Elimination of nicotinic acid amide reduces the hydroxylation activity by 60 per cent when NADP acted as the coenzyme. When NAD acted as the coenzyme removal of nicotinic acid amide did not cause any significant change whereas when NADH was used the hydroxylation activity was slightly reduced.

The microsomal hydroxylation activity of rabbit liver seemed to be about ten times greater than the activity of dog liver. The cofactor studies gave the same results in dog and rabbit so that the hydroxylation was more effective in the presence of NADP and nicotinic acid amide. In dog the hydroxylation capacity of the duodenum was found to be approximately the same as in the liver.

In previous studies we have found that cinchophen is hydroxylated *in vivo* also to 8-hydroxycinchophen in addition to 4′-hydroxycinchophen. In these *in vitro* studies no 8-hydroxycinchophen was found.

*Acknowledgements*—This study has been supported by a Public Health Service Grant AM-6018.

*Department of Physiology,*
*University of Turku,*
*Turku, Finland*

TUULIKKI TERHO
K. HARTIALA

## REFERENCES

1. C. MITOMA, A. POSNER, H. REITZ and S. UNDENFRIEND, *Arch. S. Biochem.* **61**, 431 (1956).
2. J. BOOTH and E. BOYLAND, *Biochem. J.* **66**, 73 (1957).
3. B. BRODIE, J. GILLETTE and B. LA DU, *A. Rev. Biochem.* **27**, 427 (1958).

---

Biochemical Pharmacology, Vol. 16, pp. 600–603. Pergamon Press Ltd. 1967. Printed in Great Britain

# The effect of diphenylhydantoin on sodium-, potassium-, magnesium-stimulated adenosine triphosphatase activity of rat brain*

(*Received* 1 *September* 1966; *accepted* 15 *November* 1966)

IT HAS been reported that diphenylhydantoin has a specific effect upon mechanisms in the neural membrane by increasing the rate of transport of $Na^+$ from brain cells.[1, 2]

There is evidence that Na- K- Mg-activated ATPase (Na K Mg-ATPase) is either the carrier mechanism involved in the active transport of $Na^+$ and $K^+$ or is closely related to it.[3] The present study was designed to ascertain whether or not diphenylhydantoin has any effect upon the Na K Mg-ATPase of rat brain *in vivo* or *in vitro*.

*Preparation of brain tissue.* Albino rats were decapitated and the entire brain, including cerebellum and medulla, was removed, weighed, and homogenized at 0° in 9 volumes of 0·32 M sucrose solution.

*Adenosine triphosphatase assay.* The reaction mixture contained 30 mM KCl, 3 mM $MgCl_2$, 3 mM ATP (Sigma), and 100 mM NaCl buffered at pH 7·4 with 25 mM Tris-HCl in a 1·0 ml volume. At times NaCl was omitted, and ouabain in varying concentrations was added. The mixture was incubated for 10 min at 37°. The reaction was stopped by the addition of 5·0 ml of cold 10% $HClO_4$. Reaction blanks were prepared by adding $HClO_4$ without incubation. The mixture was centrifuged, and inorganic phosphate was measured in 1·0 ml of supernatant fraction by the method of Fiske and Subbarow.[4] The Na K Mg-ATPase activity was estimated by subtracting non-Na K Mg-ATPase values from total ATPase values. Non-Na K Mg-ATPase activity was estimated by phosphate

* This work was supported by Grants 5T1-NB5030 and ROI-NB 00940 from the National Institute of Neurological Diseases and Blindness.

released in incubations to which $Na^+$ had not been added or vessels to which ouabain had been added. Total ATPase activity was estimated by phosphate released in incubations containing $Na^+$ but from which ouabain was omitted.

*Determination of the effect of diphenylhydantoin on Na K Mg-ATPase.* Acute experiments *in vivo*: Male albino rats were given diphenylhydantoin in a dose of 50 mg/kg, i.p. Two hours later the animals were sacrified and the brains analyzed for Na K Mg-ATPase. These results were compared with those of unmedicated control animals.

Chronic experiments *in vivo*: Male albino rats were given diphenylhydantoin in doses of 50 mg/kg daily for 5 consecutive days. Two hours after the last injection, the animals were sacrified. Na K Mg-ATPase values were compared with non-injected controls.

Experiments *in vitro*: In this series of experiments, the effect of diphenylhydantoin upon the ouabain-induced inhibition of Na K Mg-ATPase was determined. Ouabain in concentrations varying from $10^{-6}$ M to $10^{-2}$ M was placed in the $Na^+$-containing incubation medium and the $P_i$ content after incubation was compared with an identical control sample to which no ouabain was added. The difference represented the ouabain-inhibited (i.e. Na K Mg-ATPase) fraction. At the same time an identical set of incubations was performed to which diphenylhydantoin as well as ouabain was added. All samples were done in duplicate at each concentration. Ten animals were used in experiments in which diphenylhydantoin was tested at $10^{-4}$M, three animals at $10^{-5}$ M, and two at $10^{-6}$ M.

The characteristics of ATPase activities found in this work were similar to those previously described.[5]

*Effects of diphenylhydantoin on Na K Mg-ATPase activity* in vivo. The effect of diphenylhydantoin upon the Na K Mg-ATPase activity of five male albino rats was determined in acute experiments 2 hr after administration of the drug, and the results were compared with five controls (see Table 1).

TABLE 1. INFLUENCE OF DIPHENYLHYDANTOIN ON NA K MG-ATPASE ACTIVITY OF RAT BRAIN

| Dose and duration | No. | ATPase activity ($\mu$moles $P_i$/g/10 min) Control Mean ± S.E., range | Treated Mean ± S.E., range |
|---|---|---|---|
| 50 mg/kg, once | 5 | 160 ± 40, 30-230 | 160 ± 50, 50-310 |
| 50 mg/kg, daily for 5 days | 8 | 140 ± 10, 100-200 | 160 ± 30, 80-320 |

The injected animals had Na K Mg-ATPase values of 160 ± 50† $\mu$moles $P_i$/g/10-min incubation as compared with 160 ± 40 units in the controls. In the chronic experiments the Na K Mg-ATPase values in eight diphenylhydantoin-treated animals was 170 ± 30 units as compared with 140 ± 10 units in six controls. This tendency of diphenylhydantoin to increase Na K Mg-ATPase levels was not statistically significant ($P < 0.2$).

*Effects of diphenylhydantoin on Na K Mg-ATPase activity* in vitro. The effect of diphenylhydantoin upon the Na K Mg-ATPase activity of five male albino rats was compared with that of five controls (Table 1). In the experimental samples, diphenylhydantoin ($10^{-4}$ M) was added to the brain homogenate and to the incubation mixture. The Na K Mg-ATPase value in the experimental group was 120 ± 10 and in the control group 140 ± 10. The tendency toward depression of Na K Mg-ATPase values in the treated sample was not statistically significant ($P < 0.1$).

This experiment was repeated with five animals which served as their own controls. In these, incubations were carried out with the same brain sample in the presence and in the absence of diphenylhydantoin ($10^{-4}$ M). The Na K Mg-ATPase values were obtained by measuring the difference in $P_i$ production of incubation solutions containing added $Na^+$ and those to which none was added. The values for the experimental samples were 160 ± 30 and for the controls 180 ± 30. In this series the tendency toward depression of ATPase values in the treated sample was not significant ($P < 0.1$).

† Expressed as mean values ± standard error.

As can be seen from these data, diphenylhydantoin added *in vitro* had the possible effect of diminishing, slightly and inconclusively, Na K Mg-ATPase activity. The degree of diminution ranged from 10 to 20 m-moles $P_i$/g/10 min at $10^{-4}$ M. No effect was apparent at $10^{-5}$ M or $10^{-6}$ M. In the presence of ouabain, however, diphenylhydantoin increased the inactivation of Na K Mg-ATPase by an amount approximately two to three times that which could be obtained by the use of diphenylhydantoin alone. When diphenylhydantoin at $10^{-4}$ M was used, this phenomenon was observed in all incubations containing ouabain at $10^{-4}$ M and $10^{-5}$ M ($P < 0{\cdot}05$) and in 75 per cent of the incubations containing ouabain at $10^{-2}$ M, $10^{-3}$ M, and $10^{-6}$ M (see Fig. 1). Diphenylhydantoin alone at $10^{-5}$ M had no effect on Na K Mg-ATPase; in combination with ouabain, however, an effect similar to that described above was seen but it was quantitatively smaller and less consistent. At $10^{-6}$ M, diphenylhydantoin had no effect on Na K Mg-ATPase in the presence or absence of ouabain.

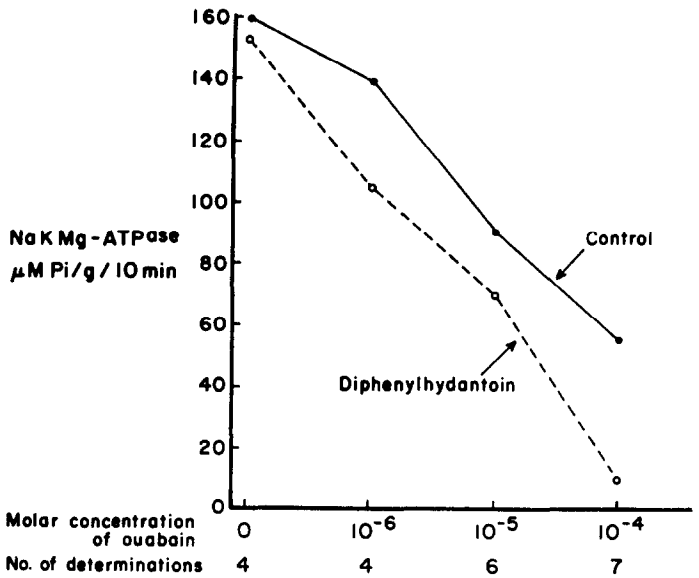


FIG. 1. Mean activity of Na K Mg-ATPase, as measured by ouabain inactivation, in the presence and absence of diphenylhydantoin $10^{-4}$ M.

Na K Mg-ATPase seems to be remarkably stable and unaffected by many neurophysiologically active drugs. Preliminary experiments in this laboratory *in vivo* with metrazole (75 mg/kg), pentobarbital (25 mg/kg), and a neurotoxic thiamine antagonist (pyrithiamine) indicated that these substances have no effect upon Na K Mg-activated ATPase. The central nervous system depressant and anesthetic adjuvant, γ-hydroxybutyric acid (GHB), in lethal doses (2 g/kg), produced an enhancement of Na K Mg-ATPase activity, but this was not noted at lower, anesthetic levels (600 mg/kg). Similarly, the sulfur analogue of GHB, 4-mercaptobutyrate, a potent convulsant, produced an increase in Na K Mg-ATPase only in lethal doses (500 mg/kg). Experiments done *in vitro*, however, indicated, that neither GHB ($10^{-2}$ M) nor 4-mercaptobutyrate ($10^{-2}$ M) had any effect upon this enzyme system; nor did prochlorperazine ($10^{-4}$ M), lysergic acid diethylamide ($10^{-8}$ M), tetrodotoxin (10/ml), or pyrithiamine ($5 \times 10^{-4}$ M) have any effect. Other drugs have similarly been shown by other investigators to have little effect on this system. For example, amytal, tribromethanol, and propanol at concentrations that inhibit cationic stimulation of brain respiration have little effect on ouabain-sensitive ATPase.[6, 7] In addition, acetazoleamide, aldosterone, and hydrocortisone have been found to produce no change.[8]

Diphenylhydantoin, administered parenterally in chronic experiments, had no demonstrable effect; for although it seemed to have a tendency to increase the activity of Na K Mg-ATPase over that of control animals, this was inconsistent and not statistically significant.

The studies carried out *in vitro*, which demonstrated inhibition of Na K Mg-ATPase, are of interest since the degree of inhibition caused by diphenylhydantoin alone was minimal and not statistically significant. This inhibition was markedly augmented by the presence of ouabain especially in concentrations of $10^{-4}$ M and $10^{-5}$ M. This effect of diphenylhydantoin in inhibiting Na K Mg-ATPase in the presence of ouabain was statistically significant and dose related and indicates that diphenylhydantoin *in vitro* may inhibit Na K Mg-ATPase to a small extent but that the inhibition is augmented by the presence of other inhibitors.

These results suggest that under certain circumstances diphenylhydantoin may act upon the Na K Mg-ATPase fraction of brain.

*Departments of Pharmacology and Medicine (Neurology),*
*Yale University School of Medicine,*
*New Haven, Conn., U.S.A.*

J. H. PINCUS
N. J. GIARMAN

## REFERENCES

1. D. M. WOODBURY, *J. Pharmac. Exp. Ther.* **115**, 74 (1955).
2. D. A. BRODIE and D. M. WOODBURY, *Am. J. Physiol.* **192**, 91 (1958).
3. J. C. SKOU, *Physiol. Rev.* **45**, 596 (1965).
4. C. H. FISK and Y. SUBBAROW, *J. biol. Chem.* **66**, 375 (1925).
5. A. SCHWARTZ, H. S. BACHELARD and H. MCILWAIN, *Biochem. J.* **84**, 636 (1962).
6. O. GONDA, S. L. CHAN and J. H. QUASTEL, *Proc. Can. Fed. biol. Soc.* **8**, 51 (1965).
7. J. H. QUASTEL, *Proc. R. Soc. B.* **163**, 169 (1965).
8. S. C. BONTING, K. A. SIMON and N. M. HAWKINS, *Archs Biochem. Biophys.* **95**, 416 (1961).

---

Biochemical Pharmacology, Vol. 16, pp. 603–604. Pergamon Press Ltd. 1967. Printed in Great Britain

## Anesthesia LXXII: Anesthesia with deuterochloroform*

(*Received* 15 *September* 1966; *accepted* 14 *October* 1966)

IN OUR previous studies[1] we showed that deuterated ethylene, evoked anesthesia in dogs similar to that induced by ethylene, and also that it did not sensitize the heart to challenging doses of epinephrine. In the latter respect it also resembles ethylene. Recently, our attention has again been focused on deuterated compounds[2] and their anesthetic properties. Having available a sample of deuterated chloroform we became interested in determining the character of the anesthetic syndrome it elicits, whether or not deuterium is exchanged for hydrogen during anesthesia, and whether deuterochloroform sensitizes the heart to epinephrine as does chloroform.

The deuterated chloroform was obtained from Volk and had an isotope purity better than 99·5 atom per cent.

The chloroform was "Chloroform for Anesthesia," N. F. Merck. The isotopic analyses were made with a CEC model 21103C mass spectrometer.

Anesthesia was conducted on Swiss-Webster albino mice ICR strain and mongrel dogs.

The mice, in groups of five, were placed in a 4-l. jar of oxygen with a gauze bag of soda lime. The anesthetic agent was introduced through a stopcock, and anesthesia was allowed to continue for 30 min. The gaseous contents of the jar were then drawn off by mild suction through a trap chilled with $CO_2$ ice and ethylene glycol monoethyl ether acetate. This was continued for 30 min. The frozen gases from the jar were thawed and subjected to mass spectrometric analysis.

The dogs were anesthetized via a mask with a closed-circuit Ohio infant inhalation set. The anesthetic was injected through a specially devised mask onto gauze.

The initial dose was 0·1 ml/kg. Additional chloroform was given during the course of the experiment to maintain anesthesia, as far as possible, in stage 3, plane 3. Control electrocardiograms (ECGs) (lead II) were obtained with a Sanborn Cardiette.

* Supported by a grant from the Life Sciences Division, Army Research Office.