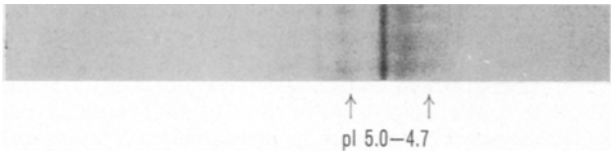

Fig. 3. Gel electrofocusing of N-acetyl-β-D-hexosaminidase according to AWDEH et al.[10]. The enzyme and carrier ampholytes (range pH 3–10) were set in the gel. Gels were removed and fixed in 5% trichloroacetic acid after focusing for 150 min at 50 mA (decreasing to 28 mA) at a voltage of 210 V (increasing to 1080 V). After the run, the pH gradient established was determined with a combined microsurface electrode.

During the purification procedure an appreciable amount of protein precipitated in the affinity gel. Despite this fact we had an overall recovery of more than 40% of the enzymatic activity.

The dialyzed fraction No. 13 was lyophilized and further characterized. On a Sephadex G 200 column, calibrated according to ANDREWS[8], the purified enzyme separated in 2 distinct peaks of MW 140,000 and 65,000 daltons. Most of the enzyme activity was associated with the peak of 140,000 daltons.

Polyacrylamide-gel electrophoresis[9] showed it still to be heterogenous and revealed 2 major bands in close vicinity (Figure 2.) After cutting the gel into 1 mm discs and eluting them with 0.1 *M* CPB pH 4.5, two fractions with enzyme activity were detectable, which corresponded with the protein bands in the gel. Isoelectrofocusing in thin-layer-polyacrylamide-gel[10] revealed microheterogeneity of the enzyme preparation, showing a pI region of pH 4.7–5.0 (Figure 3).

BULLOCK and WINCHESTER[11] and WINCHESTER[12] demonstrated several components of N-acetylhexosaminidase in ram epididymis by isoelectric focusing. They found in distinct anatomical sections of the ram epididymis different predominant N-acetylhexosaminidase components. In our case, using a *p*-aminophenyl-N-acetyl-β-D-thioglucosaminid as the inhibitor coupled to Sepharose 4 B, we gained a 35-fold purification of N-acetylhexosaminidase from boar epididymis.

With this method of affinity chromatography, we devized a technique to isolate minute amounts of N-acetylhexosaminidase to a fair degree of purity from the different segments of the boar epididymis and to study their possible significance on sperm maturation.

Zusammenfassung. Aus dem Nebenhoden vom Eber gewonnene N-Acetylhexosaminidase (EC 3.2.1.30) wurde mittels Affinitätschromatographie etwa 35fach gereinigt. An einen Träger gebundenes *p*-Aminophenyl-Thioglucosaminid diente als reversibles Adsorbens für das Enzym. Einige physicochemische Charakteristika wurden bestimmt: Molekulargewicht 140.000; pI-Bereich 4,7–5,0.

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Enhanced Calcium Accumulation Related to Increased Protein Phosphorylation in Cardiac Sarcoplasmic Reticulum Induced by Cyclic 3′, 5′-AMP or Isoproterenol

Catecholamines have been demonstrated to increase Ca-transport in cardiac sarcoplasmic reticulum (SR), as shown by EBASHI and ENDO[1], SHINEBOURNE et al.[2] and others. This effect involved the following sequence of processes: activation of adenylate cyclase, cyclic adenosine 3′, 5′-monophosphate (cAMP) synthesis and a cAMP and protein kinase (PK) EC.2.7.1.37 PK-mediated phosphorylation of SR protein[3–5]. The latter step is believed to be directly responsible for changes in the rate of calcium transport[6–8].

In the present study, evidence is given of the quantitative relationship between demonstration of the above sequence of processes in vitro, when applying cAMP to the isolated vesicles of SR and the effects of in vivo application of isoproterenol (ISO).

Material and methods. Male adult mongrel dogs were used, injected with a single dose of 7.5 mg/kg ISO in saline. Cardiac microsomes were prepared from the ventricles by the method of HARIGAYA and SCHWARTZ[9]. Electron microscopic examination of the membranes showed homogenous vesicles without evidence of mitochondria.

The activities of the marker enzymes glucose-6-phosphatase EC.3.1.3.9, 5′-nucleotidase EC.3.1.3.5 and cytochrome c oxidase EC.1.9.3.1 were determined according to the method of BERGMEYER[10], MUIR et al.[11] and SMITH and CAMERINO[12].

The activities of these enzymes in preparations of high purity showed no significant differences ($p \geqslant 0.05$) between the preparations isolated from the controls and from

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hearts influenced by ISO. Lack of mitochondrial contamination was also demonstrated by the insensivity of the Ca binding (Table I) to 5 m*M* azide and 2.5 µg/ml oligomycin[13].

Ca-binding (in absence of oxalate) and Ca-uptake (in presence of 2.5 m*M Tris* oxalate) was measured according to KATZ and REPKE[5] by the Millipore filter technique, using for incubation 40 and/or 10 µg of protein/ml at 25 °C and 37 °C respectively. The protein content of the preparations was established according to LOWRY's method[14].

Results and discussion. In our experiments we have found double the original amount of Ca-binding and Ca-uptake by SR 2 h after ISO application in vivo (Table II).

Our results concerning Ca-uptake are in agreement with those of others[2, 5, 15]. The increase of Ca-binding may be explained by nonspecific high-affinity binding sites involved in or coupled with some Ca^{2+}-phosphate complex formation[16, 17]. In experimental conditions similar to those under which the ISO-induced changes in Ca-accumulation by cardiac SR were studied, the Ca^{2+}-independent cAMP- and PK-mediated phosphorylation of the SR protein reached a 3-times higher value (Table II, Figure B) in comparison with the extent of Mg^{2+}-dependent phosphorylation, i.e. the self-phosphorylation of this protein in the controls (Table II, Figure A). The addition of beef heart PK did not appreciably influence the rate and the extent of phosphorylation (Figure A and

Table I. Influence of oligomycin and azide on the calcium binding activities of heavy microsomes (sarcoplasmic reticulum) isolated from control and ISO-treated dog hearts

	Calcium binding (% of values without inhibitors)	
	Oligomycin (2.5 µg/ml)	Sodium azide (5 m*M*)
Control	94 ± 3.6	98 ± 0.2
2 h ISO	95 ± 3.4	97 ± 1.8

The results are means ± SE of 3 experiments. Microsomes (sarcoplasmic reticulum) 40 µg protein/ml were incubated at 25 °C in a medium containing 100 m*M* KCl, 10 m*M* $MgCl_2$, 4 m*M* Na-ATP, 20 m*M Tris*-HCl, pH 6.8, and 0.1 m*M* $Ca^{45}Cl_2$. The incubation time was 5 min. The inhibitors were added 2 min before starting the reaction by ATP; it was terminated by Millipore filtration at the times indicated above.

Table II. Ca-binding, Mg^{2+}-dependent phosphorylation and Mg^{2+}-dependent and exogenous cAMP-stimulated phosphorylation of the membrane protein (SR)

	Control	2 h ISO
Ca-binding (nmoles Ca^{2+}/mg^{-1} protein/5 min incubation)	70 ± 8	140 ± 15
Ca-uptake (nmoles Ca^{2+}/mg^{-1} protein/30 min incubation)	3300 ± 150	6318 ± 210
Mg^{2+}-dependent phosphorylation (nmoles P_i incorporated/mg^{-1} protein)	0.202	0.638
Mg^{2+}-dependent cAMP-stimulated phosphorylation (nmoles P_i incorporated/mg^{-1} protein)	0.500	0.701

The results are means ± SE of 3–5 experiments. Experimental conditions for measurement of Ca-binding (see Table I). Ca-uptake was measured in the same medium as Ca-binding, however the incubation time was 30 min at 37 °C with 10 µg membrane protein/ml. ^{32}P-incorporation into SR protein was measured in conditions for Ca-binding in the following medium: 100 m*M* KCl, 15 m*M* $MgCl_2$, 4 m*M* ATP (γ-^{32}P), 2 m*M* EGTA, 0.2 mg/ml beef heart protein kinase, 0.15 µ*M* cAMP and 40 µg/ml of membrane protein. Reaction was stopped with 10% TCA + 1 m*M* KH_2PO_4 and labelled protein washed and counted using a Packard Tri-Carb Liquid Scintillation Spectrometer.

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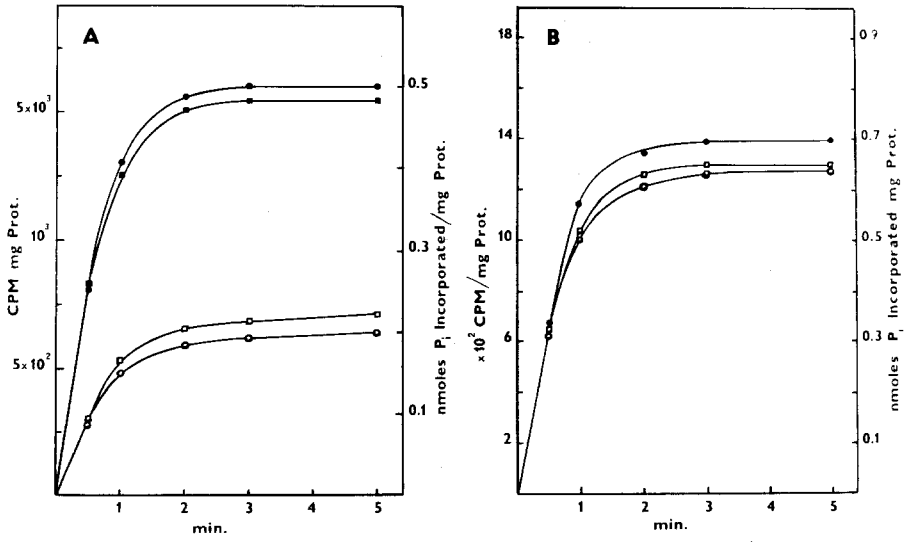

Left hand panel A). Time dependence of phosphorylation of the microsomal fraction enriched with SR in the control group. Symbols used for various additions: ●, cAMP + PK; ○, PK; ■, cAMP; □, basal. Right hand panel B). Time dependence of phosphorylation of the microsomal fraction enriched with SR 2 h after isoproterenol application. Symbols used for various additions: ●, cAMP + PK; ○, PK; □, basal. Experimental conditions are described in Table II.

B). On the contrary, addition of cAMP without exogenous PK stimulated phosphorylation almost to the same extent as cAMP and PK together (Figure A). This finding may serve as evidence for sufficiently high endogenous PK activity in our preparations.

The cAMP concentration responsible for the half maximal stimulation of the Mg^{2+}-dependent phosphorylation of cardiac SR in the controls in vitro amounted to 0.15×10^{-6} *M*. The corresponding increase of phosphorylation represented the 2.5-fold of the basic Mg^{2+}-dependent value (Figure A).

In preparations from hearts pretreated with ISO in vivo, phosphorylation without addition of cAMP or PK exceeded by 30% the extent of the half maximal stimulation reached with cAMP in the controls in vitro. However, this phosphorylation would represent not only the basic Mg^{2+} but, to a certain extent, also a cAMP-dependent incorporation of ^{32}P into phosphoserine[18].

However, subsequent addition of 0.15×10^{-6} cAMP to preparations from ISO-treated hearts increased phosphorylation by a further 8% only (Figure B). This poor response to exogenous cAMP suggests a stimulatory effect on the cAMP-producing systems obtained already by the application of ISO in vivo. The latter stimulatory effect corresponds to an amount of cAMP produced, which would stimulate the phosphorylation obtained by in vitro application of cAMP to the controls, approximately by 74% of the maximal stimulation (Figure A).

The present results appear to confirm the validity of our supposition concerning the mechanism of regulating the SR calcium transport by catecholamines (isoproterenol) both in vivo and in vitro.

Zusammenfassung. Die cAMP-abhängige Inkorporation von ^{32}P in das sarkoplasmatische Reticulum (SR) wurde im Zusammenhang mit Ca-Akkumulation in Kontrollherzen und in Herzen 2 h nach in vivo Verabreichung von 7,5 mg/kg Isoproterenol gemessen und gezeigt, dass die Katecholamine den intrazellulären Kalziumtransport in vitro sowie in vivo über die cAMP-stimulierte und PK-abhängige Phosphorylierung einer Proteinfraktion des SR regulieren.

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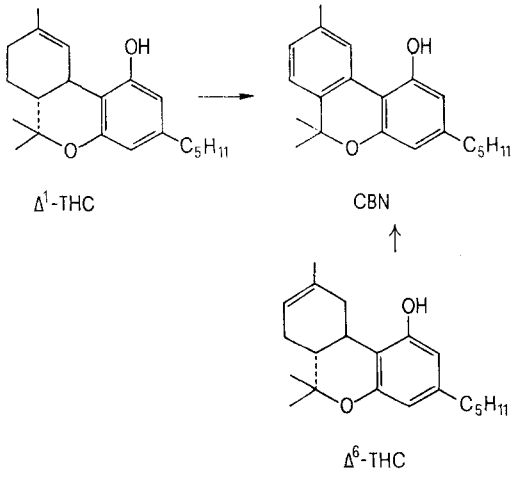
Cannabinol: a Rapidly Formed Metabolite of Δ^1- and Δ^6-Tetrahydrocannabinol

The primary metabolic pathway of Δ^1-tetrahydrocannabinol (Δ^1-THC) and Δ^6-THC is considered to be a mono-oxygenation, mostly at allylic positions[1]. Recently, several indications that cannabinol (CBN) and its oxidized derivatives may be Δ^1-THC metabolites have also been reported. Relatively large amounts of CBN were found[2] by one of us (N. McC.) in the blood of Δ^1-THC smokers. Later, WIDMAN et al.[3] observed high concentrations of CBN relative to Δ^1-THC (but in less than 0.1% overall yield) in rat bile after the i.v. injection of Δ^1-THC. BEN ZVI et al.[4] have isolated CBN-7-oic acid from the urine of rhesus monkeys administered with Δ^1-THC.

It has been shown[5] with rats that the presence of CBN in administered Δ^1-THC causes an increased rate of disappearance of Δ^1-THC from the blood. This results in higher levels of CBN relative to Δ^1-THC than would be expected from the metabolism of the pure compounds. The possibility that both MCCALLUM[2] and WIDMAN et al.[3] could have been observing a similar phenomenon, combined with the fact that the amount of CBN determined in blood by both these authors was less than the amount of CBN administered as an impurity in the Δ^1-THC, prompted us to do a careful re-evaluation of CBN as a possible Δ^1-THC metabolite.



Methods and materials. Male rats were injected with the cannabinoid (in 5–10 µl propylene glycol) via a tail vein. Their blood was collected and mixed with heparin and the internal standard[6]; it was then extracted with light petroleum, acidified (1 *N* HCl), reextracted and centrifuged[2]. The petroleum fraction was analyzed by gas chromatography (GLC) (glass column filled with 4% SE 30 on Gas Chrom Q 100 mesh, at 200°, with 60 ml/min nitrogen carrier gas using flame ionization detection). Calibration

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