# Mechanism of Biogenic Amine Transport

## II. Relationship between Sodium and the Mechanism of Ouabain Blockade of the Accumulation of Serotonin and Norepinephrine by Synaptosomes

ANJA H. TISSARI,[1] PETER S. SCHÖNHÖFER, DONALD F. BOGDANSKI, AND BERNARD B. BRODIE

*Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda, Maryland 20014*

(Received May 26, 1969)

SUMMARY

When ouabain and labeled serotonin ($^{14}$C-5-HT) or norepinephrine ($^{3}$H-NE) are simultaneously introduced into a suspension of isolated nerve endings (synaptosomes) in Krebs-bicarbonate solution, the initial accumulation of $^{3}$H-NE by the synaptosomes proceeds for a few minutes, then abruptly stops. When ouabain is first incubated with synaptosomes in the absence of substrate, the initial accumulation of $^{14}$C-5-HT subsequently added is blocked. Thus, a lapse of time is required for the inhibitory effect of ouabain to develop. Moreover, $Na^+$ must also be present with the ouabain. In contrast, ouabain inhibits ($Na^+ + K^+$)-ATPase activity immediately. It appears, therefore, that ouabain blocks the accumulation of amine by an indirect process resulting from the inhibition of ($Na^+ + K^+$)-ATPase. The indirect process requires $Na^+$ and is probably the increase in the intracellular $Na^+$ concentration which occurs in the presence of ouabain.

### INTRODUCTION

Recent studies have shown that sodium ions are essential for the uptake and storage of tritiated norepinephrine ($^{3}$H-NE) by sympathetic nerve endings in the rat heart (1–4). Moreover, these $Na^+$-stimulated processes are competitively inhibited by $K^+$ (5). The uptake and storage of both $^{3}$H-NE and labeled serotonin ($^{14}$C-5-HT) by rat brain synaptosomes (pinched-off nerve endings) also require $Na^+$, and these processes likewise are inhibited by $K^+$ (6, 7). Our findings have led us to postulate that catecholamines are transported across the neuronal membrane by a transport mechanism involving a carrier having a high affinity for the amine at the outer neuronal membrane surface, where the $Na^+$ concentration is high and the $K^+$ concentration is low, and a low affinity for the amine at the inner surface, where the $K^+$ concentration is high and the $Na^+$ concentration is low (5, 6). The $Na^+$ and $K^+$ gradients upon which this mechanism depends are maintained by the $Na^+$ pump associated with ($Na^+ + K^+$)-ATPase in the membrane (8, 9).

The present report describes the effects of ouabain on the uptake and accumulation of NE and 5-HT by synaptosomes from rabbit brain. The results suggest that ouabain does not inhibit the amine transport system directly, but acts indirectly by reducing the $Na^+$-$K^+$ gradient through the inhibition of ATPase. The model transport system proposed by this laboratory for NE (5) is also applicable to 5-HT.

[1] Present address, Department of Pharmacology, University of Helsinki, Helsinki 17, Finland.

## METHODS

Male New Zealand rabbits, weighing 2.0–2.5 kg, were killed by cervical dislocation. Their brains were immediately homogenized in a homogenizer with a loosely fitting pestle having a clearance of 0.25 mm (10) in 9 volumes of ice-cold 0.32 M sucrose containing $10^{-6}$ M $Ca^{++}$ (11). The homogenate was centrifuged at $1000 \times g$ for 10 min, and the sediment was washed once with 0.32 M sucrose. The supernatant fluid and washings were combined and centrifuged at $10,000 \times g$ for 20 min. The pellet was suspended in 0.32 M sucrose, and 5–10 ml of the suspension were layered over a discontinuous sucrose gradient consisting of 5 ml of 1.4 M, 7 ml of 1.2 M, 7 ml of 1.0 M, and 5 ml of 0.8 M sucrose. The gradient was centrifuged at $55,000 \times g$ for 120 min in a model L2-65B Beckman ultracentrifuge equipped with a SW 25.1 rotor. The synaptosomes that equilibrated at the 1.0–1.2 M interphase were removed by aspiration, suspended with an equal volume of water, and allowed to stand for 20 min. The suspension was then centrifuged at $15,000 \times g$ for 20 min, the supernatant fluid was discarded, and the synaptosomes were finally suspended in 16 ml of various media. The final synaptosome suspension averaged 0.2 mg of protein per milliliter. The synaptosome concentration was kept low to prevent rapid disappearance of substrate. For example, suspensions containing 1.5 mg of protein per milliliter metabolized 80% of $^{14}$C-5-HT (20 ng/ml) within 5 min.

The accumulation and total uptake of $^{14}$C-5-HT (Nuclear-Chicago, 56 mCi/mmole) or $^{3}$H-NE (New England Nuclear, 7.4 Ci/mmole) were determined after introduction of the biogenic amines into synaptosome suspensions and incubation of the latter in an Eberbach water bath shaker in an atmosphere of 95% $O_2$–5% $CO_2$ at 37.5°. Ouabain was added before or simultaneously with the amines. Details of time schedules for the addition of inhibitor and amine are given with the tables and figures.

Aliquots (4 ml) of the suspension were removed after various periods of incubation and cooled in an ice bath, with shaking, to stop the transport of amines. The aliquots were centrifuged for 5 min at $10,000 \times g$; the supernatant fluid was saved, and the pellets were rinsed three times with Krebs-bicarbonate solution and again centrifuged. The pellets were homogenized in 3.5 ml of distilled water for the estimation of radioactivity and protein. Two-milliliter samples of the original supernatant fluid and pellet extract were acidified with 2 N HCl to 0.2 N, and the amines and metabolites were separated as described below for counting by liquid scintillation spectrometry. To measure protein, a 1-ml portion of the original aliquot was assayed by the method of Warburg and Christian (12).

Serotonin was extracted by the method of Bogdanski *et al.* (13), and 5-hydroxyindoleacetic acid was extracted by the method of Udenfriend *et al.* (14). After incubation, the sum of $^{14}$C-5-HT and $^{14}$C-5-hydroxyindoleacetic acid was less than the amount of $^{14}$C-5-HT added at the beginning of the incubation. The difference was accounted for by radioactive material showing the chromatographic properties of $^{14}$C-5-hydroxytryptophol (15). This radioactivity was included in the total for the deaminated products. Radioactive NE was isolated by the method of Haggendal (16).

ATPase activity in rabbit synaptosomes was estimated by the method of Hosie (17). Synaptosomes in 0.32 M sucrose were frozen and stored overnight with little or no effect on ATPase activity compared with nonfrozen synaptosomes. The temperature of the 2-ml suspension of synaptosomes was allowed to rise to 37° in the water bath; ouabain was then introduced in various concentrations, and 5 min later 0.2 ml of Tris-ATP (17) was introduced into the incubation mixture to give the final concentration of 3 mM. In testing for a delayed action of ouabain, ATP and ouabain were introduced together into the suspension after the usual warming period. The synaptosome suspensions in the ATPase experiments contained 0.1–0.3 mg of protein per sample.

After various periods of incubation, the

TABLE 1

*Effect of ouabain on metabolism of $^{14}$C-5-HT*

Synaptosomes were incubated for 10 min in Krebs-bicarbonate solution in the presence of various concentrations of ouabain. $^{14}$C-5-HT was then added to the suspension to a final concentration of 20 ng/ml, and the synaptosomes were again incubated for 20 min. The data represent the amount of metabolism, expressed as a percentage of the amine originally present; the accumulation of $^{14}$C-5-HT, expressed as the concentration ratio of intracellular to extracellular amine; and the total transport, which is defined as $^{14}$C-5-HT accumulated by the synaptosomes plus total $^{14}$C-deaminated metabolites in the suspension, expressed as a percentage of the amine originally present. Figures in parentheses refer to number of experiments.

| Medium | 5-HT metabolized ±SD | Accumulation ratio ±SD | Total transport ±SD |
|---|---|---|---|
| | % | | % |
| Krebs-bicarbonate, no ouabain (5) | 32.1 ± 13 | 32 ± 15 | 47.2 ± 13 |
| $10^{-6}$ M ouabain (3) | 24.6 ± 11 | 24 ± 6 | 44.9 ± 5 |
| $10^{-5}$ M ouabain (6) | 15.5 ± 7 | 5.2 ± 3 | 17.0 ± 8 |
| $10^{-4}$ M ouabain (6) | 14.6 ± 6 | 3.5 ± 1 | 16.6 ± 6 |
| $10^{-3}$ M ouabain (4) | 10.5 ± 8 | 4.2 ± 2 | 12.6 ± 7 |

enzyme reaction was stopped with 0.5 ml of 20% trichloracetic acid. Hydrolysis of ATP was determined from inorganic phosphate in 2 ml of the suspension. Phosphate was estimated by the method of Berenblum and Chain (18) and expressed as micromoles per minute per milligram of protein. For the ATPase experiments, protein was measured by the method of Sutherland *et al.* (19). All ATPase experiments were run in duplicate.

## RESULTS

*Effect of ouabain on $^{14}$C-5-HT metabolism in synaptosomes.* The following studies were carried out to determine whether ouabain blocks the accumulation of 5-HT in synaptosomes by an action on the membrane transport or on the storage vesicles. This question can be answered by comparing the effects of drugs on accumulation and metabolism of exogenous amines, provided that both processes take place in the same cell. To show that both processes take place in the same cell we take advantage of the fact that only nerve tissue can accumulate exogenous amines, causing the internal concentration to exceed greatly the external concentration. Moreover, monoamine oxidase is known to be located in nerve endings (20). Thus, ouabain or a lack of $Na^+$ inhibits both accumulation and metabolism, indicating that the same cells are involved in both processes (5, 6). For additional proof we have made use of the fact that in synaptosomes, reserpine in-

TABLE 2

*Effect of ouabain on metabolism of $^3$H-NE*

Synaptosomes were incubated for 10 min in Krebs-bicarbonate solution with and without $10^{-4}$M ouabain. Tritiated NE was then added to the suspension to a final concentration of 5 ng/ml, and the synaptosomes were again incubated for 20 min. The data represent the amount of metabolism, expressed as a percentage of the amine originally present; the accumulation of $^3$H-NE, expressed as the concentration ratio of intracellular to extracellular amine; and the total uptake, which is defined as $^3$H-NE accumulated by the synaptosomes plus total $^3$H-deaminated metabolites in the suspension. Figures in parentheses refer to number of experiments.

| Medium | $^3$H-NE metabolized ±SE | Accumulation ratio ±SE | Total uptake ±SE |
|---|---|---|---|
| % | % | | % |
| Krebs-bicarbonate (4) | 8.5 ± 2.7 | 29.0 ± 4.2 | 19.0 ± 2.5 |
| $10^{-4}$ M ouabain (4) | 3.3 ± 2.1 | 2.6 ± 0.8 | 5.2 ± 4.0 |

hibits accumulation but, unlike ouabain, also increases metabolism. In the presence of a monoamine oxidase inhibitor, metabolism of amines is blocked and the ability of synaptosomes to accumulate amines is restored (6). Thus, reserpine prevents the cell from storing amines and blocks accumulation by enabling monoamine oxidase to metabolize free amines (6, 20). When the monoamine oxidase in nerve endings is blocked, the intact transport process located in the cell membrane causes the cell to accumulate amines.

In accord with the above discussion, Table 1 shows that various concentrations of ouabain produced a marked decrease in the metabolism, accumulation, and total uptake of exogenous $^{14}$C-5-HT, indicating that membrane transport had been inhibited. Table 2 shows similar effects of ouabain on the metabolism and accumulation of $^{3}$H-NE.

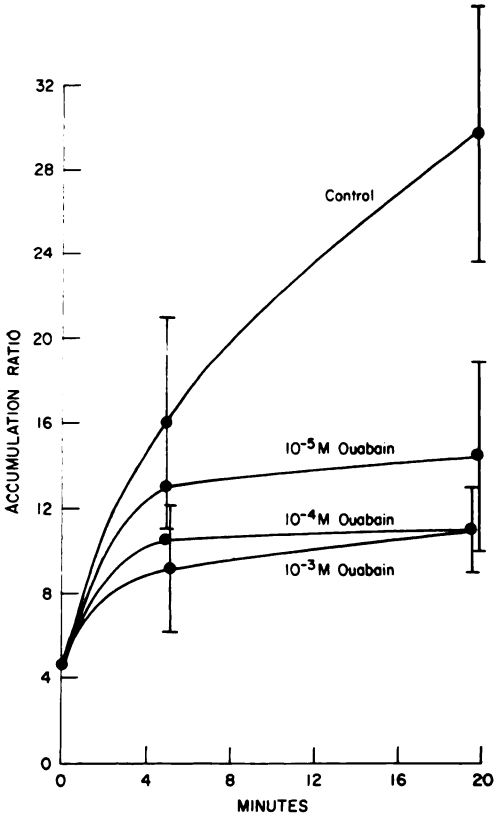


FIG. 1. *Effect of various concentrations of ouabain on accumulation of $^{14}$C-5-HT in rabbit synaptosomes suspended in Krebs-bicarbonate solution*

Synaptosomes were warmed at 37° for 10 min; $^{14}$C-5-HT (20 ng/ml) and various concentrations of ouabain were then added simultaneously. The synaptosome suspension was sampled for assay of 5-HT and protein at various time periods. The curves represent the average and standard deviation of four experiments.

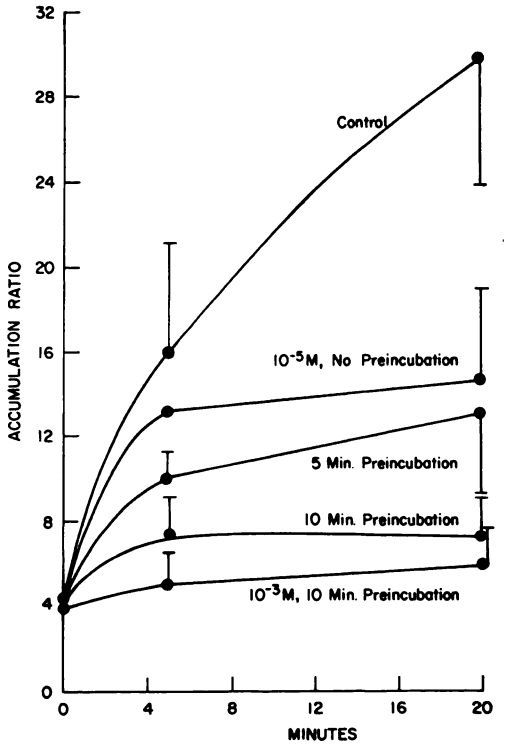


FIG. 2. *Effect of prior incubation of synaptosomes with ouabain on the accumulation of subsequently added $^{14}$C-5-HT by rabbit synaptosomes suspended in Krebs-bicarbonate solution*

Synaptosomes were warmed at 37° for at least 5 min and then incubated with ouabain ($10^{-5}$ M) for various time periods. The schedule of preliminary incubations with ouabain was arranged so that all samples were exposed to the warm environment for the same length of time (15 min) before the substrate was introduced into the suspension. $^{14}$C-5-HT (20 ng/ml) was then pipetted into the suspensions, which subsequently were sampled for assay of $^{14}$C-5-HT and protein at various periods of time. The effect of a 10-min incubation with $10^{-3}$ M ouabain on accumulation of 5-HT is also shown to indicate maximum effect of inhibitor. The curves represent the average and standard deviation of five experiments.

*Effect of ouabain on $^{14}$C-5-HT accumulation.* Figure 1 shows the effects of various concentrations of ouabain on the accumulation of $^{14}$C-5-HT by synaptosomes when substrate and inhibitor were simultaneously added to the suspensions. At a concentration of $10^{-5}$ M, ouabain only partially blocked the accumulation of 5-HT during the first 5 min. After 5 min, however, the accumulation of 5-HT was almost completely inhibited. These results suggest that ouabain did not act immediately, but required some time to block the process responsible for the accumulation of 5-HT. Higher concentrations of ouabain also failed to block the accumulation of the amine immediately. These data indicate that the initial rate of accumulation was not appreciably affected by ouabain and that the effect of the inhibitor on amine accumulation was delayed.

*Effect of prior incubation with ouabain on $^{14}$C-5-HT accumulation.* Figure 2 shows that when synaptosomes were incubated with ouabain ($10^{-5}$ M), the initial accumulation of $^{14}$C-5-HT by nerve endings was decreased by a factor proportional to the duration of the prior incubation. Accumulation was virtually blocked by incubation for 10 min with a high concentration ($10^{-3}$

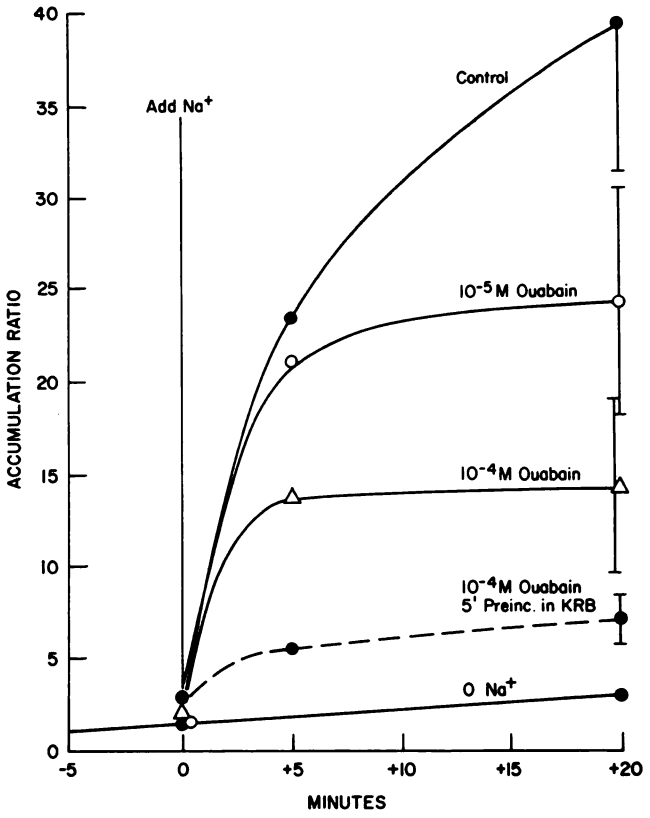


FIG. 3. *Effect of $Na^+$ on accumulation of $^{14}$C-5-HT by synaptosomes incubated in a $Na^+$-free medium containing various concentrations of ouabain*

Synaptosomes were incubated for 5 min with ouabain in a $Na^+$-free solution (isotonicity maintained by sucrose) containing all other electrolytes normally present in Krebs-bicarbonate solution (KRB). At zero time, $Na^+$ and/or $^{14}$C-5-HT were added to final concentrations of 20 ng/ml and 143 mM, respectively. Accumulation of $^{14}$C-5-HT in the $Na^+$-free medium without subsequent addition of $Na^+$ is also shown (bottom curve, designated 0 $Na^+$). For comparison, the dashed curve shows the effect of a 5-min preliminary incubation of synaptosomes with $Na^+$ and ouabain on accumulation of $^{14}$C-5-HT added at zero time. The curves represent the average and standard deviation of five experiments.

M) of the drug. These data again indicate that a measurable time lag precedes the full effects of ouabain.

*Effect of $Na^+$ lack during prior incubation with ouabain on $^{14}C$-5-HT accumulation.* Previous studies have shown that 5-HT is not accumulated by synaptosomes in a $Na^+$-free suspension (6), but that this process is restored by the addition of the ion, as shown in Fig. 3. In the present studies synaptosomes were incubated for 5 min in a $Na^+$-free medium in the presence of ouabain ($10^{-4}$ or $10^{-5}$ M); 5 min later $^{14}C$-5-HT and $Na^+$ (143 mM) were added to the suspension. At first, considerable amounts of 5-HT were accumulated by the synaptosomes, but the rate of accumulation slowed considerably within 5 min (compare with Fig. 1). These results should also be compared with those in Fig. 2, which show that synaptosomes do not accumulate 5-HT after they have been incubated with ouabain in the presence of $Na^+$ (normal Krebs solution).

Thus, if synaptosomes were incubated with ouabain in the presence of $Na^+$, there was no accumulation when the amine was subsequently added. If synaptosomes were incubated with ouabain in the absence of $Na^+$, there was temporary accumulation of amine when substrate and $Na^+$ were added. Therefore, incubation with ouabain alone was not sufficient to block accumulation when amine was introduced into the medium. These facts show that the development of the blocking effect of ouabain on the transport of subsequently added amine depends upon the simultaneous presence of $Na^+$ with ouabain.

*Effect of prior incubation with ouabain on $^3H$-NE accumulation.* Figure 4 shows that when ouabain and $^3H$-NE were simultaneously added to the suspension the amine accumulated initially, but the process stopped after 5 min (compare with Fig. 1). The amount of $^3H$-NE initially accumulated in the presence of ouabain decreased as the duration of the preliminary incubation was increased (compare with Fig. 2). Accumulation was almost completely

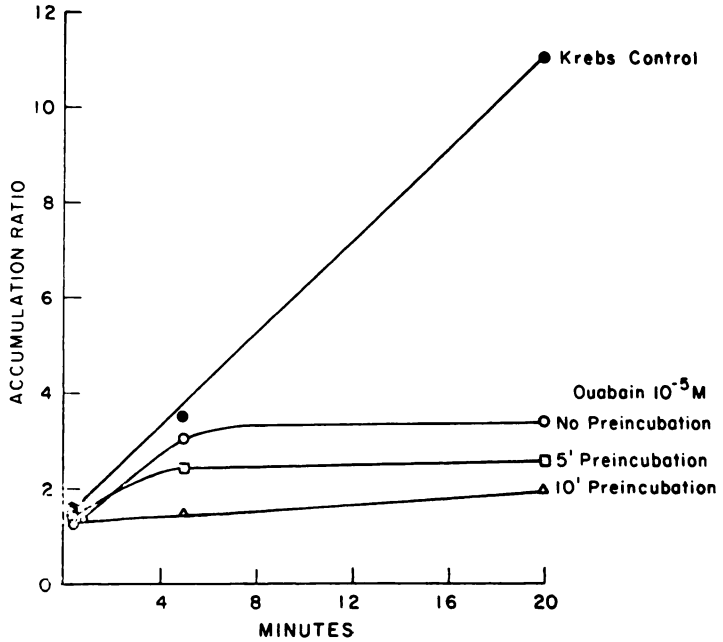


FIG. 4. *Effect of prior incubation of synaptosomes on the accumulation of subsequently added $^3H$-NE by rabbit synaptosomes suspended in Krebs-bicarbonate solution*

The experimental design was the same as described in Fig. 2. The [$^3H$-NE] was 5 ng/ml. The curves represent the average of three experiments.

blocked by a 10-min incubation with $10^{-5}$ M ouabain. These experiments indicate that a lapse of time is required for the full effects of ouabain to develop.

*Effect of $Na^+$ lack during prior incubation with ouabain on $^3$H-NE accumulation.* After incubation of synaptosomes with ouabain in a $Na^+$-free medium or a medium low in $Na^+$ (25 mM), additional $Na^+$ (143 mM) did not restore the process of accumulation of $^3$H-NE (Table 3). The reason why ouabain affects NE accumulation differently from 5-HT accumulation (Fig. 3) is not known, although it is possibly related to the fact that the addition of $Na^+$ to a $Na^+$-free synaptosome suspension did not completely restore the ability of the synaptosomes to accumulate NE even in the absence of ouabain.

TABLE 3

*Effect of prior incubation with ouabain on restoration of $^3$H-NE accumulation on adding $Na^+$ to $Na^+$-deficient medium*

Synaptosomes were incubated for 5 min in $Na^+$-deficient medium ($Na^+$-free or 25 mM $Na^+$ made isotonic with sucrose). Ouabain, $10^{-4}$ M or $10^{-5}$ M, was present during this period. Then $Na^+$ was added at zero time to a final concentration of 143 mM, together with $^3$H-NE (4 ng/ml), and the synaptosomes were incubated for 20 min. $Na^+$-deficient media contained all other ions normally contained in Krebs-bicarbonate solution. For comparison, the effect of a 5-min preliminary incubation of ouabain (in Krebs-bicarbonate solution) on the accumulation of $^3$H-NE is also represented. Numbers in parentheses refer to the number of experiments. The low [$Na^+$], 25 mM, was added because accumulation in the $Na^+$-free medium was only partially restored by the addition of $Na^+$.

| Preliminary incubation medium | | Accumulation ratio ± SD |
|---|---|---|
| [Ouabain] | [$Na^+$] | |
| M | mM | |
| 0 | 0 | 8.3 ± 4.1 (7) |
| $10^{-4}$ | 0 | 2.3 ± 0.3 (7) |
| 0 | 25 | 17.6 ± 5.5 (7) |
| $10^{-4}$ | 25 | 2.8 ± 0.3 (7) |
| 0 | 143[a] | 18.2 ± 5.2 (6) |
| $10^{-4}$ | 143[a] | 2.0 ± 0.4 (7) |

[a] Krebs-bicarbonate solution.

TABLE 4

*Effect of various inorganic ions on ATPase activity in synaptosomes*

Synaptosomes were incubated in plastic tubes in a standard medium of 150 mM Tris buffer (pH 7.4), 1 mM EDTA, and 4 mM $Mg^{++}$. $Na^+$ (120 mM) and $K^+$ (20 mM) were added (as indicated in the table) 5 min before or together with ATP (3 mM, final concentration). The latter time schedule is identified by the word "added." To compare the effect of ouabain on ATPase activity with that of $Na^+ + K^+$ deficiency, ouabain ($10^{-4}$ M) was incubated with synaptosomes in the standard medium for 5 min before the addition of ATP. Figures in parentheses refer to number of experiments.

| Incubation medium | ($Na^+ + K^+$)-ATPase activity |
|---|---|
| | *μmole $P_i$/min/mg* (±*SD*) |
| $Mg^{++}$, $Na^+$, $K^+$ | 0.41 ± 0.05 (3) |
| $Mg^{++}$ only | 0.24 ± 0.05 (3) |
| $Mg^{++}$ ($Na^+ + K^+$ added) | 0.55 ± 0.02 (2) |
| $Mg^{++}$, ouabain ($Na^+ + K^+$ added) | 0.17 ± 0.03 (2) |
| $Mg^{++}$ ($K^+$ added) | 0.20 ± 0.01 (2) |
| $Mg^{++}$ ($Na^+$ added) | 0.14 ± 0.01 (2) |

TABLE 5

*Effect of ouabain on ($Na^+ + K^+$)-ATPase activity*

Synaptosomes were incubated in the standard Tris buffer medium (pH 7.4) containing 1 mM EDTA, 4 mM $Mg^{++}$, 120 mM $Na^+$, 20 mM $K^+$, and ouabain at various concentrations. After 5 min, ATP was introduced at 3 mM. The percentage inhibition is relative to ($Na^+ + K^+$)-ATPase activity, which is defined here as the difference between total ATPase activity in the standard medium and no ouabain, and ATPase activity in the standard medium containing $10^{-3}$ M ouabain (see the text). Figures in parentheses refer to number of experiments.

| [Ouabain] | ($Na^+ + K^+$)-ATPase activity | Inhibition of ($Na^+ + K^+$)-ATPase activity |
|---|---|---|
| M | *μmole $P_i$/min/mg* (±*SD*) | % |
| 0 (4) | 0.27 ± 0.03 | 0 ± 12 |
| $10^{-7}$ (2) | 0.26 ± 0.01 | 5 ± 2 |
| $10^{-6}$ (2) | 0.21 ± 0.04 | 31 ± 9 |
| $10^{-5}$ (4) | 0.11 ± 0.02 | 84 ± 8 |
| $10^{-4}$ (4) | 0.09 ± 0.01 | 95 ± 2 |
| $10^{-3}$ (3) | 0.08 ± 0.02 | 100 ± 11 |

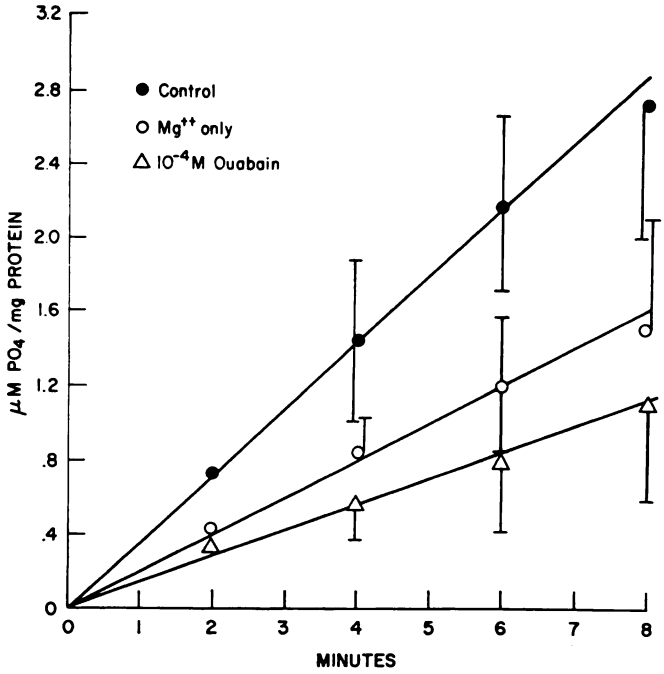


FIG. 5. *Effects of ouabain in the presence of $Na^+$ and $K^+$ on ($Na^+$ + $K^+$)-ATPase activity in synaptosomes*

Synaptosomes were first warmed for 5 min before ATP was introduced into the suspension. ●——●, control synaptosomes were incubated in 0.15 M Tris buffer, pH 7.2, containing 1 mM EDTA, 4 mM $Mg^{++}$, 120 mM $Na^+$, and 20 mM $K^+$. △——△, ATP and ouabain were added together to final concentrations of 3 mM and $10^{-4}$ M. ○——○, effect of $Na^+$ + $K^+$ omission. ($Na^+$ + $K^+$)-ATPase activity is expressed as micromoles of phosphate released from ATP per minute per milligram of protein. The curves represent the average and standard deviation of four experiments.

*Effect of ouabain on ATPase activity.* The time lag between the addition of ouabain to synaptosomes and its blockade of amine accumulation suggested that the inhibition might be a secondary effect of ouabain, perhaps related to changes in ion gradients. This possibility was investigated by comparing the time courses of the blockade of amine accumulation and the inhibition of ATPase activity, since it is well known that ouabain inhibits ($Na^+$ + $K^+$)-ATPase (21). For the purposes of this study, the difference between ATPase activity in the presence of $Na^+$, $K^+$, and $Mg^{++}$ and activity in the presence of $10^{-3}$ M ouabain was taken to indicate the ($Na^+$ + $K^+$)-ATPase activity (Table 4 and Fig. 5).

*Temporal development of the inhibition of ($Na^+$ + $K^+$)-ATPase by ouabain.* Figure 6 shows that the activity of the ($Na^+$ + $K^+$)-ATPase was completely blocked when ouabain ($10^{-4}$ M) was added together with ATP at zero time or when the ATP was added after the synaptosomes had been incubated with ouabain for 5 min. In each case, the data are represented by a single straight line which extrapolates back to zero, indicating that there was no initial time lapse in the action of ouabain on ATPase comparable to that on amine accumulation (compare Fig. 6 with Figs. 1 and 2).

Table 5 shows the inhibition of ($Na^+$ + $K^+$)-ATPase by various concentrations of ouabain. At $10^{-5}$ M, ouabain blocked the ($Na^+$ + $K^+$)-ATPase by 84%; in contrast, this concentration of ouabain blocked accumulation of $^{14}$C-5-HT by only 20% after the first 5 min (Fig. 1). After a 10-min incubation, however, this same concentration of ouabain blocked amine accumula-

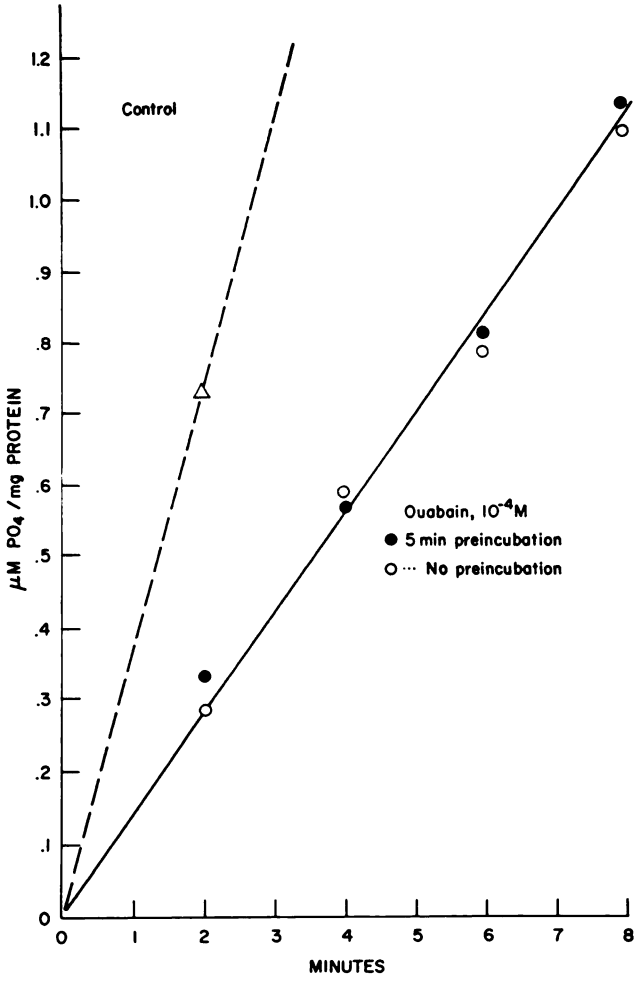


FIG. 6. *Effect of prior incubation of synaptosomes with ouabain on* $(Na^+ + K^+)$*-ATPase activity*

The medium was the same as the control solution in Fig. 5. Ouabain ($10^{-4}$ M) was added together with ATP after 10 min of warming (○——○), or 5 min before ATP after 5 min of warming (●——●). $(Na^+ + K^+)$-ATPase activity is expressed as in Fig. 4. The curves represent the average of four experiments.

tion by 75% (Fig. 2). The maximum inhibitory effect of ouabain on $(Na^+ + K^+)$-ATPase and on the accumulation of $^{14}$C-5-HT was produced by a concentration of $10^{-4}$ M.

## DISCUSSION

Dengler *et al.* (22) first demonstrated that ouabain could block the accumulation of NE by brain and heart slices. Carlsson *et al.* (23) suggested that ouabain exerts this effect by inhibiting the transport of NE across the cell membrane rather than by acting on storage granules. Later, Pletscher *et al.* (24) presented evidence that ouabain blocks the transport of 5-HT into platelets by an action on the cell membrane. The present studies indicate that ouabain acts on the neuronal membrane since it protects exogenous 5-HT from metabolism by monoamine oxidase within the cell.

The results of the present study also show that there is a lapse of time before ouabain blocks the transport process. The findings of other investigators that ouabain only partially inhibits the uptake of NE and metaraminol (22, 25, 26) is probably

based on the accumulation of the amine that takes place before ouabain has completely blocked the transport process. Table 1 shows that some 5-HT is metabolized by synaptosomes, even though ouabain ($10^{-3}$ M) has almost completely blocked accumulation of the amines. It is probable that this deamination is produced by monoamine oxidase from damaged synaptosomes and free mitochondria. In this regard, Leitz and Stefano,[2] using the intact perfused heart, have found that ouabain completely blocks the metabolism of infused NE.

To elucidate the mechanism by which ouabain blocks the accumulation of biogenic amines by synaptosomes, the possibility was considered that this effect might result from inhibition of membrane $(Na^+ + K^+)$-ATPase. The present study is an attempt to answer the question whether ouabain blocks amine transport directly through the inhibition of ATPase or indirectly through the effects of ATPase inhibition on ion distribution. In the first place, the inhibitory effects of ouabain on $(Na^+ + K^+)$-ATPase and on amine transport differ in their temporal development. Although ouabain at concentrations of $10^{-5}$ M or more blocks ATPase almost immediately, it produces complete inhibition of the process that accumulates 5-HT only after a delay of 5–10 min. However, after incubation with ouabain for 10 min, both ATPase and transport of the amines are almost completely blocked. The relatively slow onset of the blockade of amine accumulation supports the view that this action is secondary to the inhibition of $(Na^+ + K^+)$-ATPase.

The question arises whether the inhibition of metabolism of exogenous ATP is a measure of ATPase inhibition within the nerve endings. It is known that ATP is metabolized intracellularly and that exogenous ATP cannot penetrate membranes (8). It is possible, therefore, that the enzyme activity we have measured took place in fragmented synaptosomes or, alternatively, that rabbit brain synaptosomes are permeable to ATP. In any case, other studies have shown that ouabain blocks ion transport in nerve and heart almost immediately (27, 28).

In an attempt to show that the effect of ouabain on amine transport is secondary to changes in cellular electrolyte concentration, we have found that blockade of amine accumulation by ouabain depends upon the presence of $Na^+$ in the medium. For example, if synaptosomes are incubated for 5 min with ouabain in the presence of $Na^+$, the transport of 5-HT is completely blocked. However, if synaptosomes are incubated for the same length of time with ouabain in the absence of $Na^+$, the introduction of $Na^+$ into the external medium restores the transport of the amine for a period of about 5 min. In contrast, ouabain completely blocks ATPase whether $Na^+$ is present or absent during prior incubation with the inhibitor (Fig. 5 and Table 3). These findings suggest that in order to inhibit transport, ouabain must have time to cause an increase in the absolute intracellular $[Na^+]$, thus eliminating the transmembrane $[Na^+]$ gradient that is established when exogenous $Na^+$ is added to the $Na^+$-free preparation. However, when 5-HT is added to synaptosomes that have been incubated previously with ouabain in the presence of $Na^+$, the amine does not accumulate, presumably because the normal $Na^+$ and $K^+$ concentration gradients are drastically altered. This type of experiment is similar to that reported by Iff and Wilbrandt (29) in their study of the uptake of iodide by the perfused thyroid gland. To our knowledge, our experiment is the first of this design in which an organic molecule was used as substrate.

The evidence we have presented supports our model of the amine transport mechanism located in the neuronal membrane (5, 6). This model is patterned after similar models for sugar (30) and amino acid transport (31). Amine transport is visualized as being carrier-mediated (32, 33), and the affinity of the carrier for the amine is regulated by electrolytes. Outside the cell, the affinity between carrier and amine is high, since affinity is increased by high

[2] F. Leitz and F. Stefano, unpublished observations.

[$Na^+$] and low [$K^+$] (5, 6).[3] Intracellularly, the affinity between carrier and amine is low, because the intracellular [$Na^+$] is low and the high [$K^+$] readily antagonizes the effect of the $Na^+$ on the carrier (5).[3] Therefore, at the inner membrane surface both amine and $Na^+$ are released from the carrier; the amine is stored or metabolized, and the $Na^+$ is pumped out of the cell by the ($Na^+ + K^+$)-ATPase mechanism. Thus, the normal asymmetrical distribution of $Na^+$ and $K^+$ across the cell membrane causes the affinity of the carrier for amine to be less at the inner membrane surface than at the outer surface. In accord with the concepts of Wilbrandt and Rosenberg (34), the uneven distribution of affinity favors active transport. It is therefore possible that the energy for active transport of amines is provided by the asymmetrical distribution of $Na^+$ across the cell membrane, as postulated for other substances (30, 35). If so, the transport would ultimately be regulated by ($Na^+ + K^+$)-ATPase, which maintains the ionic gradients.

Ouabain blocks amine transport by increasing the intracellular [$Na^+$] and decreasing [$K^+$] through the inhibition of ($Na^+ + K^+$)-ATPase. This hypothesis is in accord with the well-known fact, recently also shown for isolated nerve cells (36), that ouabain increases the intracellular [$Na^+$] and reduces [$K^+$]. The transport process can be blocked even before the cytoplasmic $Na^+$ reaches a new steady state, as shown by the finding that the $Na^+$ may preferentially accumulate at the membrane, at least during the early stages of ouabain action (37). As a result, the affinity of the carrier for amine at the inner membane surface would be increased, eliminating the uneven distribution of affinity; hence the amine would tend to remain on the carrier. Active transport would now come to a halt, because the cycle of amine attachment and release has been stopped.

One difficulty with the above model is the failure of NE transport, unlike that of 5-HT, to be restored when $Na^+$ is added to synaptosomes that have previously been incubated with ouabain in the absence of $Na^+$. The significance of this difference is under investigation. One possible explanation is that NE is transported by a carrier which must first be phosphorylated before it can transport NE. When $Na^+$ is added subsequently, transport remains inhibited because the carrier cannot be phosphorylated when ATPase has been inactivated by ouabain.

[3] Unpublished observations.

REFERENCES


1. D. F. Bogdanski and B. B. Brodie, *Life Sci.* **5**, 1563 (1966).
2. L. L. Iversen and E. A. Kravitz, *Mol. Pharmacol.* **2**, 360 (1966).
3. C. N. Gillis and D. M. Paton, *Brit. J. Pharmacol. Chemother.* **29**, 309 (1967).
4. W. D. Horst, I. J. Kopin and E. R. Ramey, *Amer. J. Physiol.* **215**, 817 (1968).
5. D. F. Bogdanski and B. B. Brodie, *J. Pharmacol. Exp. Ther.* **165**, 181 (1969).
6. D. F. Bogdanski, A. Tissari and B. B. Brodie, *Life Sci.* **7** (Part I), 419 (1968).
7. R. W. Colburn, F. K. Goodwin, D. L. Murphy, W. E. Bunney, Jr., and J. M. Davis, *Biochem. Pharmacol.* **17**, 957 (1968).
8. P. C. Caldwell, A. L. Hodgkin, R. D. Keynes and T. I. Shaw, *J. Physiol. (London)* **152**, 561 (1960).
9. P. J. Garrahan and I. M. Glynn, *J. Physiol. (London)* **192**, 217 (1967).
10. E. G. Gray and V. P. Whittaker, *J. Anat. (London)* **96**, 79 (1962).
11. G. Rodriguez de Lores Arnaiz and E. de Robertis, *J. Neurochem.* **11**, 213 (1964).
12. O. Warburg and W. Christian, *Biochem. Z.* **310**, 384 (1941).
13. D. F. Bogdanski, A. Pletscher, B. B. Brodie and S. Udenfriend, *J. Pharmacol. Exp. Ther.* **117**, 82 (1956).
14. S. Udenfriend, H. Weissbach and B. B. Brodie, *Methods Biochem. Anal.* **6**, 95 (1958).
15. A. Pletscher, *Brit. J. Pharmacol. Chemother.* **32**, 1 (1968).
16. J. Haggendal, *Scand. J. Clin. Lab. Invest.* **14**, 128 (1958).
17. R. J. A. Hosie, *Biochem. J.* **96**, 404 (1965).
18. I. Berenblum and E. Chain, *Biochem. J.* **32**, 295 (1938).
19. E. W. Sutherland, C. F. Cori, R. Haynes and N. S. Olsen, *J. Biol. Chem.* **180**, 825 (1949).
20. I. J. Kopin, *Pharmacol. Rev.* **16**, 179 (1964).
21. J. C. Skou, *Biochem. Biophys. Acta* **42**, 6 (1960).

22. H. J. Dengler, H. E. Spiegel and E. O. Titus, *Science* **133**, 1072 (1961).
23. A. Carlsson, N.-Å. Hillarp and B. Waldeck, *Acta Physiol. Scand.* **215** (Suppl.), 59 (1963).
24. A. Pletscher, W. P. Burkard, I. P. Tranzer and K. F. Gey, *Life Sci.* **6**, 273 (1967).
25. S. B. Ross and A. L. Renyi, *Life Sci.* **5**, 639 (1966).
26. F. Berti and P. A. Shore, *Biochem. Pharmacol.* **16**, 2091 (1967).
27. P. C. Caldwell and R. D. Keynes, *J. Physiol. (London)* **148**, 8P (1959).
28. M. Weatheral, *in* "Enzymes and Drug Action" (J. L. Mongar and A. V. S. de Reuck, eds.), p. 115. Little, Brown, Boston, 1962.
29. H. W. Iff and W. Wilbrandt, *Biochim. Biophys. Acta* **68**, 711 (1963).
30. R. K. Crane, *Fed. Proc.* **24**, 1000 (1965).
31. D. M. Kipnis and J. E. Parrish, *Fed. Proc.* **24**, 1051 (1965).
32. H. J. Dengler, I. A. Michaelson, H. E. Speigel and E. O. Titus, *Int. J. Neuropharmacol.* **1**, 23 (1962).
33. L. L. Iversen, *Brit. J. Pharmacol. Chemother.* **21**, 523 (1963).
34. W. Wilbrandt and T. Rosenberg, *Pharmacol. Rev.* **13**, 109 (1961).
35. G. A. Vidaver, *Biochemistry* **3**, 795 (1964).
36. E. Giacobini, S. Hovmark and Z. Kometiani, *Acta Physiol. Scand.* **71**, 391 (1967).
37. G. I. Kaye, N. Lane, J. D. Cole and A. Donn, *Anat. Rec.* **154**, 366 (1966).