Wie die Tabelle zeigt, lassen sich alle angeführten Gibberelline auf zwei Chromatogrammen trennen: (a) Entwicklung mit einem der Gemische I–IV oder VI unter Auftrennung von A_8, $(A_3 + A_1)$, $(A_7 + A_4 + A_5)$ und A_9; (b) Durchlaufchromatographie mit Gemisch V zur weiteren Differenzierung der Gruppen $(A_3 + A_1)$ und $(A_7 + A_4 + A_5)$. Entwicklungsgemisch VII eignet sich vor allem zur Trennung von Gibberellinsäurederivaten mit $R_{Standard}$-Werten unter 1,0.

$R_{Standard}$-Werte[a] von Gibberellinen bei der Dünnschichtchromatographie an Kieselsäuregel

Gibberellin	Entwicklungsgemisch[b]						
	I	II	III	IV	V	VI	VII
A_1	1,1	1,1	1,1	1,1	1,5	1,0	1,0
A_3	1,0	1,0	1,0	1,0	1,0	1,0	1,0
A_4	2,1	2,3	2,6	3,9	4,9	1,5	1,2
A_5	2,1	2,4	2,7	4,2	5,3	1,6	1,2
A_7	2,0	2,3	2,6	3,6	4,6	1,5	1,2
A_8	0,6	0,5	0,5	0,4		0,7	0,8
A_9	2,5	3,3	4,3	7,6		2,2	1,3

[a] Bezogen auf Rf-Wert von Gibberellin $A_3 = 1{,}0$.
[b] I: Chloroform/Essigester/Eisessig (60:40:5), Rf von $A_3 \sim 0{,}35$; II: Chloroform/Essigester/Eisessig (70:30:5), Rf von $A_3 \sim 0{,}24$; III: Chloroform/Essigester/Eisessig (80:20:5), Rf von $A_3 \sim 0{,}16$; IV: Chloroform/Essigester/Eisessig (90:10:5), Rf von $A_3 \sim 0{,}10$; V: Chloroform/Essigester/Eisessig (80:20:1), Laufstrecke von $A_3 \sim 2{,}8$ cm; VI: n-Butanol/3n Ammoniak (5:1), Rf von $A_3 \sim 0{,}25$; VII: n-Propanol/3n Ammoniak (5:1), Rf von $A_3 \sim 0{,}55$.
Mit I–IV 2stündige, mit VI und VII etwa 7stündige aufsteigende Entwicklung bei 20°C. Mit V wurde bei 20°C 15–18 h absteigend und durchlaufend entwickelt[8,10].

Nach unseren Erfahrungen dürfte die Dünnschichtchromatographie für Trennung und Identifizierung der Gibberelline gute Dienste leisten, besonders im Hinblick auf die im Vergleich zur Papierchromatographie grundsätzlichen Vorteile dieser Methode[6,9], wie kürzere Entwicklungszeiten, erhöhte Nachweisempfindlichkeit, grössere Kapazität der Trägerschicht sowie saubere Elutionsmöglichkeit für mikropräparative Gewinnung (Ermittlung der physikalischen Konstanten), quantitative Bestimmung und biologische Testung[10].

Summary. Thin-layer chromatography on silica gel is described as a new method for the separation and identification of the gibberellins A_1, A_3, A_4, A_5, A_7, A_8, and A_9.

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Deutsche Akademie der Wissenschaften zu Berlin, Institut für Kulturpflanzenforschung Gatersleben, Kreis Aschersleben (Deutschland), 13. August 1962.

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[9] K. Randerath, *Dünnschichtchromatographie* (Weinheim/Bergstrasse 1962).

[10] Inzwischen wurde uns bekannt, dass von M. Kutáček, J. Rostmus und Z. Deyl, Biol. plant. (Prag) *4*, 226 (1962), für eine Trennung von Gibberellin A_1 und A_3 ebenfalls die Dünnschichtchromatographie, allerdings an Al_2O_3, mit Erfolg angewendet worden ist. Mit dem von diesen Autoren empfohlenen Entwicklungsgemisch Benzol/Eisessig (10:3) erzielten wir an Kieselgel G (Merck) folgende Ergebnisse: Rf von Gibberellin A_3 0,15 = $R_{St.}$ 1,0; $R_{St.}$ von A_1 1,0, A_4 4,0, A_5 3,5, A_7 3,8, A_8 0,4, A_9 6,0.

STUDIORUM PROGRESSUS

Dose-Response Relations for some Synthetic Analogues of Oxytocin, and the Mode of Action of Oxytocin on the Isolated Uterus[1]

Since the synthesis of oxytocin was accomplished by du Vigneaud et al.[2] in 1953, more than 50 analogues of this hormone and of vasopressin have been synthesized and examined for their biological properties. The conclusions which have hitherto emerged from this work (recently summarized in part[3]) have, to our mind, been disappointing in that they give no information about the mechanism of action of such compounds at the molecular level. As was to be anticipated, the hormonal activities expressed in different biological systems differed in their response to particular structural changes, and structural modifications at different sites in the molecule proved to be of unequal importance for biological activity. Perhaps more surprising was the finding that even slight structural changes in chemically nondescript, non-functional side-chains—in particular that of isoleucine in position 3 of the peptide chain[4–6]—caused a marked loss of activity in the assays regarded as typical of oxytocin, whereas even such chemically obtrusive functional groups as the amino group of the terminal half-cystine residue[7], or the phenolic hydroxyl of tyrosine[8,9] could be omitted altogether with only partial loss, or no loss, of activity.

Recent emphasis on the importance of lipophilic interactions between non-functional amino acid side-chains in proteins and related compounds as factors in intra- and intermolecular interactions in aqueous environments[10] has suggested a possible interpretation of this

[1] Presented at the General Meeting of the Österreichische Biochemische Gesellschaft, Vienna (May 7th, 1962).

[2] V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, J. Amer. chem. Soc. *75*, 4879 (1953). – V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, J. Amer. chem. Soc. *76*, 3115 (1954).

[3] R. A. Boissonnas, S. Guttmann, B. Berde, and H. Konzett, Exper. *17*, 377 (1961).

[4] J. Rudinger, J. Honzl, and M. Zaoral, Coll. Czech. chem. Comm. *21*, 770 (1956).

[5] R. A. Boissonnas, S. Guttmann, P.-A. Jaquenoud, and J.-P. Waller, Helv. chim. Acta *39*, 1421 (1956). – P.-A. Jaquenoud and R. A. Boissonnas, Helv. chim. Acta *44*, 113 (1961). – B. Berde, W. Doepfner, and H. Konzett, Brit. J. Pharmacol. *12*, 208 (1957).

[6] H. Nesvadba, J. Honzl, and J. Rudinger, Coll. Czech. chem. Comm., in press.

[7] V. du Vigneaud, G. Winestock, V. S. Murti, D. B. Hope, and R. D. Kimbrough, J. biol. Chem. *235*, PC 64 (1960).

[8] M. Bodanszky and V. du Vigneaud, J. Amer. chem. Soc. *81*, 1258, 6072 (1959).

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situation based on the assumption that the side-chain in position 3 participates to an important extent in the binding of the hormone molecule to the receptor, without sharing in the function of the hormone-receptor complex. In order to test this idea, we have now carried out a more detailed examination of dose-response relations for a number of oxytocin analogues on the isolated rat uterus, structurally the simplest biological system suitable for the present purpose.

Experimentally, this was done by the cumulative dose procedure[11] successfully applied to a number of other biological preparations. The uterine muscle was suspended in an organ bath (25 ml) under the standard conditions used in oxytocin assays[12], except that the lever used to record the isotonic contractions was rather more heavily loaded. The concentration of the compound to be examined in the organ bath was increased logarithmically by doubling the dose at one-minute intervals (without rinsing). In control experiments the same doses were applied with rinsing between each application. Generally speaking, results conforming to the expected relations were found, as illustrated in Figure 1 for a cumulative dose experiment. The qualitative picture obtained with individual organ preparations was reproducible, though there was appreciable variation in the numerical results[13]. The measurement of isotonic contractions may not be the most suitable technique for studies of this type[14] and we are examining alternative techniques in order to increase the quantitative significance of the results.

Five analogues modified in position 3 of the peptide chain were examined, with the isoleucine (Ile) replaced by alloisoleucine (aIle), valine (Val), leucine (Leu), norleucine (Nle), and norvaline (Nva). These compounds were obtained by a synthetic route originally developed for the preparation of oxytocin[15], purified by countercurrent distribution and characterized by elemental analysis and quantitative amino-acid analysis[6]. The log dose-response relations were determined with oxytocin as reference for each organ preparation. In all cases the curves for the analogues were parallel to that of oxytocin, displaced toward higher concentrations, and at sufficiently high dose levels reached the same maximum effect as oxytocin; examples are shown in Figure 2a, b. LINEWEAVER–BURK plots[16] were linear, with identical intercepts on the reciprocal activity axis (Figure 3). In terms of the receptor theory of pharmacological action[17–21], as recently formulated by ARIËNS et al.[21],

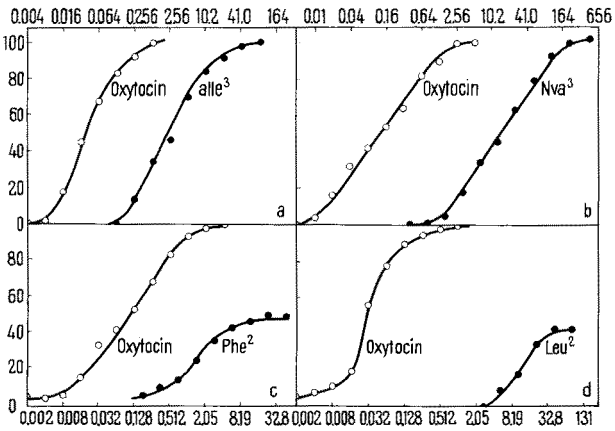

Fig. 2. Log dose-response curves for the isotonic contraction of the isolated rat uterus under the influence of oxytocin (○) and analogues (●). For designation of the analogues see text. Ordinates: contraction in % of maximum, abscissae: dose in mμmoles (logarithmic scale).

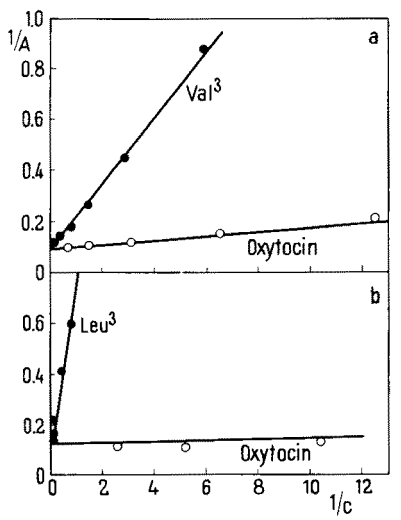

Fig. 3. Reciprocal plot of dose-response relations for oxytocin and two analogues. For designation of the analogues see text. Ordinates: reciprocal of response (cm^{-1}), abscissae: reciprocal of dose ($m\mu mole^{-1}$).

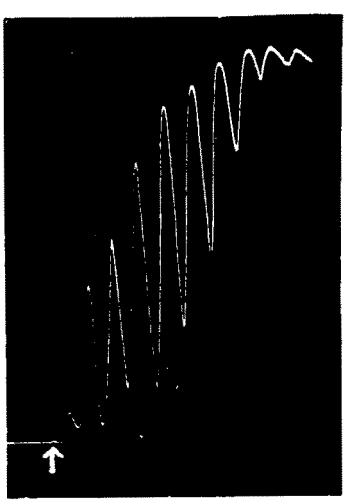

Fig. 1. Contractions of the isolated rat uterus under the influence of increasing concentrations of Val3-oxytocin. Organ bath volume 25 ml, 33°C. Dose doubled at one-minute intervals starting from 0.156 mμmoles (arrow).

[11] E. J. ARIËNS and W. M. DE GROOT, Arch. int. Pharmacodyn. *99*, 193 (1954).
[12] P. HOLTON, Brit. J. Pharmacol. *3*, 328 (1948).
[13] In certain experiments, contracture (tonic contraction) supervened before the rhythmic (tetanic) contractions had reached maximum amplitude. For purposes of the present study such experiments were disregarded.
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[15] M. ZAORAL and J. RUDINGER, Coll. Czech. chem. Comm. *20*, 1183 (1955). – J. HONZL and J. RUDINGER, Coll. Czech. chem. Comm. *20*, 1190 (1955). – J. RUDINGER, J. HONZL, and M. ZAORAL, Coll. Czech. chem. Comm. *21*, 202 (1956).
[16] H. LINEWEAVER and D. BURK, J. Amer. chem. Soc. *56*, 658 (1934).
[17] A. J. CLARK, *The Mode of Action of Drugs on Cells* (Arnold, London 1933); *General Pharmacology*, in *Heffters Handbuch der experimentellen Pharmakologie*, Vol. 4 (Springer, Berlin 1937).
[18] J. H. GADDUM, J. Physiol. *89*, 7P (1937).
[19] E. J. ARIËNS, Arch. int. Pharmacodyn. *99*, 32 (1954).
[20] R. P. STEPHENSON, Brit. J. Pharmacol. 11, 379 (1956).
[21] E. J. ARIËNS, J. M. VAN ROSSUM, and A. M. SIMONIS, Arzneimittel-Forschung *6*, 282 (1956); Pharmacol. Rev. *9*, 218 (1957).

this means that these analogues differ from oxytocin in their affinity for the receptor but possess the same intrinsic activity[19,21] as the prototype.

The same type of approach was next used with 2-O-methyltyrosine-oxytocin (Tyr(Me)^2-oxytocin), a compound prepared independently in our laboratory[22,23] and by LAW and DU VIGNEAUD[24]. We had found this derivative to be a potent inhibitor of the action of oxytocin on the isolated uterus[25]. The dose-response relations for oxytocin in the presence of varying amounts of Tyr(Me)^2-oxytocin, plotted according to LINEWEAVER and BURK (Figure 4), clearly demonstrate that the O-methyl derivative behaves as a typical competitive antagonist of oxytocin, with an inhibition index of less than 10. In terms of the receptor theory, this implies a rather high affinity for the receptor, with very low or no intrinsic activity; and this in turn suggests that the phenolic hydroxyl group may in some way be implicated in the actual mechanism by which oxytocin exerts its biological effect in this system.

This conclusion is in apparent contradiction to the relatively high specific activity on the isolated rat uterus recorded by two independent groups of investigators[8,9] for 2-phenylalanine-oxytocin (Phe^2-oxytocin), a derivative lacking the phenolic hydroxyl group altogether. We have therefore prepared[23] and reinvestigated this compound, with the results shown in Figure 2c. The relatively high affinity of this analogue for the oxytocin receptor accounts for the high specific activity, determined, as is customary, in the lower dose range; however, the maximum effect at full saturation – the intrinsic activity – is only about one-half that of oxytocin. This, indeed, supports the conclusion that the free hydroxyl group of the tyrosine residue is implicated in the action of oxytocin on the uterine muscle but shows that it is not critically important for this effect. Replacement of the aromatic side-chain in position 2 by an aliphatic one in 2-leucine-oxytocin (Leu^2-oxytocin)[22,23] leads to a further drop in specific activity, but this is due to a decrease in affinity as against Phe^2-oxytocin, with little or no further decrease in the intrinsic activity (Figure 2d). This finding indicates that the side-chain of tyrosine is involved both in attachment of the hormone to the receptor and in the function of the hormone-receptor complex, and incidentally emphasizes the necessity for considering the hormone molecule as a topochemical whole.

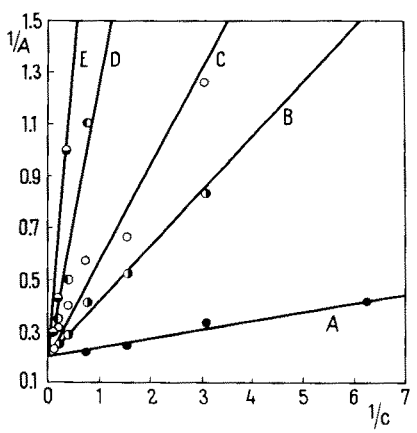

Fig. 4. Reciprocal plots of dose-response relations for oxytocin in the presence of varying amounts of Tyr(Me)^2-oxytocin. Coordinates as in Figure 3. Amount of inhibitor (mμmoles): *A* 0, *B* 6, *C* 10, *D* 20, *E* 50.

In general, the actual significance of the terms 'receptor', 'affinity', and 'intrinsic activity' of the receptor theory is open to some doubt since dose-response relations such as those on which the theory relies for experimental support might be produced by saturation phenomena at a number of stages leading up to the manifestation of the biological effect, and by mechanisms other than the formation of active drug-receptor complexes[14]. However, some encouragement may be derived from the studies of RICHARDS and VITHAYATHIL[26] on the 'ribonuclease S' system: The two fragments obtainable from ribonuclease A by the action of subtilisin – one an eicosapeptide (S-peptide), the other a protein-like molecule (S-protein) – though each separately devoid of enzymic activity and different from the native enzyme in important physical and chemical properties have been shown to recombine to a complex (ribonuclease S) which closely resembles native ribonuclease in physical, chemical, and enzymic properties. As the authors have pointed out[26] this system provides a model of hormone action, with an inactive peptide combining relatively firmly with a biological macromolecule (the 'receptor'), forcing upon it a change of conformation and yielding a complex of defined biological activity. Moreover, certain modifications of the S-peptide lead to a partial loss of enzymic activity in the system as a whole, and it has been possible to trace the effect of particular structural modifications either to a lowered tendency to complex formation (decreased 'affinity') or to lowered enzymic activity of the complex formed (decreased 'intrinsic activity'). Bearing in mind this plausible model of hormone action on the receptor principle, it is possible to speculate on hormone-receptor relations of the compounds which form the subject of this paper.

The decreased affinity resulting from alterations in the side-chain structure at position 3 might be due in principle to steric factors or to changes in lipophilic interactions; in either case the effect could be intramolecular – making the conformation of the hormone necessary for reaction with the receptor less readily attainable – or intermolecular, directly affecting the hormone-receptor interactions. On the evidence available it is not possible to distinguish which of these effects are operative and, indeed, their relative importance may vary from case to case; but steric hindrance effects of either kind can hardly be responsible for the decreased affinity of those analogues which are derived from the prototype by omission of methylene or methyl groups (Val^3-oxytocin and Nva^3-oxytocin), and it is these cases which provide the best evidence for the importance of lipophilic interactions.

Formation of the hormone-receptor complex, which may (but need not) involve conformational changes in either component, leads to the appearance or completion of the topochemical feature responsible for activity ('active site'). As has been argued above, the tyrosine hydroxyl group may normally form part of this topo-

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chemical region though even in its absence (Phe²-oxytocin) the remaining groups suffice for activity. The inhibitory effect of Tyr(Me)²-oxytocin, as against the mere partial loss of intrinsic activity in Phe²-oxytocin, can be rationalized on the assumption that O-methylation not only blocks participation of the hydroxyl group but that the methyl group projects into the space required for whatever process is responsible for activity, and prevents the remaining groups of the 'active site' from exerting their effect.

An alternative interpretation could be based on a similar receptor model, but without assuming direct participation of any part of the hormone molecule in the topochemical entity responsible for activity, the function of the hormone molecule being confined to inducing the conformational change in the receptor which gives rise to the arrangement of groups (belonging in this case to the receptor alone) constituting this topochemical entity. Modifications in the structure of the hormone molecule could in certain cases lead to differences in the extent of deformation induced by the hormone analogue in the receptor, and these in turn might affect the exact topochemistry of the 'active site' sufficiently to cause partial or complete loss of activity in the hormone-receptor complex, while the stability of this complex might not be appreciably impaired by the same structural modifications. Such a state of affairs would also result in a decreased intrinsic activity (combined with relatively high affinity), and the appearance of competitive inhibition.

In the present instance the first explanation appears more attractive, because it permits a rational correlation between the actual chemical modification involved and the resultant changes in pharmacological behaviour.

It should be noted that these considerations require no assumptions about the actual process responsible for activity (except insofar as this process is thought of as linked with the existence[21], rather than the formation[14], of the hormone-receptor complex). As to the nature of this process, no direct evidence is available. It has been shown that, in the presence of calcium, oxytocin acts at the membrane of the uterine muscle cell and these observations have been summarized by CSAPO[27] as being "compatible with the assumption that the Ca ion is the myoplasmic activator, and that oxytocic substances act as 'Ca carriers' or that they modify the distribution of membrane Ca by their special affinity for some component of the myometrial cell membrane"[29]. On this basis it is tempting to speculate that the 'active site' of the oxytocin-receptor complex might be a grouping capable of chelating calcium. A more conventional (unspecified) type of enzymic activity might also be envisaged, in direct analogy with the ribonuclease S model. In any case, it is relevant to point out that, inasfar as other oxytocic substances do act by the same mechanism as oxytocin, they appear to do so at different receptor sites since the antagonism by Tyr(Me)²-oxytocin is directed specifically against oxytocin and the compound does not inhibit the uterine contractions induced by acetylcholine, adrenalin, serotonin, or ergometrine[29] or by the peptide bradykinin[30]. Incidentally, this finding lends support to the criticism recently directed by SCHWYZER[31] against the diffuse concept of structural specificity advanced by WOOLLEY et al.[32].

Finally, it should be stressed that hormone-receptor relations, as revealed by studies on the simple *in vitro* systems, are but one aspect of the complex events leading to the observed action of the hormone in more complicated systems. This is illustrated by the mere fact that the specific activities of many oxytocin analogues, referred to the natural hormone, differ when assayed, e.g. on the rat urerus *in vitro*, and on the same organ *in situ*[3,5]. No doubt the analysis of such differences[33] will expose further factors operative in hormonal regulation; nevertheless it is our belief that the approach to structure-activity relations outlined in this paper can yield information of fundamental importance to an understanding of the mechanism of hormone action.

Zusammenfassung. Eine auf der Rezeptorentheorie begründete Analyse der Dosis-Wirkungs-Beziehung bei Analogen des Oxytocins hat gezeigt, dass die verringerte spezifische Aktivität in der Seitenkette des Isoleucins modifizierter Analogen nur auf eine verringerte Affinität für die Rezeptoren zurückzuführen ist, wogegen die Verbindungen, in denen das Tyrosin des Oxytocins durch Phenylalanin oder Leucin ersetzt ist, zusätzlich eine verringerte maximale Aktivität bei Absättigung der Rezeptoren ('intrinsic activity') aufweisen. Das 2-O-Methyltyrosin-Oxytocin wirkt als typisch kompetitiver Antagonist des Hormons. Eine auf Modellsysteme gestützte Erklärung dieser Resultate wird erwogen.

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Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, and Research Institute for Natural Drugs, Prague (Czechoslovakia), July 31, 1962.

[27] A. CSAPO, in *Oxytocin*. Proceedings of an International Symposium held in Montevideo 1959 (Pergamon Press, 1961), p. 111.

[28] If the calcium concentration in the organ bath is increased above 0.4 m*M*, Tyr(Me)²-oxytocin begins to show an uterotonic effect; eventually, at sufficiently high concentrations of calcium, all the analogues discussed above behave as full agonists of oxytocin.

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