Material und Methoden. Zur Isolierung wurde Phallolysin aus kalt-wässerigen Extrakten lyophilisierter Pilze mit 40% Ammoniumsulfat gefällt und durch Ionenaustauschchromatographie an DEAE-Cellulose (0,03 *M Tris*-HCl-Puffer pH 8,0; 2°C) und wiederholte Gelchromatographie an Biogel P-30 (0,1 *M* Na-Phosphatpuffer pH 5,5, NaCl ad 0,3 *M*; 2°C) gereinigt. Zur Reinheitsprüfung diente Celluloseacetat-Elektrophorese (pH 8,5), Standard-Disk-Elektrophorese (pH 4,0; pH 8,5) und Natriumdodecylsulfat (SDS)-Disk-Elektrophorese; gefärbt wurde mit Coomassie Brilliant Blue. Das apparente Molekulargewicht ermittelten wir durch gelchromatographischen Vergleich mit Standardproteinen an Sephadex G-75, den Isoelektrischen Punkt durch Elektrofokussierung in Ampholine. Phosphat wurde nach AMES[2] bestimmt.

Ergebnisse und Diskussion. Die hämolytische Aktivität liess sich aus Celluloseacetat-Elektropherogrammen und Polyacrylamidgelen eluieren und entsprach einer mit Coomassie Blue färbbaren Bande. Bei der SDS-Disk-Elektrophorese erwies sich das isolierte Phallolysin als zu etwa 50% rein.

Bei der Elektrophorese verhielt es sich basisch, der Isoelektrische Punkt lag bei 8,25. Es war nicht dialysabel. Für das native Hämolysin wurde ein Molekulargewicht von 30.000 ermittelt.

Phallolysin wurde durch 0,1% Natriumdodecylsulfat (60 min/37°C) vollständig und irreversibel inaktiviert. Durch 8 *M* Harnstoff wurde es langsam inaktiviert (nach 24 h bei 20°C Restaktivität < 1–15%). Dabei verschwand mit der hämolytischen Aktivität auch die Toxizität. Der inaktivierte Anteil liess sich nicht reaktivieren durch Dialyse, Verdünnung oder gelchromatographische Abtrennung des Harnstoffs.

Es war thermolabil und verlor bei 65°C innerhalb von 5 min 90% seiner Aktivität. Es war säurelabil und wurde bei pH 2,0 bei 37°C innerhalb von 10 min inaktiviert; in der Kälte war es gegen Säure beständiger. Gegen Alkali war es stabiler.

Obwohl die physikalisch-chemischen Eigenschaften von Phallolysin auf ein Protein hinwiesen, war die hämolytische Aktivität resistent gegen Pepsin, Trypsin, Chymotrypsin, Subtilisin und Pronase (48h/37°C). Dabei hemmte es die Aktivität dieser Proteasen gegen andere Substrate nicht; auch änderte unter der Enzymbehandlung die der hämolytischen Aktivität entsprechende Bande ihr disk-elektrophoretisches Verhalten nicht. Die hämolytische Aktivität war auch resistent gegen Bromelin und Proteinase K (48 h/37°C). Auch durch α-Amylase oder Pankreatin (24 h/37°C) wurde Phallolysin nicht inaktiviert.

Pro Molekül eines etwa 50% reinen Phallolysin (Mol-Gew. 30.000) wurden nur 3–4 Moleküle Phosphat nachgewiesen, die Reste nicht abgetrennter Puffersalze darstellen konnten.

Phallolysin hämolysierte gewaschene Erythrocyten von Mensch, Maus, Kaninchen, Meerschweinchen, Ratte, Hund und Schwein direkt; gegen menschliche Erythrocyten war es 20 mal aktiver als Digitonin; Rinder- und Hammelerythrocyten waren weitgehend resistent. Es wurde durch Cholesterin nicht gehemmt, zeigte keine Phospholipase A-Aktivität und auch keine Phospholipase C-Aktivität[3].

Es wirkte cytotoxisch auf HeLa-Zellen und Mäuse-Ascites-Tumorzellen in vitro[4]. Es war i.p. hoch toxisch. Die mit verschieden weit gereinigtem Phallolysin bestimmte Toxizität war nach Abtrennung der toxischen Cyclopeptide der hämolytischen Aktivität korreliert. Unabhängig vom Reinheitsgrad betrug die LD_{50} für Mäuse (i.p.) ca 1000 hämolytische Einheiten/kg. (Eine Hämolytische Einheit hämolysiert 1 ml 1%ige Suspension gewaschener Kaninchenerythrocyten bei 37°C innerhalb von 2 h total.); je nach Aktivität des Materials waren dies 0,3–0,6 mg isoliertes, lyophilisiertes Phallolysin.

In grünen Knollenblätterpilzen von verschiedenen Fundorten und Jahren wurde Phallolysin konstant und in hoher Aktivität nachgewiesen[5]. Infolge seiner Thermo- und Säurelabilität dürfte dieses Toxin jedoch an der Knollenblätterpilzvergiftung des Menschen nicht beteiligt sein. Eine eingehende Darstellung erfolgt an anderer Stelle.

Summary. Phallolysin, a toxic hemolysin of high molecular weight, was isolated from aqueous extracts of *Amanita phalloides*. Its properties are discussed.

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10. Februar 1973.

	LD_{50} (mg/kg[a]) mit Vertrauensgrenzen	
Pilztrockenpulver (in Agar suspendiert)	26,0	(22,2–30,5)
Äthanolischer Extrakt (50% Äthanol, 25°C)	112,0	(102,0–123,0)
Wässriger Extrakt (2×24 h, 2°C)	20,2	(15,6–26,1)

[a] Ausgedrückt als Pilztrockenpulver.

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7 Ein Teil der Ergebnisse entstammt der Dissertation von H. SCHARRER.

Changes of the β-Receptor Binding Sites of the Rabbit Ileum under the Influence of High Temperature

Several authors observed that an elevation of temperature was accompanied by a decrease of the affinity for sympathomimetic drugs to the β-receptors on the isolated guinea-pig atrium[1–5] and on the rabbit ileum[6]. On rabbit ileum, this decrease of affinity was much more pronounced than on guinea-pig atrium. The diminution of affinity caused by high temperature was found to be completely reversible by the metabolic inhibitor iodoace-

tic acid on atrium as well as on ileum[7]. Furthermore, experiments on atria showed the affinity for β-sympathomimetics to be nearly identical either at 25°C under control conditions or at 25°C after recooling from 42°C[3]. The contractile amplitude and the maximal increase of the amplitude induced by sympathomimetics were not altered by this procedure.

These observations on atria are in accordance with the idea that in this organ the increase of metabolic rate is the main factor responsible for the diminution of the affinity for β-sympathomimetic drugs. We were, therefore, interested to see whether in the ileum additional factors for the observed diminution of β-sympathomimetic affinity have to be taken into consideration.

Material and methods. Pieces from rabbit ileum were kept in Krebs-Henseleit solution containing 6.3×10^{-7} *M* phentolamine in order to block the α-receptors. The spontaneous motility was registered by a strain gauge. Dose-response curves for the β-sympathomimetic drugs isoprenaline and Th 1165a[6] were determined by adding single doses. The equilibration time for every new temperature step was 60 min.

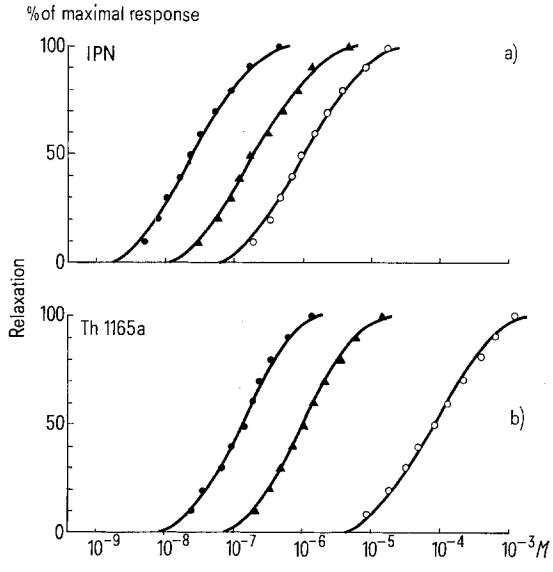
After completion of the dose-response curves at 25°C, the organ bath was heated up to 42°C and maintained for 120 min at this temperature without further experimental procedure. The system was then recooled to 25°C again and another dose-response curve was determined at 25°C. Also a dose-response curve 5 h after recooling to 25°C was performed for isoprenaline. In a parallel set of experiments, dose-response curves for the sympathomimetics at 42°C were determined. The intrinsic activity (α) and the pD_2-values were estimated according to ARIENS[8] and VAN ROSSUM[9], resp. For further details of the methods see prior publications[3,6,7].

Results. On the isolated rabbit ileum an increase of the bath temperature from 25° to 42°C resulted in a shift of the dose-response curves for isoprenaline (IPN) and Th 1165a to the right (Figure). The resulting diminution of affinity is expressed by means of the pD_2-values. For IPN they declined significantly from 7.70 at 25°C to 6.05 at 42°C and even more pronounced for Th 1165a from 6.80 to 4.06 (Table).

In order to prove whether the diminution of affinity described was reversible or not, recooling experiments were performed. After 120 minutes at 42°C recooling to 25°C increased the affinities of the two sympathomimetics investigated, but not to their initial values at 25°C: recooling from 42°C increased the pD_2-value for IPN from 6.05 to 6.82 and for Th 1165a from 4.06 to 6.03. When the dose-response curve for IPN was repeated 5 h after recooling at 25°C, no further reshift occurred as is indicated by the pD_2-value of 7.03 ± 0.07 ($n = 7$).

These findings imply that the temperature-induced diminution of affinity consists of an irreversible and a reversible part. The irreversible decrease of affinity, which is defined by the difference of the dose-response curves at 25° and at 25°C after recooling, is of the same order of magnitude for both amines as can be derived from the dose-response curves for IPN and Th 1165a (Figure). The distances between the dose-response curves at 42° and at 25°C after recooling, determining the reversible part, show, on the contrary, a clear cut difference, i.e. the reversible part for Th 1165a amounts to about 2 decades, that for IPN less than one.

Since we observed that the diminution of affinity evoked by high temperature is partly due to an irrever-



Dose-response curves for isoprenaline (IPN) and Th 1165a on rabbit ileum at 25°C before (●—●) and after heating up the organ bath to 42°C (▲—▲), and at 42°C (○—○). Number of experiments are given in the Table.

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pD_2-values and intrinsic activity (α) of isoprenaline (IPN) and Th 1165a at 25°C after recooling from 42°C and at 42°C

		25°C	25°C after recooling from 42°C	42°C
IPN	pD_2-values	7.70 ± 0.08 (7)	6.82 ± 0.10[b] (7)	6.05 ± 0.11[b] (5)
	Intrinsic activity (α)	1	0.71	0.55
Th 1165a	pD_2-values	6.80 ± 0.11 (5)	6.03 ± 0.20[a] (5)	4.06 ± 0.11[b] (5
	Intrinsic activity (α)	1	0.94	0.90

pD_2-values: means ± S.E.; number of experiments in brackets. [a] $P < 0.05$; [b] $P < 0.001$ compared with 25°C.

sible diminution, it was of special interest to examine the intrinsic activities of the two sympathomimetics under our experimental conditions. At 25°C both amines relaxed the preparation completely, that means the intrinsic activity amounted to 1.0. Troughout the experimental procedure, no change of the intrinsic activity for Th 1165a occured (Table). The intrinsic activity for IPN, on the other hand, decreased to 0.55 at 42°C and was only partly restored to 0.71 after recooling.

Discussion. Our results showed that the shift to the right of the dose-response curves for IPN and Th 1165a caused by an elevation of temperature from 25° to 42°C could not be completely reversed, i.e. an irreversible shift of the dose-response curves for the β-sympathomimetics remained after recooling to 25°C. This could be due to a denaturation of the contractile apparatus or to an actual conformational change of the β-receptors on rabbit ileum. The denaturation can be excluded since papaverine showed neither a diminution of affinity nor any alteration of the intrinsic activity at high temperature on rabbit ileum (unpublished results). We would like to suggest, therefore, that β-receptor sites undergo irreversible conformational changes. That accounts not only for adrenergic β-receptors but also for receptors mediating the responses for angiotensin, vasopressin[10] and serotonin[10,11] since on rat fundus strip, recooling from 47° to 37.5°C showed an irreversible shift of their dose-response curves to the right and also a decrease of the intrinsic activities. On the other hand, heating is without any influence on the dose-response curves for acetylcholine, KCl and bradykinin[10]. These results are in favour of the existence of heat-labile and -stable receptors on smooth muscle of the digestive tract.

According to our results, the irreversible part of the diminution of the sympathomimetic affinity is nearly equal for both amines. This implies that the population of the β-receptors which undergo an alteration of their active sites by our procedure is of the same order of magnitude.

On the other hand, the extent of the reversible diminution of affinity showed great differences for both amines. This part is greater for Th 1165a than for isoprenaline. That was not surprising since the decrease of affinity for Th 1165a[12] evoked by high temperatures was much more pronounced than for IPN. These results are in accordance with the observation that preincubation with the metabolic inhibitor, iodoacetic acid, increased the affinity of Th 1165a to a higher degree than for isoprenaline[7].

From the present study the conclusion can be drawn that in rabbit ileum the decrease of affinity induced by alteration of temperature is not only dependent upon an increase of the metabolic rate, as for instance in the guinea-pig atrium[3], but in addition by an irreversible impairment of the β-receptor sites.

The alteration of the intrinsic activity observed for isoprenaline cannot be explained so far and needs further elucidation by experiments.

Zusammenfassung. Am Kaninchen-Ileum besteht die durch Temperaturerhöhung bedingte Rechtsverschiebung der Dosis-Wirkungs-Kurven von Isoprenalin und Th 1165a aus einem reversiblen und einem irreversiblen Anteil.

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Ascorbic Acid: Effect of High Doses on Brain and Heart Catecholamine Levels in Guinea-Pigs and Rats

The growing interest in megavitamin therapy for mental illness[1–4] has prompted us to study the effect of relatively high doses of ascorbic acid on catecholamine levels in the heart and brain of the guinea-pig, a species which like man, cannot synthesize its own ascorbic acid. THOA et al.[5] have shown that heart norepinephrine is decreased in the scorbutic guinea-pig while more recently LOKOSHKO and LESNYKH[6] have reported that in guinea-pigs with hypovitaminosis C, heart epinephrine is no different from normal healthy animals. In the present communication we report the effect of a relatively high dietary intake of vitamin C on heart and brain levels of catecholamines in the guinea-pig. By way of comparison, we also report the effect of a high dietary intake of ascorbic acid in the rat, a species which is capable of synthesizing its own ascorbic acid.

Materials and methods. Ascorbic acid-free powdered Reid-Briggs diet, purchased from General Biochemicals, Chagrin Falls, Ohio, was supplemented with vitamin C sufficient to supply guinea-pigs with 150 or 1000 mg/kg/day of ascorbic acid. For the rat studies, powdered Purina Rat Chow was supplemented with ascorbic acid so as to supply the animals with 1000 mg/kg/day. The diets were prepared by mixing in a rotary mixer for 3 h; fresh diet was prepared every 4 days. The animals were allowed food and water ad libitum. In these studies guinea-pigs and rats consumed 20–25 and 18–20 g of food per animal per day, respectively.

Male, Sprague-Dawley rats (200–250 g) or male, Hartley strain guinea-pigs (300–400 g) were used in our studies. When the guinea-pigs were received from the supplier, they were acclimated to our animal room for 10 days on a scorbutagenic diet which was supplemented with ascorbic acid so as to supply the animals with 150 mg/kg/day of the vitamin. This amount of ascorbic acid is approximately equivalent to the amount of vitamin C which guinea-pigs ingest when they are fed normal guinea-pig lab chow supplemented with a daily ration of lettuce. Rats were acclimated on standard Purina lab chow. The animals were then placed on the

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