.

2.1. Sepharose-linked testosterone binding proteins

3 g of Sepharose activated with cyanogen bromide (purchased from Pharmacia or prepared according to [1]) were suspended in 10 ml of buffer I and kept for 20 min at room temp. The gel was transferred to a porous glass filtration funnel and washed for not more than 15 min with approx. 200 ml of 0.001 N HCl to remove soluble impurities. The material on the filter was then washed with approx. 200 ml of buffer I, sucked almost dry, and transferred to a solution of 2 ml of human late pregnancy serum (third trimester) in 5 ml of buffer I contained in a 100 ml round bottomed flask. The mixture was gently stirred by slow rotation of the flask at 4° for 15 hr. The protein-modified Sepharose was collected on a porous glass filter and washed with 250–500 ml of buffer I, followed by 250–500 ml of buffer II. The gel was then suspended in 200 ml of buffer II and stored until used at approx. 1–4° in a beaker sealed with "Parafilm".

2.2. Assay of "testosterone-like substances" in blood plasma

2.2.1. Preparation of plasma extracts and blanks

The heparinized blood was immediately centrifuged and the plasma stored at –20° in plastic vials. 1.0 ml of the plasma (or a smaller amount diluted to 1.0 ml with water) was placed, together with 5.0 ml of spectroscopic grade dichloromethane, into a glass-

stoppered vial and agitated for 1 min. The aqueous phase was pipetted off and replaced with 0.5 ml 1 N Na_2CO_3 solution. After agitation (30 sec) and centrifugation, the aqueous phase was removed, and the organic phase washed neutral with two to three successive 0.75 ml volumes of water (test with pH paper). Four 0.50 ml aliquots of the organic phase were pipetted into four Eppendorf vials and kept in a vacuum desiccator for at least 1 hr at approx. 10 Torr. For the estimation of blank values, 1.0 ml of water was treated in exactly the same manner as the plasma samples.

2.2.2. Calibration

For every assay (double estimations of the unknowns) a calibration curve was constructed, using 50 μl each of ethanolic solutions containing in this volume 5.0, 1.25, 0.32, 0.008, and zero ng of testosterone in Eppendorf vials. The solvent was removed as in sect. 2.2.1.

2.2.3. Binding assay

100 μl of [1,2- ^3H]testosterone (Amersham) in water (= 10 nCi) were added to each of the desiccated vials (mechanical shaking for 30 sec dissolved the dried extract or blank). The protein-modified Sepharose in the storage beaker was evenly suspended by slow magnetic stirring, and 200 μl each of the suspension pipetted into the vials. After mechanical agitation for 5 sec, equilibration was achieved by standing at room temp. for unspecified times between 20 and 120 min. The contents of the vials were then pipetted onto 35 μm polyester nets (Müllereigaze Estal mono PE 35, No. 19176, Schweizerische Seidegaze-fabrik AG., Thal, Switzerland) contained in suction filter funnels. The vials were rinsed with 1 ml each of buffer II, and the materials on the filters washed once or twice with approx. 1 ml of the same buffer (volumes aren't critical). The nets were transferred into the counting vials and covered with 5 ml of the scintillant solution (Insta-Gel, Packard). After agitation, the radioactivity was determined by scintillation counting (Packard Tri-Carb instrument). It was usually expressed in percentage of a 100% value (= 100 μl of the above [1,2- ^3H]testosterone solution added to the scintillant).

3. Results

3.1. Properties of the "Sepharose-linked testosterone binding proteins"

The mean association constant for [1,2- ^3H]testosterone in 33 mM Tris-HCl buffer, pH 7.4, at 20°, and the Hill coefficients, n , were determined according to the method described in [1]: $K_{\text{ass}} = 9.87 \times 10^8$ l/mole ($K_{\text{diss}} = 1.01 \times 10^{-9}$ mole/l); $n = 1$. The non-specific adsorption of [1,2- ^3H]testosterone to Sepharose and to the polyester net is less than 1% of the total tracer concentration added.

3.2. Water blank

The water blank is considerably lower with the present method ($\bar{x} = 6.8$ ng/100 ml, $S_{\bar{x}} = 2.2$, $n = 5$) than with Florisil 2 ($\bar{x} = 64.6$ ng/100 ml, $S_{\bar{x}} = 4.6$, $n = 13$). In both cases, the extraction was carried out with dichloromethane, the solvent giving the lowest "reagent blank" [3].

3.3. Plasma testosterone values

Results obtained with the present method from samples of normal subjects and patients with various

Table 1
Plasma testosterone values in normal pubertal and adult males.

| Subject No. | Age (yr) | Plasma testosterone (ng/100 ml) |
|--------------------|----------|---------------------------------|
| 1 | 15 | 644 |
| 2 | 16 | 680 |
| 3 | 16 | 685 |
| 4 | 17 | 410 |
| | | 1000* |
| | | 1025* |
| | | 1300* |
| 5 | 17 | 465 |
| 6 | 17 | 470 |
| 7 | 19 | 530 |
| 8 | 20 | 800 |
| 9 | 21 | 455 |
| 10 | 54 | 980 |
| Mean value \pm s | | 612 \pm 172 |

* = 2, 4 and 6 days after administration of human chorionic gonadotropin (5000 U per square meter of body surface area).

Table 2
Plasma testosterone values in patients with various disorders.

| Subject No. | Age (yr) | Sex | Diagnosis | Plasma testosterone (ng/100 ml) |
|--------------------|----------|-----|---|---------------------------------|
| <u>High values</u> | | | | |
| 1 | 3 | M | Idiopathic precocious puberty, untreated | 750 |
| 2 | 6 | M | Idiopathic precocious puberty untreated | 1140 |
| | | | Under treatment with cyproterone acetate | 410 |
| 3 | 19 | M | Testicular teratoma, untreated | 600 |
| | | | After surgery | 235 |
| 4 | 23 | F | Idiopathic hirsutism | 210 |
| 5 | 23 | F | Idiopathic hirsutism | 380 |
| 6 | 30 | F | Idiopathic hirsutism | 450 |
| 7 | 27 | M | Congenital adrenal hyperplasia (21-hydroxylase deficiency), untreated | 1265 |
| 8 | 30 | F | Virilizing adenoma of the left adrenal: | |
| | | | Left adrenal venous plasma | 6500 |
| | | | Right adrenal venous plasma | 1600 |
| <u>Low values</u> | | | | |
| 9 | 0.6 | M | Tall stature within normal limits | 33 |
| 10 | 4 | M | Hereditary defect of testosterone biosynthesis (17,20-desmolase deficiency) | 20 |
| 11 | 6 | M | Congenital anorchia | 10 |
| 12 | 52 | M | Castrate, untreated | 25 |
| 13 | 16 | M | Delayed puberty | 105 |
| 14 | 16 | M | Delayed puberty | 210 |
| 15 | 17 | M | Delayed puberty | 260 |
| 16 | 17 | M | Delayed puberty | 345 |
| 17 | 18 | M | Delayed puberty | 130 |
| 18 | 19 | M | Delayed puberty | 171 |
| 19 | 17 | M | Klinefelter's syndrome | 165 |
| 20 | 35 | M | Hypogonadism | 120 |
| 21 | 40 | M | Hypogonadism | 175 |

endocrine disorders are displayed in tables 1 and 2. They agree well with those reported by others using different techniques [4, 5]. The mean value obtained in normal pubertal and adult males was 612 ± 172 ng/100 ml ($n=10$), while a compiled average from the literature [5] is 670 ± 230 ng/100 ml. The highest figure was obtained for adrenal venous blood from a patient with virilizing adrenal adenoma (6500 ng/100 ml, patient No. 8, table 2). High values were also found in untreated patients suffering from congenital adrenal hyperplasia due to a 21-hydroxylase deficiency and from idiopathic precocious puberty. In the latter condition, there was, in one case studied, a definite reduction of the level during treatment with cyproterone acetate. Low values were obtained in an adult, unsubstituted castrate male, in a boy with congenital anorchia, and in one child with a congenital defect of testosterone biosynthesis (17,20-desmolase deficiency, proven by incubation of testicular tissue with testosterone precursors [6]). Moderately low levels were found in cases with benign delayed puberty, Klinefelter's syndrome, and hypogonadism, as one would expect.

4. Discussion

The competitive protein binding technique for the assay of hormones in biological fluids was introduced by Ekins (1960) for thyroxin [7]. Subsequently, Murphy [8, 9] adapted the principle for the estimation of steroids. A large number of methods for the determination of various steroids using CPB has since been published (for references and discussion see [10]). These techniques have the advantage of relative simplicity and high sensitivity. Their specificity, however, depends on the prepurification steps applied rather than on the CPB itself.

Among other difficulties, one of the major problems of CPB techniques is the separation of the free from the protein bound steroid fraction. For this purpose, various methods have been used, including gel filtration (Sephadex G25 [11], dextrane coated charcoal [12], ammonium sulfate for protein precipitation [13], and Florisil, p.e. [2, 14, 15]). We have, in the Dept. of Pediatrics, been previously using a modification of the relatively simple and non-specific method of Anderson [2] for the determination of "testosterone-like" substances in plasma, where no prepurifica-

tion is carried out and the separation of the free from the bound fraction is done with Florisil. Our modifications consisted of extraction with methylene chloride instead of ether (lower non-specific "reagent" blank, cf. [3]), of a different CPB step, cf. [16], and of minor changes of shaking.

Although for clinical use, the results obtained with this previous method agreed well with those of urinary steroid determinations in the same subjects by highly specific gas chromatographic techniques (Zachmann [17]), the separation step with Florisil was a constant source of difficulties and did impair the reproducibility of the calibration curves due to its sensitivity to even minimal changes of shaking time and other methodological factors. In addition, in our experience and in that of others [9, 15], too high testosterone values were obtained with Florisil in plasma samples from prepubertal children and females, and the water blanks were relatively high.

Similar difficulties had been encountered in the CPB assays of *cAMP* and had been overcome by the use of relatively very stable, Sepharose-linked *cAMP* binding proteins [1]. The results presented in this paper show that analogous improvements can be achieved for steroid assays with the help of immobilized binding proteins. The advantages consist in: i) rapid and facile separation of bound and free steroid fractions by simple filtration, ii) the constant equilibrium between 20 min and at least 3 hr, resulting in improved reproducibility of the standard curves and relative insensitivity to changes in handling times, iii) reduction of the water blank, iv) no observable desorption of testosterone during filtration and washing procedures, v) the negligible nonspecific adsorption of testosterone to Sepharose and polyester net, and vi) the stability of the Sepharose-linked protein.

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