Biochemical Pharmacology, Vol. 26, pp. 589–594. Pergamon Press, 1977. Printed in Great Britain.

$(Na^+ + K^+)$ATPase INHIBITION BY *PALYTHOA* EXTRACTS—

CHEMICAL NATURE OF THE INHIBITOR AND KINETICS OF INHIBITION

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(*Received* 11 *August* 1975; *accepted* 2 *August* 1976)

Abstract—Crude toxic extracts obtained by ethanol extraction from the coelenterate *Palythoa caribaeorum* were shown to possess strong $(Na^+ + K^+)$ATPase inhibitory activity on enzyme preparations from the electroplax of *Electrophorus electricus*. The toxic and inhibitory effects were found to be separable. Chromatographic, spectrophotofluorimetric, electrophoretic and biological data demonstrate that the inhibitor is serotonin. It is a non-competitive inhibitor for Na^+ and ATP but is a competitive inhibitor for K^+. In enzyme preparations of a specific activity of 1.5 μM P_i/min, I_{50} is of the order of 1 mM.

In the course of our studies with crude preparations of palytoxin, a powerful marine toxin isolated from the zoanthid *Palythoa caribaeorum* [1], we were able to detect strong inhibitory effects on ouabain-sensitive ATPase isolated from the electric organ of *Electrophorus electricus*. Using crude extracts of ciguatoxin, another powerful marine toxin, Rayner and Szekerczes [2] reported a similar observation. They failed to find a correlation between toxicities of the extracts and inhibitory activity. After a similar experience with crude palytoxin, we were able to separate the toxic constituent from the ATPase inhibitor, which was shown to be an indole [3]. Other indoles, such as the hallucinogenic drugs harmaline, harmalol, harmine and LSD, are known to inhibit NaK ATPase from several tissues. Among this group, harmaline has been studied in greater detail [4].

Because of the wide distribution of indoles in marine animals, studies were undertaken to identify the inhibitor, study its effects on the kinetics of $(Na^+ + K^+)$ATPase and attempt to explain its mechanism of action. An enzyme preparation isolated from *E. electricus* was used for these studies.

MATERIALS AND METHODS

Trizma–HCl, Trizma-Base, ouabain, ATP, tryptophan (TRY), 5-hydroxytryptamine (5-HT, serotonin), 5-methoxytryptamine (5-MeO-T), 5-methoxyindoleacetic acid (5-MeO-IAA), 6-hydroxytryptamine (6-HT), *N*-acetyl-5-hydroxytryptamine (*N*-acetyl-5-HT), *p*-nitrophenylphosphate (pNPP) and CM-Cellulose were obtained from Sigma; 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptophan (5-HTRY) were obtained from Pfaltz and Bauer; and indole-3-acetic acid (IAA) was obtained from Nutritional Biochemicals. All other reagents were either U.S.P. or C.P. grade. Sephadex G-15 was a product of Pharmacia. Fluorescence measurements were done in a Perkin–Elmer model 204 spectrophotofluorometer. Ultraviolet measurements were done using either a Cary 17 or a Gilford 2400 spectrophotometer.

Precoated Silica gel plates from Kontes/Quantum and polyethylimine P EI-Cellulose plates from Brinkman were used for thin-layer chromatography (t.l.c.).

Ascending paper chromatography (p.c.) was done using Whatman 3 MM paper and butanol–acetic acid–water (4:1:5, superior phase) as the solvent system. Chromatograms were visualized under u.v. light or stained with the reagents of Sprince [5] or Procházka [6].

A Shandon apparatus was used for high voltage electrophoresis (HVEP). The solvent system used was water–acetic acid (14:1) adjusted to pH 4.0 with pyridine. Samples were allowed to run for 50 min at 60 mA and spots stained as described above.

Silica plates were developed in isopropanol–ethyl acetate–28% ammonia–dimethylformamide (35:45:20:5) and polyethylimine (PEI)-Cellulose plates in 0.5 M (5 min), 1.0 M (10 min) and 1.5 M (30 min) LiCl [7]. After drying, the plates were stained with one of the above-mentioned staining reagents.

Toxicity of crude palytoxin extracts was tested in adult mice by intraperitoneal injection of 0.2 ml of appropriate dilutions. The time of death was recorded and toxicity expressed as the reciprocal of survival time $(min^{-1}) \times 1000$.

Preparation and assay of $(Na^+ + K^+)$*ATPase.* The electric organ of *E. electricus* was used as the source of $(Na^+ + K^+)$ATPase. The procedure for the isolation of the enzyme was essentially that described by Albers *et al.* [8] with the modification that disruption of the tissue was achieved in 5 mM EDTA by repeated homogenization instead of sonication. A preparation with a specific activity of approximately 1.5 μmoles $P_i \times min^{-1} \times mg^{-1}$ and 96 per cent sen-

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sitive to ouabain was used. After lyophilization, this preparation was stored at −15 for prolonged periods (over 2 years) without appreciable loss of activity.

ATPase assays were performed at 37 for 20 min, after the addition of the substrate, during which period the activity was found to be linear with time. A typical incubation mixture contained 150 μmoles Na^+, 20 μmoles K^+, 5 μmoles Mg^{2+} (as chloride salts), enough enzyme to hydrolyze 10 per cent of the substrate during the incubation period, the desired amounts of inhibitor (whenever ouabain was used its final concentration was 1 mM) and 2.5 μmoles ATP as substrate, added to start the reaction after equilibration of the mixture for 10 min. The total volume was 1 ml. The reaction was stopped by the addition of 2 ml of a 10% trichloroacetic acid (TCA) solution containing 2% ascorbic acid.

Enzyme activity was measured in terms of the inorganic phosphate produced, which was determined after the procedure described by Baginski *et al.* [9]. Protein was determined by the method of Lowry *et al.* [10].

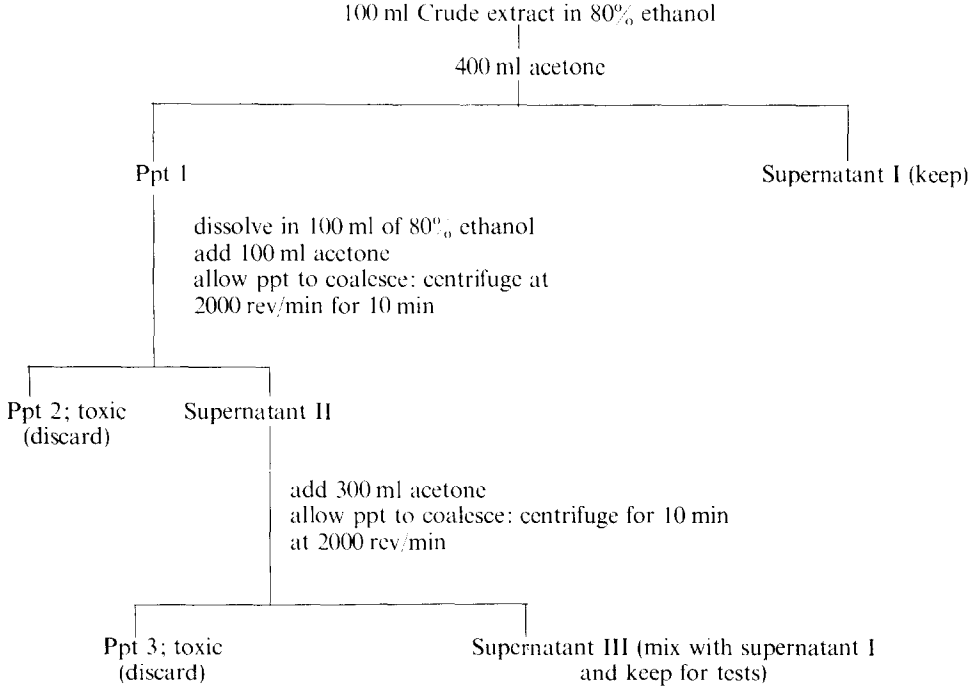
Assay of K-dependent phosphatase. Potassium-activated neutral phosphatase was assayed essentially as described by Nagai *et al.* [11] using *p*-nitrophenylphosphate (pNPP) as the substrate. The enzyme is referred to as pNPPase. Its activity was calculated as the difference between the activity measured in the presence of Mg^{2+} plus K^+ (5 and 10 mM) and the activity detected with Mg^{2+} alone. The reaction was started by adding the substrate to a final concentration of 5 mM and allowed to proceed for 15 min at 37 and was stopped by adding 2 ml of 0.05 M NaOH. The mixtures were centrifuged and the *p*-nitrophenol (pNP) liberated was read at 410 nm. Blanks were included to correct for absorption due to the presence of the inhibitor.

A heart preparation of guinea pig ventricle was used for testing the effect of serotonin (creatinine sulfate complex) and the inhibitor. The preparation consisted of an isolated strip of right ventricle wall mounted vertically in a transparent cylindrical chamber containing a solution of the following composition (mM): NaCl, 138; KCl, 2.7; $CaCl_2$, 2.7; $MgCl^2$, 0.5; $NaHCO_3$, 12; NaH_2PO_4, 3.6; and glucose, 5.5. The temperature was kept at 37 . The concentrations of serotonin and ATPase inhibitor used were 42 and 47 μg/ml respectively. The preparation was stimulated by current pulses of 3 msec duration and twice the threshold at 1 c/s. A resting tension of 350 mg was given by stretching the muscle. Peak tension was recorded with a strain gauge and displayed in a Grass Polygraph before and after addition of the substances being tested [12].

RESULTS AND DISCUSSION

Isolation of the ATPase inhibitor. Portions of *P. caribaeorum* colonies, collected off the eastern coast of Puerto Rico, were placed in 95% ethanol and extraction was allowed to proceed in the cold for 1 week or longer. This extract was dried by evaporation and redissolved in 80% ethanol for further steps in the purification scheme that follows.

The supernatant fractions I and III from the above scheme were evaporated in a rotary evaporator. The toxicity (per unit weight) of the dried residue was negligible compared to that of the precipitate. After resuspension in 25 ml of distilled water an insoluble, gummy, strongly red fluorescent (due to chlorophyl) precipitate was removed by filtration. The clear filtrate was applied to a Sephadex G-15 column (45 × 4.5 cm) previously washed with distilled water. The sample was eluted stepwise with water, 0.9% saline and 8% NaCl. The eluted fractions were read for absorbancy at 275 nm and for fluorescence emission at 338 nm (excitation at 300 nm). A total of 16



peaks could be identified by absorbancy measurements (Fig. 1).

The material in peak 16 was found to exert the most pronounced inhibitory activity. Judging by the symmetry of the peak and the approximately constant O.D. ratios between selected points of the u.v. spectrum read at different levels of the peak, it appeared to be homogeneous.

Since peak 16 is eluted in an NaCl gradient and because indoles are unstable in aqueous solutions, it was considered convenient to remove the salts and keep the material in dried form. The material in solution was dried by flash evaporation and suspended in acetone, taking advantage of the low solubility of NaCl in acetone. The acetone solution was dried again, yielding a yellow brown residue, which was kept in the freezer for further testing.

Inhibition of NaK–ATPase by the crude extract and the material in peak 16. The dose-dependent inhibitory effect of the crude extract (ethanol evaporated, residue dissolved in water) of palytoxin is shown in the lower curve of Fig. 2, where the per cent inhibition is plotted against the amount of crude extract added (expressed on a dry-weight basis). Less than 2 mg/ml of crude extract caused a 50 per cent inhibition of the enzyme activity.

The upper curve in Fig. 2 shows the results of an experiment in which the desalted material from peak 16 was tested for inhibition. Inhibition of 50 per cent was obtained by an inhibitor concentration of 200 μg/ml. For the desalted peak 16 material, routine calculation of concentration for inhibition experiments was done after establishing an $E_{1\%}$ of 232 $\pm$ 20 at 275 nm (1 cm length path cell), not very different from the calculated $E^{1\%}_{1\,cm}$ of 5-hydroxyindoleamines, reported to be 277 [13]. This represents roughly a 10-fold purification of the crude extract.

Evidence of serotonin as the inhibitor. Table 1 summarizes the results of the chromatographic, electrophoretic and spectrophotofluorimetric tests performed in the identification of the inhibitor prior to

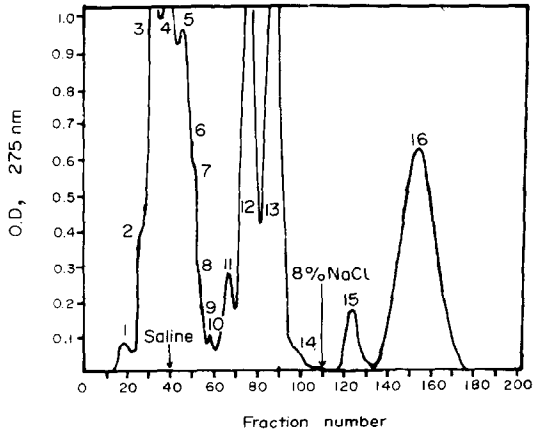


Fig. 1. Sephadex G-15 fractionation of the acetone supernatant. The elution profile was redrawn from the original record taken at 254 nm in a UA-2 Isco u.v. monitor. The elution begins with water and is shifted to 0.9 and 8% NaCl at the arrows. This record was used for establishing the nomenclature of the peaks in all subsequent experiments.

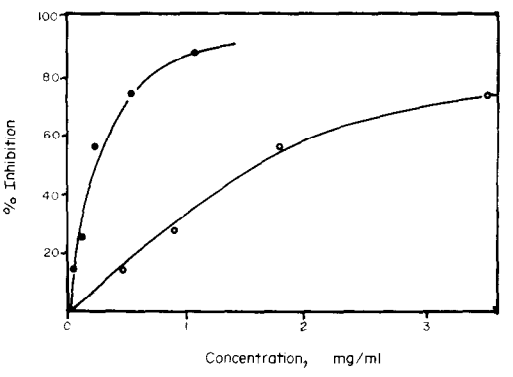


Fig. 2. Electroplax ATPase inhibition by the crude extract of *P. caribaeorum* (lower curve) and by the material in desalted peak 16 (upper curve). The abscissa scale represents the final concentration of the inhibitor in the assay mixture, which in addition contained: 2.5 mM ATP, 150 mM Na^+, 20 mM K^+ and 5 mM Mg^{2+}. Crude extract concentration was derived from dry weight determinations. Desalted peak 16 concentration was derived from O.D. measurements at 275 nm and an $E^{1\%}_{1\,cm}$ of 232 at that wavelength.

desalting. Although chromatographic mobility does not rule out the identity of 6-HT as the inhibitor, the two compounds stain differently. In addition, the spectrophotofluorimetric properties of these two compounds are markedly different, leaving 5-HT as the compound with properties closest to those of the inhibitor. The shift in the emission peak of serotonin from 338 to 535 nm when placed in a strongly acid medium (3 M HCl) is a widely used criterion for its identification, since it is characteristic of hydroxyindoles [14]. The other hydroxyindole tested, *N*-acetyl-5-HT, showed a markedly different electrophoretic mobility from that of the inhibitor (16 mm as compared to 83 mm). Preliminary mass spectrographic data indicate that the inhibitor is an entity of a molecular weight of about 172. This additional information supports the existing data used in the identification of the inhibitor.

Further analysis of the material from peak 16 and evidence of its instability. The desalted material obtained by acetone extraction from the dried fraction 16 was tested for homogeneity by p.c. and t.l.c. in silica gel plates. The material appeared to be heterogeneous and two main components were identified. The second component showed a faster mobility than serotonin, indicating a lower polarity. Identical results were obtained by high voltage electrophoresis. Thin-layer chromatography on PEI-Cellulose leads to the separation of three spots. In all tests, the most abundant component showed chromatographic behavior identical to that of serotonin. Interestingly enough, when free serotonin was dissolved in 8% NaCl, dried and extracted with acetone, its chromatographic heterogeneity was identical to that shown by the desalted material from peak 16.

Effect of the isolated inhibitor on NaK-ATPase kinetics. Figures 3 and 4 summarize the effects of the inhibitory material from peak 16 on the kinetic properties of ouabain-sensitive ATPase under varying experimental conditions.

Table 1. Electrophoretic, chromatographic and spectrophotofluorimetric data on the ATPase inhibitor from peak 16 and related indoles

Compound	HVEP (mm)	Thin-layer chromatography (Silica) (R_f)	Paper chromatography (R_f)	Thin-layer chromatography (PEI) (R_f)	Emission in H_2O (nm)	Emission in 3 M HCl (nm)
TRY		0.39	0.54		350	No shift
5-MeO-T	85	0.88				
5-MeO-IAA		0.43				
N-acetyl-5-HT	16	0.95			339	552
6-HT	82	0.84			353	No shift
5-HTRY	15	0.30				
5-HT	84	0.81	0.52	0.37	338	535
Material from peak 16	83	0.82	0.52	0.37	338	535

Figure 3A shows enzyme activity at varying Mg^{2+} concentrations, all other experimental conditions remaining constant. An inhibitor concentration of 200 μg/ml is enough to induce 50 per cent inhibition. The extent of the inhibition does not depend on Mg^{2+} concentration.

Figure 3B shows enzyme activity as a function of Na^+ concentration, at constant K^+, Mg^{2+} and substrate concentrations, both in the absence and presence of inhibitor. The per cent inhibition does not vary appreciably with the variation of Na^+ concentration in the range employed. The constancy of the per cent inhibition obtained when using a fixed concentration of inhibitor in the presence of varying concentrations of these ligands suggests a non-competitive type of kinetics.

Additional evidence of non-competitive kinetics between the inhibitor and Na^+ was obtained by tracing a double reciprocal plot of the data presented in Fig. 3B ($1/v$ vs $[1/Na^+]$) in the absence and in the presence of the inhibitor. Extrapolation in both cases gave an identical value for K_m (not shown). Analysis of the same data by the non-linear regression method of Wilkinson [15] yielded values for K_m of 12.98 $\pm$ 2.77 and 11.75 $\pm$ 3.44 mM in the absence and in the presence of inhibitor respectively. Statistically this is not a significant difference, since values of K_m fall within the standard deviation of each other.

Potassium partially abolishes the effects of the inhibitor. Varying concentrations of K^+ in the presence of a constant amount of inhibitor, all other parameters remaining constant, yield the results shown in Fig. 4. The double reciprocal plot indicates a mixed type inhibition. Analysis of the data by the method of Wilkinson [15] gave values for K_m of 1.47 $\pm$ 0.38 mM in the absence of and 6.45 $\pm$ 0.89 mM in the presence of the inhibitor. This is a highly significant difference in K_m, since each K_m falls out of the 99 per cent confidence limits of the other. This is in contrast to the observation made with other ligands (ATP, Na^+) where non-competitive effects occur.

Figure 5 shows a double reciprocal plot where the substrate concentration is varied at constant ionic concentrations of Mg^{2+}, Na^{2+}, K^+ in the presence and absence of the inhibitor. Two concentrations of inhibitor (0.1 and 0.2 mg/ml) were used. The type of inhibition is clearly non-competitive. It has been reported that tryptamine, serotonin, histamine and the cathecholamines form complexes with ATP in solution and in their storage vesicles [16]. This complex formation is expected to lead to competitive inhibition rather than the observed non-competitive inhibition reported here.

Determination of K_i. The fact that inhibition does not increase with time (data not shown) was taken

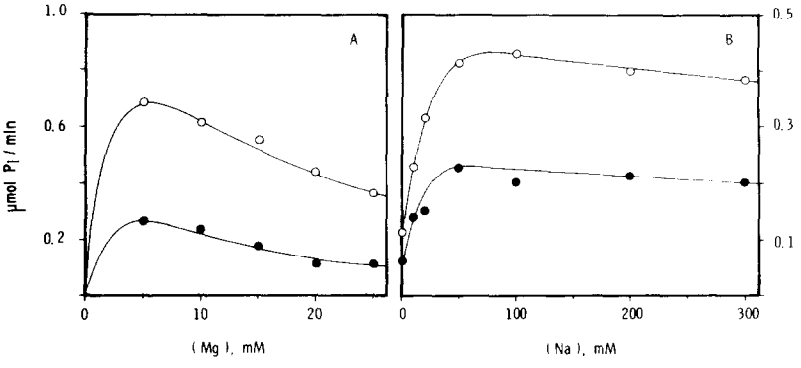


Fig. 3. Effect of Mg^{2+} (A) and Na^+ (B) concentrations on electroplax ATPase in the absence (○) and in the presence (●) of desalted peak 16. The contents of the assay in A were: 4 mM ATP, 150 mM Na^+, 20 mM K^+ and 0.23 mg/ml of inhibitor. Contents of the assay in B were: 2.5 mM ATP, 20 mM K^+, 5 mM Mg^{2+}, and 0.2 mg/ml of inhibitor. The Na^+ concentration scale does not take into account the sodium present in ATP. This explains the high enzymatic activity even when Na^+ was not added to the assay medium.

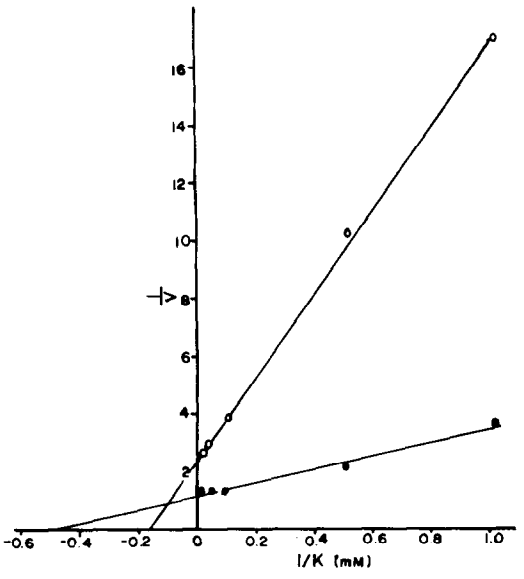


Fig. 4. Double reciprocal plot of the effect of K concentration on electroplax ATPase in the absence (●) an in the presence (○) of desalted material from peak 16.

as indirect evidence of the reversible combination of inhibitor with the enzyme. From the same data shown in Fig. 5, a value for K_i was calculated. For an enzyme preparation with a specific activity of 1.5 μM P_i/min/mg of protein, K_i is approximately 220 μg/ml. Similar results were obtained when K_i was calculated by the method of Dixon [17], using two different substrate concentrations (data not shown).

Effect on pNPPase. A currently postulated sequence of reactions for NaK-ATPase-catalyzed hydrolysis of ATP is as follows:

Step 1 Step 2 Step 3

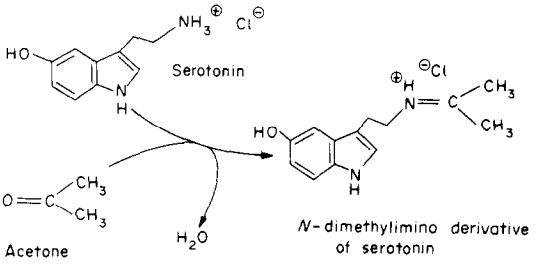
$$E_1 \underset{ATP \quad ADP}{\overset{Na^+, Mg^{2+}}{\rightleftharpoons}} E_1P \overset{Mg^{2+}}{\rightleftharpoons} E_2P \underset{H_2O \quad Mg^{2+}}{\overset{K^+}{\rightleftharpoons}} E_1 + P_i$$

where the E_2 form has marked affinity for K^+, but not for Na^+. pNPPase activity is claimed to represent step 3 of the above scheme [18].

The inhibitor from peak 16 was tested on pNPPase activity and, under the experimental conditions used, 750 μg/ml of the inhibitor caused 95 per cent inhibition of the enzyme activity. This concentration represents maximal inhibitory activity on NaK-ATPase (Fig. 2). This can be interpreted as indirect evidence that the inhibitor described here exerts its effects on NaK-ATPase by inhibiting step 3 of the above scheme. We have found that increasing concentrations of K^+ partially abolish the inhibitory effect (Fig. 4). Maximal inhibitory activity is found at K^+ concentrations of 1–2 mM. A 10-fold increase in K^+ concentration reduces the inhibition by 25 per cent.

Inotropic effects of serotonin creatinine sulfate and purified material from peak 16 on heart muscle. The similarities in biological activity of serotonin and the desalted material from peak 16 are apparent from the fact that both substances induce a positive inotropic response (Fig. 6). This is additional evidence to support the contention that the inhibitor is serotonin. When serotonin (as the creatinine sulfate complex) is tested, the inotropic response is accompanied by spontaneous arrythmic activity, not observed on addition of the inhibitor to the medium.

Probable explanation for the presence of other components in acetone-extracted material from peak 16. It can be inferred that the other constituents detectable in the material from peak 16 after desalting are probably hydroxyindoles, products of degradation of serotonin. Hydroxyindoles are known to be rather unstable substances. We have observed that commercial preparations of serotonin are stable when in the form of a complex with creatinine sulfate, but are rapidly degraded when isolated, yielding a mixture of components. In the absence of water, and in a slightly acid medium (just as it happens when serotonin hydrochloride is lyophilized and extracted with acetone), the following reaction can occur.



This reaction may well partially explain the presence of at least another component in the serotonin solution isolated from peak 16 after desalting. The compound described above would have a more apolar nature than serotonin, thus explaining the appearance of a component migrating faster than serotonin in Silica gel t.l.c. and p.c.

As additional evidence that extraction with acetone caused the partial breakdown of serotonin, the material from peak 16 was dried *in vacuo* and extracted

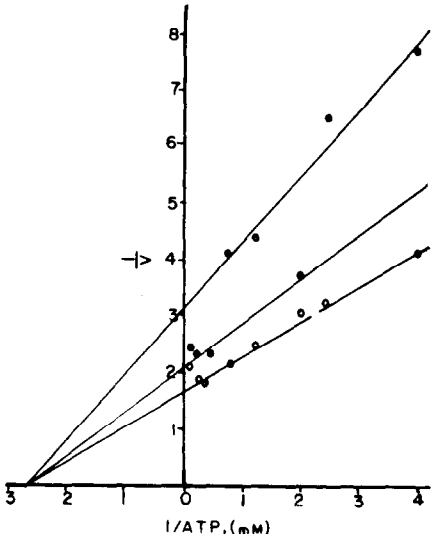


Fig. 5. Double reciprocal plot of the effect of ATP concentration on electroplax ATPase activity in the absence (○) and in the presence of 0.2 mg/ml (◒) or 0.1 mg/ml (●) of desalted peak 16. The assay mixture contained 150 mM Na^+, 20 mM K^+, 5 mM Mg^{2+} and the indicated concentrations of the inhibitor.

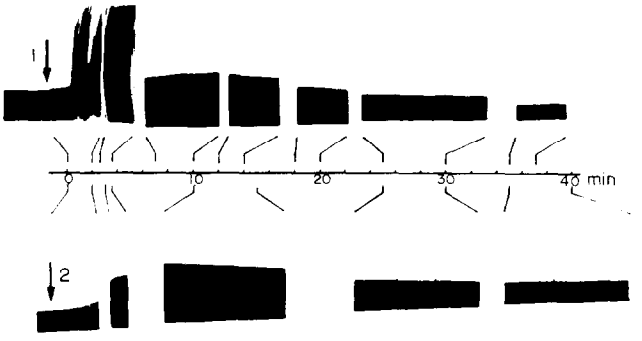


Fig. 6. Inotropic response of an isolated, electrically driven right-ventricle preparation from guinea pig heart, after adding to the chamber free serotonin (1) and desalted peak 16 (2). Final concentrations were, respectively, 42 and 47 μg/ml. An increase in the contractile force of myocardial tissue has induced many known ATPase inhibitors.

with 100 ml *n*-butanol to which five drops of ammonium hydroxide was added. The extract was dried, dissolved in water and chromatographed in PEI-Cellulose as described in Materials and Methods, using commercial serotonin as control. The extracted material was found to migrate as a single spot having the same R_f as the control.

The possibility of having other hydroxyindoleamines present in trace amounts in the material from peak 16 is not totally ruled out, but routine staining techniques fail to detect other constituents in the untreated material present in peak 16. It is our contention that the main component is serotonin and that this substance is responsible for $(Na^+ + K^+)$ATPase inhibitory activity. The chromatographic, spectrophotometric, electrophoretic and biological evidence presented here is enough to sustain the contention that serotonin is indeed the chemical entity present in peak 16, and that attempts to free it from NaCl by extraction with acetone causes its partial degradation. We have also shown that serotonin separated by cation exchange chromatography from commercial serotonin–creatinine sulfate complexes has the same specific inhibitory activity as the compound present in peak 16 [19]. Other indoles have been previously reported to act as inhibitors of $(Na^+ + K^+)$ATPases from various tissues. Canessa *et al.* [4], in describing the inhibitory activity of harmaline, a hydroxyindole isolated from the plant *Paganum harmala*, failed to detect any inhibitory activity by serotonin at concentrations as high as 10^{-4} M. These authors report that harmaline is a competitive inhibitor for Na^+. Our findings indicate that serotonin competes with K^+.

A finding opposite to the one reported here has been described by Yoshimura [20], who made the observation that serotonin, used as a serotonin–creatinine sulfate complex, acts as an activator of $(Na^+ + K^+)$ATPases from brain tissue.

The effects of serotonin on $(Na^+ + K^+)$ATPases reported here may help to explain, at least partially, the known pharmacological effects of this compound.

Acknowledgements—We thank Professor J. del Castillo for supplying the electric eels (*E. electricus*) for the isolation of the ATPases and for his encouragement and helpful observations, and Mr. Faustino MacKenzie for collecting the specimens of *P. caribaeorum*. This research was supported in part by grants GRSG 00-203-51-01, NS 07464 and K6N 14938 from the U.S. Public Health Service and by a grant-in-aid to one of us (J. I. W.) from the Maryland affiliate, American Heart Association.

REFERENCES

1. R. E. Moore and P. J. Scheuer, *Science, N.Y.* **172,** 495 (1971).
2. M. D. Rayner and J. Szekerczes, *Toxic. appl. Pharmac.* **24,** 489 (1973).
3. S. García-Castiñeiras, J. I. White and E. Toro-Goyco, *Fedn Proc.* **34,** 505 (1975).
4. M. Canessa, E. Jaimovich and M. de la Fuente, *J. mèmb. Biol.* **13,** 263 (1973).
5. H. Sprince, *J. Chromat.* **3,** 97 (1960).
6. Z. Procházka, in *Paper Chromatography, A Comprehensive Treatise* (Eds. I. M. Hais and K. Macek), p. 609. Publishing House of the Czecholslovak Acad. Sci., Prague (1963).
7. E. Randerath and K. Randerath, *Analyt. Biochem.* **12,** 83 (1965).
8. R. W. Albers, S. Fahn and G. J. Koval, *Proc. natn. Acad. Sci. U.S.A.* **50,** 474 (1963).
9. E. S. Baginski, P. P. Foa and B. Zak, *Clin. Chem.* **13,** 326 (1967).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
11. K. Nagai, F. Izumi and M. Yoshida, *J. Biochem., Tokyo* **59,** 295 (1966).
12. B. Pérez de Gracia and W. C. de Mello, *Acta physiol. latinoam.* **24,** 356 (1974).
13. S. Udenfriend, D. F. Bogdanski and H. Weissbach, *Science, N.Y.* **122,** 973 (1955).
14. S. Udenfriend and H. Weissbach, *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. VI, p. 598. Academic Press, New York (1963).
15. G. N. Wilkinson, *Biochem. J.* **80,** 324 (1961).
16. P. A. Shore, *A. Rev. Pharmac.* **12,** 209 (1972).
17. M. Dixon, *Biochem. J.* **55,** 170 (1953).
18. J. L. Dahl and L. E. Hokin, *A. Rev. Biochem.* **43,** 327 (1974).
19. E. Toro-Goyco and S. García-Castiñeiras, *Fedn Proc.* **35,** 1476 (1976).
20. K. Yoshimura, *J. Biochem., Tokyo* **74,** 389 (1973).