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STUDIES OF SODIUM-POTASSIUM ACTIVATED ADENOSINE TRIPHOSPHATASE

VII—INHIBITION BY ERYTHROPHLEUM ALKALOIDS

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Abstract—Erythrophleine and cassaine, two alkaloids occurring in the bark, leaves, and seeds of the genus Erythrophleum, were shown to be potent inhibitors of the digitalis-sensitive Na–K activated adenosine triphosphatase (Na–K ATPase) of rabbit brain, kidney, and ciliary body and cat choroid plexus. At 10^{-4} M concentration, erythrophleine inhibited Na–K ATPase activity in all four tissues completely, while cassaine at 10^{-4} M inhibited Na–K ATPase activity in the same tissues an average of 86 per cent. Neither of these compounds caused significant inhibition of Mg-activated ATPase activity.

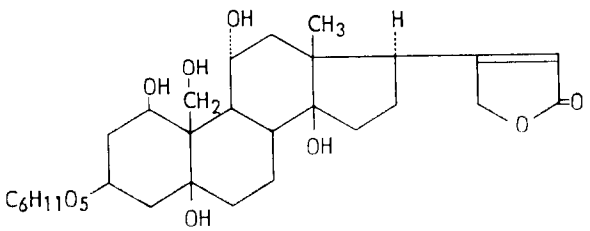
The inhibition curves for erythrophleine and cassaine in rabbit brain had the same general shape as the curve for ouabain. The negative logarithms of the molar inhibitor concentration at which 50 per cent inhibition of Na–K ATPase activity occurs were 6·58 for erythrophleine, 5·96 for ouabain, and 5·28 for cassaine. All three compounds caused a stimulation of approximately 6 per cent at about 1/300 of their half-maximal inhibition concentrations.

Increasing the K concentration in the assay medium from 0 to 40 mM caused 75 per cent reversal of the inhibition of rabbit brain Na–K ATPase by 5×10^{-7} M erythrophleine as well as by 5×10^{-7} M ouabain. No reversal was obtained of the Na–K ATPase inhibition by 10^{-4} M erythrophleine and ouabain.

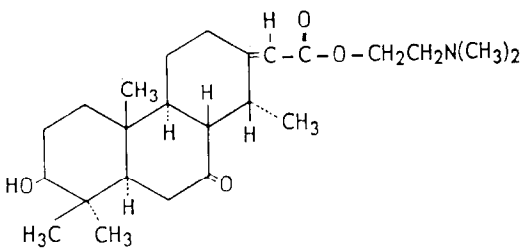
It is concluded that the similarity of the effects of the erythrophleum alkaloids and the digitalis glycosides on the Na–K ATPase system may explain the remarkable similarity between these two groups of compounds in pharmacological properties and in inhibitory effects on active cation transport.

A DIGITALIS-SENSITIVE Na–K activated adenosine triphosphatase system (Na–K ATPase) was first described by Skou,[1, 2] who postulated its relation to the Na–K transport system across the nerve membrane. Post *et al.*[3] and Dunham and Glynn[4] demonstrated the presence of this enzyme in the erythrocyte membrane and its relationship to the erythrocyte cation transport system. Studies in this laboratory have shown the wide distribution of the enzyme in animal tissues[5] and its probable role in the secretion of aqueous humor in cat and man.[6, 7] The enzyme was demonstrated in all 21 tissues with a digitalis-sensitive cation transport system.[8] A close correlation between active cation flux and Na–K ATPase activity over a 25,000-fold range was demonstrated in human erythrocytes, frog toe muscle, squid axone, frog skin, toad bladder, and electric eel electroplax.[9, 10] In further studies the Na–K ATPase system was shown to be closely related to the active cation transport system in the lens.[11] Its apparent involvement in the secretion of cerebrospinal fluid has been demonstrated more recently.[12]

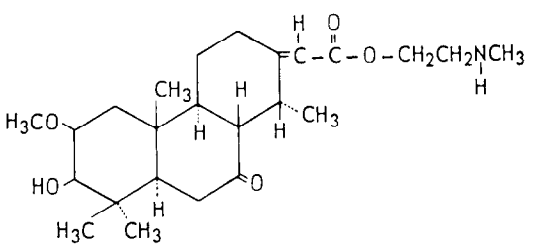
A group of alkaloids, occurring in the bark, leaves, and seeds of the genus Erythrophleum, has pharmacological properties remarkably similar to those of the digitalis compounds,[13] while differing markedly in chemical structure (Fig. 1). Ascher[16] noticed that instillation and subconjunctival injection of cassaine and erythrophleine



Ouabain ($C_{29}H_{44}O_{12}$)



Cassaine ($C_{24}H_{39}O_4N$)



Erythrophleine ($C_{24}H_{39}O_5N$)

FIG. 1. Chemical structure of ouabain and the erythrophleum alkaloids cassaine and erythrophleine. While the structure of cassaine has been established with considerable certainty,[13, 14] there is still some doubt concerning placement and configuration of the ring constituents in erythrophleine.[15]

in the eye caused a drop in the intraocular pressure. In view of the demonstrated role of Na–K ATPase in the secretion of aqueous humor, the effect on the intraocular pressure might indicate that the erythrophleum alkaloids, like the digitalis compounds, inhibit Na–K ATPase activity. This assumption was further strengthened by the recent observation of Kahn[17, 18] that these compounds inhibit active accumulation of potassium by human erythrocytes.

In this report evidence is presented that the remarkable similarity in pharmacological properties between the erythrophleum alkaloids and the digitalis compounds is indeed paralleled by an equally striking similarity in behavior toward Na–K ATPase.

METHODS

Tissues used were brain, kidney, and ciliary body from adult white rabbits, and choroid plexus from the lateral and fourth ventricles of adult cats. All tissues were taken immediately after sacrificing the animal. The rabbit tissues were homogenized in water (10% w/v) in all-glass Potter-Elvehjem tissue grinders. Aliquots of each homogenate were lyophilized at −20° and stored at −25° until use. Frozen-dried homogenates of rabbit brain, kidney, and ciliary body were reconstituted with water to contain respectively 6, 6, and 35 mg original wet weight of tissue/ml. Cat choroid plexus was frozen-dried, and immediately before use homigenized with water to contain 3 mg frozen-dried tissue/ml. Of each reconstituted homogenate 10 μl was added to 150 μl of substrate medium.

Stock solutions, 10 mM, were prepared of ouabain (USP grade, Nutritional Biochemicals Corp., Cleveland, Ohio) and erythrophleine sulfate (gift of E. Merck A.G., Darmstadt, Germany) in water, and of cassaine base (gift of E. Merck A.G.) in 0·01 N HCl.

For every tissue and medium, six 10-μl aliquots were run; three in every case were not incubated (enzyme blanks); the others were incubated for 1 hr at 37°. Trichloroacetic acid (10% w/v) was added, and the free inorganic phosphate in the supernatant was determined as described previously.[5] Neither erythrophleine and cassaine nor ouabain at 10^{-4} M concentration in the substrate medium had any effect on blank readings or phosphate standard readings.

The composition of the various substrate media is shown in Table 1. In Table 2 the relative ATPase activity for each tissue in these media is shown with the absolute

TABLE 1. SUBSTRATE MEDIA USED FOR THE ASSAY OF ATPASE*

	A	B	C	D	E
ATP	2	2	2	2	2
Mg^{2+}	1	1	1	1	1
K^+	5	5			9
Na^+	58	58	63	62	
CN^-	10	10	10	10	5
EDTA	0·1	0·1	0·1	0·1	0·1
Tris	92	91	92	91	147
Ouabain		0·1		0·1	
Erythrophleine	0·1 mM, indicated by Er after medium symbol				
Cassaine	0·1 mM, indicated by Ca after medium symbol				

* All concentrations in millimolar final concentration; only other ionic species present, Cl^-. Osmolarity 313 mOsm; pH 7·5.

activities found for each tissue. Medium A, after correction for the enzyme blank, gave the total ATPase activity; i.e. Na–K ATPase + Mg ATPase activity. The inhibition in media B, C, D, and G was approximately equal, indicating that in each of these tissues the inhibited ATPase activity indeed represents the ouabain-sensitive, Na-K activated ATPase. For each tissue the inhibition in medium C (K-free) was slightly less than in the other inhibitory media, presumably owing to partial activation of Na-K ATPase by the small amount of tissue K present in the incubation mixture. Medium G (Na-free) gave slightly more inhibition than the other media, which might

be due to the presence of a small amount of Na-stimulated, nondigitalis-sensitive ATPase.[19] It was consequently felt that the inhibited ATPase activity in medium D most closely approximated the Na–K ATPase activity, and this medium was therefore used in most experiments together with medium A.

TABLE 2. RELATIVE AND ABSOLUTE ATPASE ACTIVITIES OF TISSUES USED IN THIS STUDY

	Rabbit brain (%)	Rabbit kidney (%)	Rabbit ciliary body (%)	Cat choroid plexus (%)
Medium A (complete)	100	100	100	100
Medium B (10^{-4} M ouab.)	24·6 ± 1·0	40·2 ± 2·0	70·5 ± 1·0	65·7 ± 2·5
Medium C (no K)	32·4 ± 2·0	50·7 ± 3·7	76·2 ± 0·9	73·6 ± 2·8
Medium D (no K, + ouab.)	24·0 ± 1·3	37·3 ± 2·1	71·1 ± 1·0	65·4 ± 3·5
Medium G (no Na)	19·0 ± 2·2	30·2 ± 1·9	55·4 ± 1·8	60·8 ± 2·9
Average B, C, D, G	25·0 ± 2·8	39·6 ± 4·3	68·3 ± 4·9	66·4 ± 2·7
	(moles/kg/hr)	(moles/kg/hr)	(moles/kg/hr)	(moles/kg/hr)
Total ATPase	1·78	1·86	0·694	3·54
Mg ATPase	0·45	0·74	0·474	2·35
Na–K ATPase	1·33	1·12	0·220	1·19

In determining the inhibition of Na–K ATPase and Mg ATPase activities by 10^{-4} M erythrophleine and cassaine, media A, D, AEr, DEr, ACa, and DCa were used. The relative inhibitions of Mg ATPase and Na–K ATPase were calculated for each tissue as follows:

$$\text{per cent inhibition of Mg ATPase} = \left(1 - \frac{\text{activity in medium DEr or DCa}}{\text{activity in medium D}}\right) \times 100$$

$$\text{per cent inhibition of Na–K ATPase} = \left(\frac{1 - \text{activity in medium AEr or ACa} - \text{activity in medium DEr or DCa}}{\text{activity in medium A} - \text{activity in medium D}}\right) \times 100$$

Inhibition curves were determined for each of three compounds on rabbit brain Na–K ATPase. Reconstituted rabbit brain homogenate (6 mg wet wt/ml) was added to each substrate medium. The media used were: medium A (6 aliquots, 3 incubated, 3 nonincubated), medium D (6 aliquots, 3 incubated), and a series of media consisting of medium A to which had been added 10^{-8}, 3×10^{-7}, 10^{-7}, etc. to 10^{-4} M inhibitor (3 aliquots of each, except for the last one in which 3 nonincubated aliquots were included). The rest of the assay procedure was the same as described above. The relative Na–K ATPase activity at each inhibitor concentration was calculated as follows:

$$\text{Relative Na–K ATPase activity} = 100 \times \frac{\text{activity in medium A with added inhibitor} - \text{activity in medium D}}{\text{activity in medium A} - \text{activity in medium D}}$$

These relative Na–K ATPase activities were then plotted against the negative logarithm of the molar inhibitor concentration.

To determine whether increase of the K concentration would reverse the inhibition of Na–K ATPase activity by erythrophleine and ouabain, rabbit brain was incubated in medium C with 0, 5, 20, 30, and 40 mM K, the same media with 5×10^{-7} and 10^{-4} M ouabain, and the same media with 5×10^{-7} and 10^{-4} M erythrophleine. The conditions of the incubation and the colorimetric phosphate determination were the same as before. At each K concentration the per cent inhibition of the total ATPase activity (in media without added inhibitor) was calculated for each of the two inhibitors at the two different concentrations.

RESULTS

The relative inhibition of Na–K ATPase and Mg ATPase by 10^{-4} M erythrophleine and cassaine in all four tissues is shown in Table 3. Erythrophleine in this concentration caused complete inhibition of Na–K ATPase activity. There was no significant

TABLE 3. EFFECT OF ERYTHROPHLEUM ALKALOIDS ON ATPASE ACTIVITY*

	Erythrophleine, 10^{-4} M		Cassaine, 10^{-4} M	
	Na–K ATPase inhibition (%)	Mg ATPase inhibition (%)	Na–K ATPase inhibition (%)	Mg ATPase inhibition (%)
Brain (rabbit)	100·0 ± 1·8	−2·7 ± 2·6	81·0 ± 1·6	4·7 ± 5·3
Kidney (rabbit)	108·8 ± 5·9	−2·3 ± 1·3	88·0 ± 6·7	0·0 ± 2·2
Ciliary body (rabbit)	102·9 ± 27·2	6·8 ± 9·1	90·8 ± 2·6	0·8 ± 2·8
Choroid plexus (cat)	94·2 ± 6·1	6·3 ± 1·4	84·9 ± 2·4	5·1 ± 8·0
Average	101·5 ± 3·0	2·0 ± 2·6	86·2 ± 2·1	2·7 ± 1·3

* Each entry represents the mean of three determinations with the standard error, except for kidney, erythrophleine, where the mean of six determinations is listed.

inhibition of Mg ATPase activity except in the choroid plexus, where the effect was small (6·3 per cent). Cassaine at 10^{-4} M inhibited Na–K ATPase activity almost completely (average 86 per cent) but caused no significant inhibition of Mg ATPase activity. The extent of inhibition of Na–K ATPase activity was about the same for all four tissues.

The possible existence of an additive effect of the erythrophleum alkaloids and ouabain was studied by comparing the ATPase activities of rabbit brain and kidney in media A, D (no K, 10^{-4} M ouabain), and G (no Na). The results in Table 4 fail to indicate the presence of such an effect. The fact that the addition of 10^{-4} M erythrophleine and cassaine to medium D did not further decrease ATPase activity suggests that medium D fully inhibited Na–K ATPase activity. Hence the lower activities in medium G would be due to a slight inhibition of Mg ATPase activity in the absence of Na.

Inhibition curves for ouabain, erythrophleine, and cassaine in rabbit brain are given in Fig. 2. The general shape of the curves is quite similar. The negative logarithms of the molar inhibitor concentration at which 50 per cent inhibition of Na–K ATPase activity occurs (pI_{50}) are 6·58 for erythrophleine, 5·96 for ouabain, and 5·28 for cassaine. This means that rabbit brain Na–K ATPase is 4·2 times as sensitive to erythrophleine as to ouabain, but 4·8 times less sensitive to cassaine than to ouabain. It

will be noted that with 10^{-4} M cassaine 16% Na–K ATPase activity remained, a figure which is in good agreement with the 81 per cent inhibition shown for brain Na–K ATPase in Table 3. With 10^{-4} M erythrophleine no significant activity remained (1·4 ± 1·7%), in agreement with the 100 per cent inhibition for brain shown in Table 3.

TABLE 4. INHIBITORY EFFECT OF 10^{-4} M ERYTHROPHLEINE AND CASSAINE ON RABBIT BRAIN AND KIDNEY ATPASE ACTIVITY* IN DIFFERENT SUBSTRATE MEDIA

	Medium A (complete)	Medium D (no K, 10^{-4} M ouab.)	Medium G (no Na)
Brain, control	1·85 ± 0·02	0·45 ± 0·01	0·36 ± 0·01
erythrophleine	0·47 ± 0·01	0·47 ± 0·01	0·43 ± 0·01
cassaine	0·53 ± 0·01	0·52 ± 0·02	0·38 ± 0·01
Kidney, control	1·80 ± 0·03	0·67 ± 0·01	0·54 ± 0·01
erythrophleine	0·71 ± 0·01	0·68 ± 0·01	0·59 ± 0·01
cassaine	0·74 ± 0·01	0·69 ± 0·01	0·58 ± 0·01

* Expressed in moles per kilogram wet weight per hour; means with standard error for three determinations.

An interesting observation is the slight stimulation of Na–K ATPase activity by all three compounds at 1 to 5 × 10^{-8} M concentration. It was statistically significant in the case of ouabain (8·6 ± 0·3%), but not for erythrophleine (3·6 ± 2·7%) and cassaine (2·6 ± 1·9%). In an an additional experiment the stimulatory effect of the three compounds was determined at about 1/300 of their half-maximal inhibition concentration on rabbit brain Na–K ATPase activity. In this case all three compounds

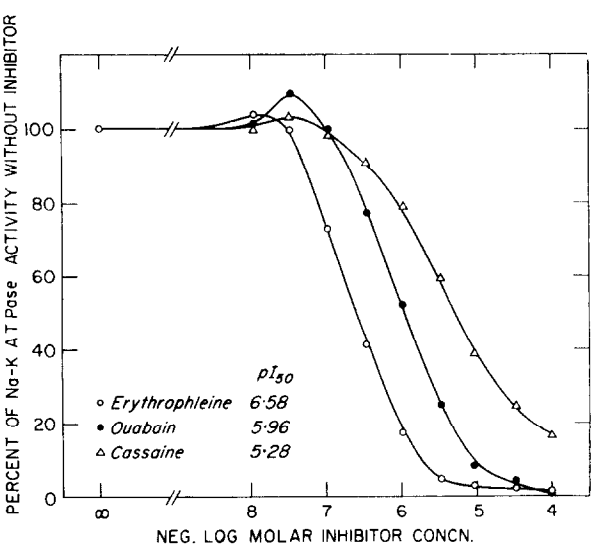


FIG. 2. Inhibition curves of rabbit brain Na–K ATPase for ouabain, erythrophleine, and cassaine. The negative logarithms of the half-maximal inhibition concentrations are listed in the bottom left corner.

caused significant stimulation ($P < 0{\cdot}05$) in triplicate determinations: ouabain (3×10^{-9} M), $5{\cdot}4 \pm 1{\cdot}6\%$; erythrophleine ($10^{-9}$ M), $6{\cdot}6 \pm 1{\cdot}7\%$; cassaine ($2 \times 10^{-8}$ M), $6{\cdot}7 \pm 2{\cdot}0\%$. No significant changes were found in the Mg ATPase activity. Further examples of this stimulation of Na–K ATPase activity at low concentrations of ouabain have been found more recently in this laboratory in cat choroid plexus[20] and herring gull salt gland[21]. It is unlikely for the following reasons that these stimulatory effects of low concentrations of ouabain or erythrophleum alkaloids are due to inhibition of phosphorylative activity in the brain homogenates. All substrate media contained cyanide (10 mM in media A–D, 5 mM in medium G), which inhibits oxidation.[22] Addition of 10^{-5} M dinitrophenol, which uncouples oxidative phosphorylation,[23] had no effect on total and Mg ATPase activities in the brain and kidney homogenates. Addition of 10 mM fluoride, which inhibits adenylate kinase,[24] caused partial inhibition of the brain and kidney ATPase activity, rather than the apparent stimulation expected if appreciable adenylate kinase activity were present in the assay systems. The low homogenate concentrations (0·4 mg wet wt brain/ml final concentration) and the absence of added substrates for phosphorylative reactions (except for the gradual formation of up to 0·8 mM ADP during the assay) further support this conclusion.

The graphs of Fig. 3 show that increasing the K concentration in the medium from 0 to 40 mM almost completely reversed the inhibition of Na–K ATPase by 5×10^{-7} M

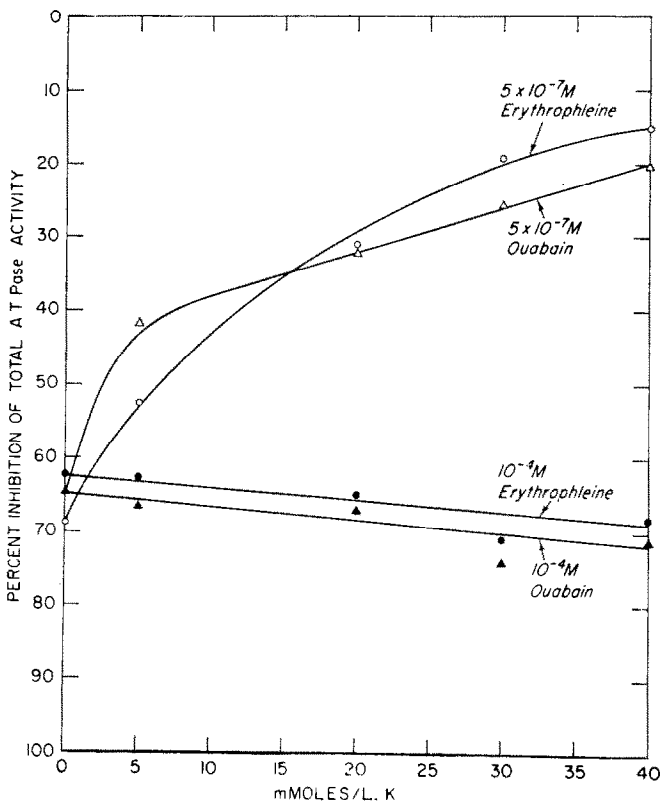


FIG. 3. Effect of increasing K concentration on the inhibition of rabbit brain Na-K ATPase by ouabain and erythrophleine.

erythrophleine as well as by 5×10^{-7} M ouabain. No reversal was obtained of the complete inhibition of Na–K ATPase activity by 10^{-4} M erythrophleine and ouabain. Note that per cent inhibition of total ATPase activity was plotted as the ordinate, hence 65 per cent inhibition represents approximately complete inhibition of Na–K ATPase activity. Thus the remaining 15 to 20 per cent inhibition at 40 mM K concentration represents about one quarter of complete Na–K ATPase inhibition. At 0 mM K the two sets of curves have converged to the same ordinate value of about 65 per cent inhibition, because in the absence of added K, complete inhibition of Na–K ATPase activity is obtained with as little as 5×10^{-7} M inhibitor.

DISCUSSION

The powerful digitalis-like cardiotoxic activity of the erythrophleum alkaloids was first noted by the discoverers, Gallois and Hardy.[25–27] Chen *et al.*[28] confirmed this finding, noting that intravenous injection of erythrophleine in the cat caused P-R prolongation, bradycardia, ectopic rhythm, secondary tachycardia, and finally ventricular fibrillation. Maling and Krayer[29] demonstrated that cassaine, erythrophleine, and coumingine (another erythrophleum alkaloid) caused a positive inotropic action in the dog heart–lung preparation in doses below those that cause cardiac irregularities. There is thus a remarkable similarity between the cardiac effects of digitalis glycosides and erythrophleum alkaloids.

In recent years ever-increasing numbers of cases have been reported of a sensitive and specific inhibition of active cation transport by digitalis glycosides (see Ref. 8 for references). Kahn[17, 18] has recently demonstrated that the erythrophleum alkaloids coumingine and cassaine have a similar effect on active K uptake in human erythrocytes. A close correlation between the occurrence of digitalis-sensitive cation transport and of the enzyme Na–K ATPase has been demonstrated.[8] In the present study it is shown that the erythrophleum alkaloids also inhibit this enzyme in brain, kidney, and ciliary body of rabbit and in cat choroid plexus. Neither compound had a significant effect on Mg ATPase activity in this concentration. The inhibition curves for erythrophleine and cassaine were similar to the curve for ouabain. All three compounds showed a slight stimulation (approximately 6 per cent) of Na–K ATPase activity at about 1/300 of their half-maximal inhibition concentration. A 3 to 6 per cent stimulation of active K uptake by erythrocytes was noted for similarly low concentrations of coumingine and cassaine by Kahn,[18] who mentions that K. Repke has observed stimulation of Na–K ATPase activity by low concentrations of digitalis glycosides. The reversal by K of the inhibitory effect of ouabain and erythrophleine on Na–K ATPase is another point of similarity between the two classes of compounds. Dunham and Glynn[4] showed K reversal of the inhibition of erythrocyte ghost Na–K ATPase by strophanthin. Raising the extracellular K concentration also reversed the inhibition by digoxin and scillaren A of the K influx in erythrocytes.[30] Intravenous administration of K counteracts cardiac glycoside toxicity in man.[31] The occurrence of K antagonism in these different effects of digitalis glycosides and erythrophleum alkaloids may be an indication that Na–K ATPase inhibition is involved in both the cardiac and noncardiac effects of the two types of compounds.

The erythrophleum alkaloids have one pharmacological property which the digitalis glycosides do not possess: they are potent local anesthetics. This was the basis of the

use of cassaine sulfate in dental practice under the name Nervocidin.[32] The side chain of the erythrophleum alkaloids has the alkylated aminoethanol ester structure (Fig. 1) which is typical of local anesthetics like procaine. This could possibly explain why the erythrophleum alkaloids have a local anesthetic effect. The side chain is also essential for the cardiac activity of erythrophleine because removal of this group by hydrolysis results in loss of pharmacological activity.[29] It is interesting to note that procaine inhibits active Na transport in the frog skin,[33] since this transport appears to be mediated by the Na–K ATPase system.[10] In the toad bladder, where the active Na transport also appears to be mediated by the Na–K ATPase system, erythrophleine and tetracaine inhibited both Na transport and the enzyme system.[34]

The local anesthetic effect of cassaine sulfate (Nervocidin) led Ascher[16] to test this compound as a corneal anesthetic. In the course of his experiments he noticed a severe decrease in intraocular pressure, both upon instillation and upon subconjunctival injection of 0·2 to 0·5 mg cassaine sulfate (0·1 % solution) in the eye of rabbit, dog, and man. The effect lasted from 5 to 23 days and occurred only in the treated eye, but not in the contralateral control eye. The maximal decrease in intraocular pressure in man was equivalent to 85 per cent inhibition of flow. He ascribed the effect to reversible 'poisoning' of the site of aqueous humor production. Evidence of a role of the ciliary body Na–K ATPase system in the secretion of aqueous humor has been presented.[6, 7] Becker[35] recently demonstrated severe and long-lasting inhibition of aqueous secretion by injection of 0·1 to 0·5 μg ouabain into the vitreous humor of the rabbit eye. A correlated inhibition of ciliary epithelial Na–K ATPase activity was found in this case (Bonting and Becker, unpublished observations). In view of the inhibition of ciliary body Na–K ATPase activity by erythrophleine and cassaine here reported, it would seem reasonable to explain Ascher's findings as an inhibition of this enzyme system. These findings suggest that the similarity of the inhibitory effects of the erythrophleum alkaloids and the digitalis glycosides on the Na–K ATPase system are responsible for the striking similarity in their pharmacological properties and in their inhibitory effects on active cation transport.

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