Biochemical Pharmacology, 1965, Vol. 14, pp. 1025–1035. Pergamon Press Ltd., Printed in Great Britain.

INTERACTION OF ETHANOL AND THYROXINE ON MITOCHONDRIA

RALPH KARLER, THOMAS S. SULKOWSKI and JAMES T. MIYAHARA

Department of Pharmacology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.

(*Received* 12 *October* 1964; *accepted* 2 *December* 1964)

Abstract—Thyroxine can stimulate mitochondrial swelling and uncouple oxidative phosphorylation. It was observed that ethanol inhibits both spontaneous and thyroxine-induced mitochrondrial swelling. The antiswelling activity of ethanol is manifested during mitochondrial respiration both in the presence and absence of phosphorylation. Ethanol was observed only to block thyroxine-induced swelling; it could not reverse the swelling. Ethanol also blocked Ca^{++}- and phosphate-induced swelling. Therefore, the antiswelling activity of ethanol is more generalized than that of serum albumin. It is concluded that the ethanol antagonism of mitochrondrial swelling produced by various agents is probably dependent on the fact that ethanol alone inhibits swelling; ethanol is therefore a pharmacological antagonist of agents that induce swelling.

In addition to inhibiting swelling, ethanol also blocks uncoupling of oxidative phosphorylation by thyroxine and Ca^{++} but not by salicylate. These observations were interpreted to indicate that ethanol affects oxidative phosphorylation only indirectly by blocking mitochondrial swelling.

The observed effects of ethanol on mitochrondria are discussed in terms of reports on the ability of thyroid hormone to antagonize acute alcohol intoxication in man, and the ability of ethanol to block effects of Ca^{++} on smooth muscle.

THE effects of thyroxine on mitochondrial structure and function have been extensively studied in recent years.[1–4] Of the effects reported, mitochondrial swelling and uncoupling of oxidative phosphorylation have received the most attention. Various investigators have observed that in the presence of appropriate experimental conditions thyroxine can stimulate mitochondrial swelling and uncouple oxidative phosphorylation.[1, 3, 5] Available evidence indicates that a sufficient degree of mitochondrial swelling can produce uncoupling;[5] in fact, agents such as thyroxine and Ca^{++} are considered by some investigators to produce uncoupling indirectly by an effect on the mitochondrial membrane that results in enhanced swelling.[6, 7] Other agents, for example, serum albumin[8] and EDTA,[9] are capable of antagonizing the swelling and uncoupling effects of thyroxine, and this provides support for the dependence of the uncoupling effect of thyroxine on primary changes produced in the mitochondrial structure.

Preliminary studies indicated that ethanol can retard both spontaneous and thyroxine-induced mitochondrial swelling.[10] The data also indicated that ethanol can block the uncoupling effect of thyroxine. These observations lend additional support to the concept that the functional properties of oxidative phosphorylation are dependent upon structural characteristics of mitochondria.

The observed antagonism between thyroxine and ethanol at the subcellular level is of particular interest because thyroid hormone has been reported to antagonize acute ethanol intoxication in man.[11, 12] In addition, Von Hagen and Hurwitz[13] observed that ethanol is capable of antagonizing the effects of Ca^{++} on smooth muscle.

In the following study, details of the influence of ethanol on the above-mentioned mitochondrial effects of thyroxine are described. Also, the reactions of ethanol with other agents known to affect mitochondrial swelling and oxidative phosphorylation are included. These agents are phosphate, Ca^{++}, and sodium salicylate.

METHODS

Preparation of mitochondria. Liver mitochondria were prepared from adult male Sprague-Dawley rats. Mitchondria were isolated from sucrose homogenates by differential centrifugation in the following manner. The tissue was pressed through a Lucite sieve to remove connective tissue, and the resulting liver mash was homogenized in isotonic sucrose solution (1:10, w/v); the homogenate was centrifuged at 750 *g* for 5 min to remove nuclei and cell debris; the resulting supernatant was centrifuged at 10,000 *g* for 10 min to collect mitochondria; and the mitochondrial pellets were washed twice by resuspension in isotonic sucrose solution (1:5 w/v), the volume of which was based on the original weight of the liver mash. After the final wash, the mitochondria were suspended in 0·25 M sucrose solution (1:5 w/v), the volume again based on the original weight of liver mash.

Determination of P/O ratios. Respiration was determined by conventional manometric technics, and a glucose-hexokinase trap was employed for the measurement of P/O ratios. Phosphate analyses were based on a modification of the method of Dryer, Tammes, and Routh.[14] The main compartment of the Warburg vessels contained 40 μmoles KH_2PO_4 buffer (pH 7·4), 40 μmoles sodium pyruvate or 20 μmoles sodium succinate, 5 μmoles ATP, 5 μmoles Mg Cl_2, 50 μmoles glucose, 80 K-M units hexokinase, 150 μmoles Tris buffer (pH 7·4), and 1·0 ml mitochondrial suspension. Total volume of reaction medium was 3·3 ml, temperature of the reaction was 30°, the gas phase was air, and the incubation period was 15 min.

Swelling studies. The swelling studies were performed in two ways. In the conventional procedure (nonphosphorylating, acceptor-deficient system), mitochondria were suspended in a 0·25 M sucrose–0·05 M Tris buffer medium (pH 7·4), and the rate of swelling was followed in a spectrophotometer at a wavelength setting of 520 μm. In other swelling studies, the suspension medium and procedure were identical with those used in the oxidative phosphorylation studies described above. The resulting suspension has a much higher optical density than that employed in the conventional swelling studies. However, relative changes in optical density could easily be measured with the use of cuvettes with a path length of 4 mm instead of the conventionally employed cuvettes with a path length of 10 mm.

Reagents. The thyroxine used in all the experiments was obtained from Sigma Chemical Co., as was the hexokinase (type III). Solutions of thyroxine were prepared fresh daily by the addition of dilute sodium hydroxide to about pH 8. Some mitochondrial preparations were not uncoupled by thyroxine; the reason for this is not known, but such preparations were discarded in the following studies. The phosphate reagent, *p*-semidine (N-phenyl-*p*-phenylenediamine monohydrochloride), was obtained from Distillation Products Industries.

RESULTS

Mitochondrial swelling

A. Thyroxine. A dose–effect relation between thyroxine and mitochondrial swelling is shown in Fig. 1. The data were obtained by the conventional procedure (non-phosphorylating, acceptor-deficient system) described under Methods for measuring mitochondrial swelling. These results indicate that between 1×10^{-5} M and 1×10^{-4} M thyroxine concentration there is a marked acceleration in the rate of mitochondrial swelling. For practical purposes, the maximal effect appeared at 1×10^{-4} M concentration, which was subsequently employed in the oxidative phosphorylation studies.

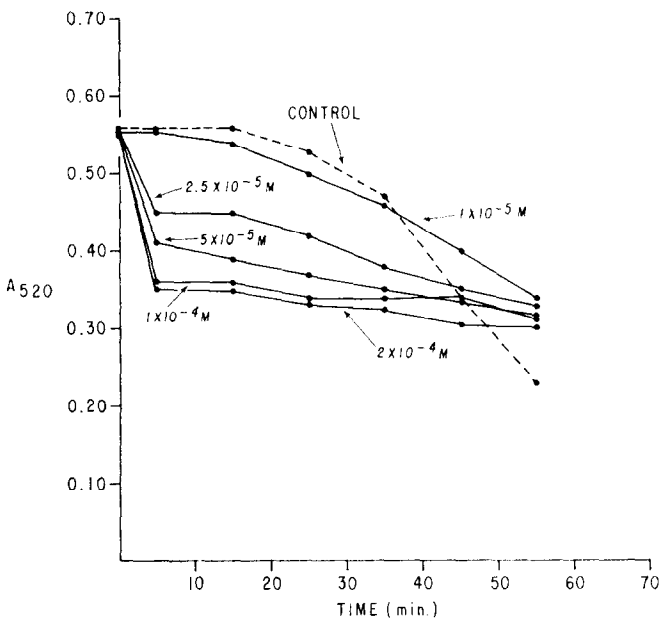

FIG. 1. Effect of various concentrations of thyroxine on rat liver mitochondria swelling. Experiment was performed on a nonphosphorylating, acceptor-deficient system as described in Methods.

B. Ethanol. Figure 2 presents a dose–effect relation between ethanol and mitochondrial swelling. The experimental procedure was identical with the above thyroxine study. Ethanol, however, produced qualitatively different results than did thyroxine; it can be seen in Fig. 2 that ethanol retards the rate of mitochondrial swelling. The maximal antiswelling effect of ethanol was attained at a concentration of 0·75 M. In the subsequent studies on oxidative phosphorylation, 0·50 M concentration of ethanol was generally employed.

C. Thyroxine and ethanol. The effect of various concentrations of ethanol on thyroxine-induced swelling is shown in Fig. 3. The data demonstrate that ethanol is capable of blocking the increased rate of mitochondrial swelling produced by thyroxine. In these experiments ethanol was added to the medium before the addition of thyroxine. In Fig. 4 are seen the results of experiments in which ethanol was added to the reaction after the addition of thyroxine and 5 min after the start of incubation. The data in

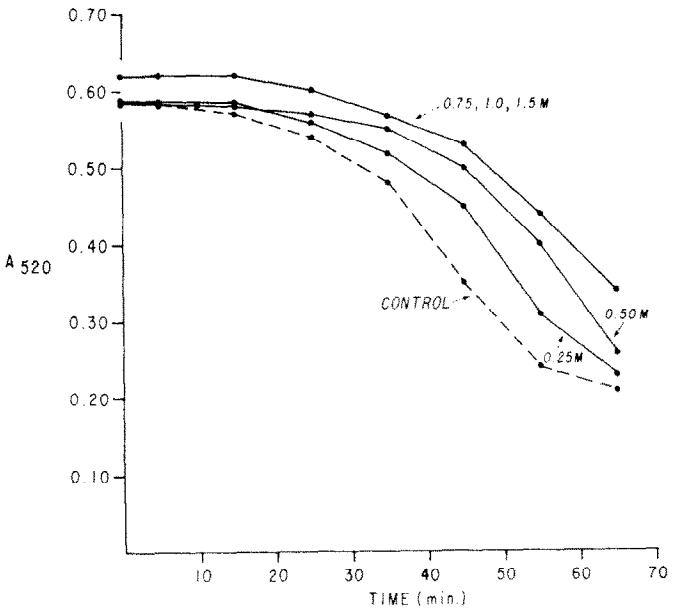

FIG. 2. Effect of various concentrations of ethanol on rat liver mitochondrial swelling. Experiment was performed on a nonphosphorylating, acceptor-deficient system as described in Methods.

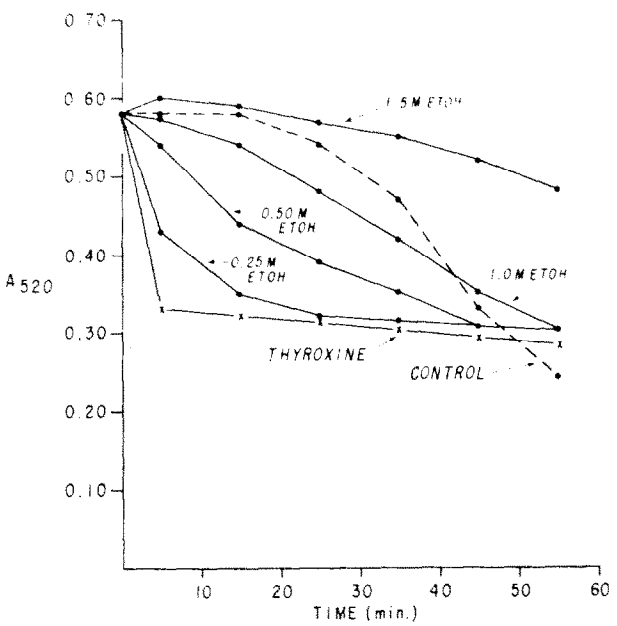

FIG. 3. Effect of various concentrations of ethanol on thyroxine-induced mitochondrial swelling. Ethanol is represented as ETOH. Ethanol was added to the reaction *before* the addition of thyroxine. Experiment was performed on a nonphosphorylating, acceptor-deficient system as described in Methods.

Fig. 4 indicate that ethanol does not reverse the mitochondrial swelling effect produced by thyroxine.

Table 1 presents data from swelling studies performed on mitochondria subjected to conditions identical with those in the oxidative phosphorylative studies. Samples were removed from Warburg flasks at the various time intervals indicated in the table,

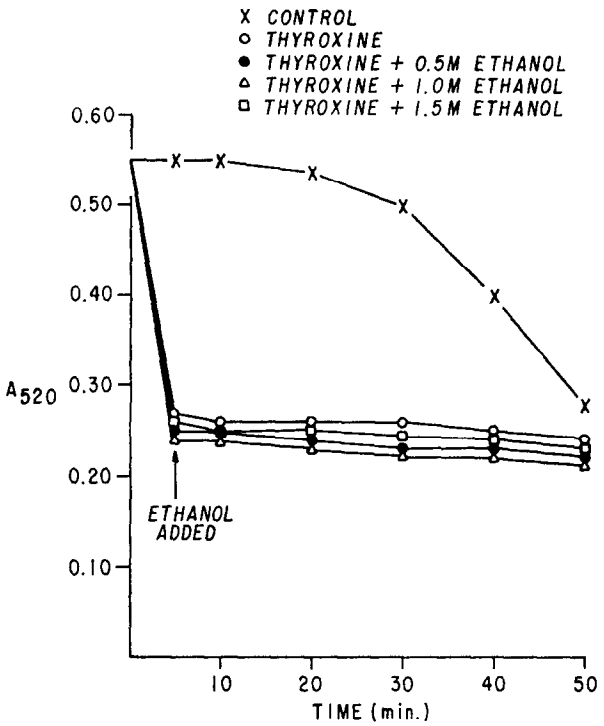

FIG. 4. Effect of ethanol on thyroxine-induced mitochondrial swelling. Ethanol was added to the reaction 5 min *after* the addition of thyroxine. Experiment was performed on a nonphosphorylating, acceptor-deficient system as described in Methods.

TABLE 1. EFFECT OF THYROXINE AND ETHANOL ON SWELLING OF RAT LIVER MITOCHONDRIA DURING PHOSPHORYLATING RESPIRATION*

Incubation time (min)	A_{520}			
	Control	Ethanol†	Thyroxine†	Thyroxine + ethanol†
0	0·68 ± 0·02	0·70 ± 0·02	0·64 ± 0·04	0·67 ± 0·03
8			0·57 ± 0·02	
13			0·50 ± 0·05	0·58 ± 0·04
18			0·47 ± 0·04	0·57 ± 0·05
23	0·64 ± 0·04	0·66 ± 0·04	0·50 ± 0·03	0·52 ± 0·03

* Values for each group are means ± S.E. of 6 experiments.

† The ethanol concentration was 0·50 M; the thyroxine concentration was 1×10^{-4} M.

and the optical density of the suspension were measured directly in a spectrophotometer as described under Methods. The data obtained during phosphorylating respiration illustrate that thyroxine promotes mitochondrial swelling and that ethanol is capable of decreasing the rate of thyroxine-induced swelling. It should be noted that ethanol in the concentration employed did not completely block the thyroxine effect; rather, it altered the rate of the effect.

D. Calcium and ethanol. Table 2 represents Ca^{++} data obtained under conditions identical with the P/O-ratio data in Table 4. It is apparent that the marked swelling effect normally produced by Ca^{++} is inhibited by the presence of ethanol in the reaction system.

TABLE 2. EFFECT OF CALCIUM AND ETHANOL ON SWELLING OF RAT LIVER MITOCHONDRIA DURING PHOSPHORYLATING RESPIRATION*

Incubation time (min)	A_{520}		
	Control	Calcium†	Calcium + ethanol†
0	0·87 ± 0·01	0·86 ± 0·01	0·86 ± 0·01
8	0·86 ± 0·02	0·74 ± 0·04	0·86 ± 0·03
23	0·86 ± 0·02	0·58 ± 0·04	0·78 ± 0·02

* Values for each group are means ± S.E. of 4 experiments.
† The calcium concentration was 2×10^{-4} M; the ethanol concentration was 0·50 M.

E. Phosphate and ethanol. The interaction of these substances with mitochondrial swelling is shown in Fig. 5. The results demonstrate that ethanol can also antagonize phosphate-induced swelling.

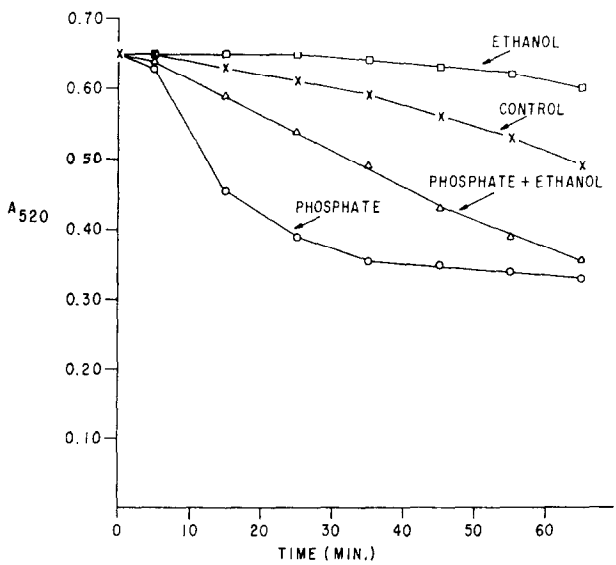

FIG. 5. Effect of ethanol on phosphate-induced mitochondrial swelling. Ethanol concentration was 0·50 M and phosphate (added as Na_2HPO_4) concentration was 2×10^{-3} M. Experiment was performed on a nonphosphorylating, acceptor-deficient system as described in Methods.

Oxidative phosphorylation

The results in Table 3 represent effects of thyroxine and ethanol on oxidative phosphorylation. The reaction medium for these experiments was identical with that employed in the swelling experiments of Table 1 except that succinate as well as pyruvate was used as a substrate. The pyruvate data indicate that ethanol alone produces little or no depression of respiration and an apparent increase in P/O ratio. An increase in P/O ratio was generally observed; however, the increase shown in Table 3 was found

TABLE 3. EFFECT OF THYROXINE AND ETHANOL ON THE P/O RATIOS OF RAT LIVER MITOCHONDRIA WITH PYRUVATE AND SUCCINATE AS SUBSTRATES*

	Pyruvate			Succinate		
Treatment	(Δ μmP$_i$)	(μA [0])	(P/O $\pm$ S.E.)	(Δ μmP$_i$)	(μA [0])	(P/O $\pm$ S.E.)
Control	23·16 (10)	9·89	2·35 $\pm$ 0·06	18·40 (7)	10·82	1·70 $\pm$ 0·01
Ethanol†	23·47 (10)	9·22	2·57 $\pm$ 0·10	15·41 (7)	8·94	1·73 $\pm$ 0·06
Thyroxine†	11·45 (10)	8·41	1·27 $\pm$ 0·23‡	5·70 (9)	10·11	0·56 $\pm$ 0·04‡
Thyroxine + ethanol†	25·15 (10)	11·12	2·17 $\pm$ 0·14‡	8·78 (9)	8·50	1·06 $\pm$ 0·06‡

* Values represent the means of the number of experiments for each group, indicated in parentheses; Δ μmP$_i$ represents μmoles phosphate uptake/15 min; μA[0] represents μatoms of oxygen uptake/15 min.

† The thyroxine concentration was 1×10^{-4} M, the ethanol concentration 0·50 M.

‡ "t" Test: $P < 0.01$ for thyroxine vs. thyroxine + ethanol.

not to be significant ($P < 0.05$) by the "t" test. Nevertheless, it is clear from the thyroxine and ethanol data that ethanol can block, at least in part, the uncoupling effect of thyroxine. The results with succinate as a substrate are essentially the same as with pyruvate.

The influence of ethanol on uncoupling produced by Ca^{++} and sodium salicylate is shown in Table 4. It can be seen that Ca^{++} greatly depresses respiration and also uncouples oxidative phosphorylation. Again, as was observed with thyroxine, ethanol

TABLE 4. EFFECT OF ETHANOL ON UNCOUPLING ACTIVITY OF CALCIUM AND SALICYLATE*

Treatment	(Δ μmP$_i$)	(μA[0])	(P/O $\pm$ S.E.)
Control (10)	24·46	10·20	2·39 $\pm$ 0·07
Ethanol (10)†	22·91	9·14	2·52 $\pm$ 0·08
Calcium (10)†	5·84	6·41	0·85 $\pm$ 0·23‡
Calcium + ethanol (10)†	15·02	7·96	1·88 $\pm$ 0·17‡
Salicylate (4)†	8·69	8·50	1·01 $\pm$ 0·05
Salicylate + ethanol (4)†	7·71	8·21	0·94 $\pm$ 0·08

* Values represent the means of the number of experiments in each group, indicated in parentheses; abbreviations as in Table 3.

† The ethanol concentration was 0·50 M, the calcium concentration 2×10^{-4} M, the salicylate concentration 1×10^{-3} M.

‡ "t" Test: $P < 0.01$ for calcium vs. calcium + ethanol.

can inhibit the usual uncoupling effect of Ca^{++}. With respect to salicylate, however, ethanol appears to be without effect on the uncoupling activity of this agent.

DISCUSSION

The characteristics of thyroxine-induced mitochondrial swelling and uncoupling of oxidative phosphorylation have been extensively investigated in recent years. The data in the present paper demonstrate that ethanol can block both of these effects of thyroxine on mitochondria. It is possible that ethanol antagonizes thyroxine by decreasing the effective concentration of thyroxine as a result of some physical or chemical interaction of the two drugs. A basis for a reaction of this type, however, is not apparent. Thyroxine is soluble in a 0·5 M solution of ethanol, so precipitation of thyroxine appears to be excluded. Any chemical reaction, for example deiodination or esterification, between these agents does not seem feasible. A spectrophotometric analysis of a solution of 1×10^{-4} M thyroxine indicated that there was no quantitative change in the absorption at 260, 270, and 280 $m\mu$ in the presence of 0·5 M ethanol. The spectrophotometric analysis, however, does not necessarily rule out a chemical change in thyroxine. Various limitations on quantitative tests make it very difficult to eliminate definitely the possibility of a chemical change. The important point here is that there is no reason to expect any chemical reaction under these conditions between ethanol and thyroxine; consequently, the ethanol antagonism of thyroxine probably occurs at the mitochondrial level.

In terms of the blocking effect of ethanol on swelling, Fig. 2 illustrates that the drug alone inhibits swelling; therefore, the ethanol antagonism of thyroxine is probably the result of the ability of ethanol to produce the opposite effect. It also appears likely that any effect of ethanol on the P/O ratio is dependent on its antiswelling property. Such an interpretation stems from the data in Table 4 which demonstrate that ethanol blocks uncoupling produced by thyroxine and Ca^{++}, but not that produced by salicylate. Both thyroxine and Ca^{++} can uncouple indirectly by inducing swelling, whereas salicylate uncouples by a direct action on the phosphorylating reactions.[15]

The mechanism of the antiswelling activity of ethanol is not clear. There are many known factors that can interfere with mitochondrial swelling, either spontaneous or thyroxine-induced. For example, swelling appears to be dependent upon respiration, and substances such as cyanide that inhibit respiration can also inhibit mitochondrial swelling.[16, 17] The inhibition of mitochondrial swelling by ethanol cannot be due to inhibition of respiration. The data shown in Table 1 indicate that, despite the relatively high ethanol concentration employed in these experiments, ethanol did not produce a significant depression of respiration. Beer and Quastel[18] also observed that high concentrations of ethanol did not depress respiration.

Most swelling studies reported are conducted in a nonphosphorylating system. Because of the influence of ATP on mitochondrial structure, it was of interest to compare the swelling of mitochondria in a nonphosphorylating system to the swelling in the system employed for the measurement of P/O ratios. The data shown in Tables 1 and 2 demonstrate that thyroxine and Ca^{++} stimulate the swelling rate in either system and that ethanol antagonizes the swelling effect produced by both agents. Therefore, the data indicate that the effects of thyroxine, Ca^{++}, and ethanol during phosphorylating respiration are similar to the effects observed in a nonphosphorylating system.

The ability of most substances to antagonize thyroxine is restricted to a blocking action; the blocking agents in general cannot reverse the thyroxine effects. The only known substance that can reverse these effects is ATP.[5] The data shown in Fig. 4 illustrate that ethanol cannot reverse thyroxine-induced swelling; therefore, the ethanol antagonism of thyroxine, at least in terms of swelling, is limited to a blocking action.

Various substances can block the swelling effect of thyroxine.[5] Serum albumin is such an agent, and it appears to antagonize thyroxine by binding U factor formed by mitochondria.[8] Both thyroxine and Ca^{++} appear to enhance mitochondrial swelling by stimulating the formation of U factor. The fact that ethanol antagonizes both thyroxine and Ca^{++} suggests the possibility that the antiswelling effects of ethanol are dependent upon an inhibitory effect on drug-induced formation of U factor. This possibility is further supported by the observations in Fig. 2 that ethanol inhibits the spontaneous mitochondrial swelling rate that has been related to the spontaneous formation of U factor. However, the ethanol effects cannot simply be due to inhibition of U-factor formation because ethanol, unlike albumin, also inhibits phosphate-induced swelling. Therefore, ethanol appears to possess nonspecific, antiswelling activity. EDTA is another substance with a wide range of antiswelling properties, and its action is considered to be dependent upon the chelating properties of the molecule.[19, 20] Such an explanation is not appropriate for the antiswelling character of ethanol.

Another possible mechanism of the antiswelling action of ethanol is that the relatively high concentration (0·5 M) employed in the experiments produces an osmotic effect on the mitochondria. Other reports have indicated that under appropriate conditions mitochondrial swelling, spontaneous or thyroxine-induced, can be inhibited and can be reversed by an osmotic effect.[21, 22] With respect to ethanol, the possibility that it exerts its antiswelling properties by an osmotic effect does not appear likely. For ethanol to exert an osmotic effect on mitochondria, it would be necessary to assume that ethanol penetrates the mitochondrial membrane slowly or not at all. Because ethanol is a relatively small, uncharged molecule that is soluble in both water and lipid, it is probable that ethanol penetrates the mitochondrial membrane rapidly. Furthermore, some of the experimental data presented in this paper indicate that ethanol does not exert an osmotic effect on mitochondrial volume. For example, the results in Fig. 4 demonstrate that ethanol cannot reverse thyroxine-induced swelling, yet osmotic effects can reverse this type of swelling. In addition, Tedeschi[22] has also shown that the mitochondrial volume is inversely proportional to the osmotic pressure of the surrounding medium. The results in Fig. 2 do not support this general relation in the presence of increasing concentrations of ethanol.

Tedeschi[22] also proposed that swelling is the result of solute penetration into mitochondria; the subsequent change in intramitochondrial osmotic pressure causes the uptake of water or swelling. It is known that sucrose penetrates mitochondria slowly,[23] which may account for swelling in a sucrose medium. If ethanol reduces the permeability of the mitochondrial membrane to sucrose, the reduction could affect the swelling rate. In addition, an effect on sucrose permeability is consistent with the fact that ethanol can block swelling but cannot reverse it. The possibility that ethanol affects the permeability of the mitochondrial membrane to sucrose is under investigation.

The demonstrated antagonism between ethanol and thyroxine and Ca^{++} at the biochemical level is of interest in terms of previous reports by others of such an antagonism *in vivo* and at the tissue level. Goldberg *et al.*[12] reported that intravenous triiodothyronine is capable of antagonizing severe alcohol depression in man. The antagonism was originally attributed to an increase in the rate of metabolism of ethanol. However, subsequent investigations failed to confirm any effect of triiodothyronine on rate of metabolism of ethanol.[24, 25] If an antagonism exists *in vivo*, the nature of it is not clear. In the present studies it can be seen that an antagonism exists between ethanol and thyroxine in terms of mitochondrial swelling and oxidative phosphorylation. How such biochemical effects are related to the described antagonism *in vivo* is not obvious. There is an effect on respiration shown in Table 3 that may be involved in the *in vivo* antagonism. The results in Table 3 demonstrate that the respiratory rate in the presence of both ethanol and thyroxine is greater than in the presence of either drug alone. The difficulty with an explanation in terms of respiratory rate is that ethanol by itself does not significantly depress respiration. In this regard, it would appear important to study the effect of both of these drugs on potassium-stimulated respiration, which has been reported by Beer and Quastel[18] to be much more sensitive to depression by ethanol than the corresponding nonstimulated respiration.

Recently, Von Hagen and Hurwitz[13] reported that ethanol is capable of antagonizing some of the effect of Ca^{++} on smooth muscle. The concentrations of ethanol they employed were approximately the same as those in the present study. Again, there is no obvious connection between the effects on mitochondrial swelling, oxidative phosphorylation, and muscle contraction. However, the effects on muscle may not be limited to the effects on mitochondria. Ca^{++} can stimulate ATPase in the microsomal fraction of muscle homogenates,[26] whereas it has been reported that ethanol inhibits ATPase activity in microsomes derived from brain[27] and cardiac tissue.[28] Therefore, it is possible that the observed antagonism between ethanol and Ca^{++} in terms of muscle excitability arises from an interaction on ATPase activity at the reticulum or cell membrane level, rather than from an interaction at the mitochondrial level.

Acknowledgements—This investigation was supported in part by research grant NB-04553 and research career program award 5-K3-NB 8641 from the National Institute of Neurological Diseases and Blindness; and Public Health Service Pharmacology Research training grant 2T1-GM 153.

REFERENCES

1. O. LINDBERG, H. LÖW, T. E. CONOVER and L. ERNSTER, in *Biological Structure and Function,* vol 2, T. W. GOODWIN and O. LINDBERG, Eds., pp. 3–29. Academic Press, London (1961).
2. A. L. LEHNINGER, in *Horizon in Biochemistry*, M. KASHA and B. PULLMAN, Eds., pp. 421–35. Academic Press, New York (1962).
3. F. L. HOCH, *New Engl. J. Med.* **266**, 446 (1962).
4. J. W. HARMAN and M. T. O'HEGARTY, *Exp. molec. Path.* **1**, 573 (1962).
5. A. L. LEHNINGER, *Physiol. Rev.* **42**, 467 (1962).
6. D. F. TAPLEY and C. COOPER, *J. biol. Chem.* **242**, 341 (1956).
7. W. C. MCMURRAY, G. F. MALEY and H. A. LARDY, *J. biol. Chem.* **230**, 219 (1958).
8. L. WOJTCZAK and A. L. LEHNINGER, *Biochim. biophys. Acta* **51**, 442 (1961).
9. D. F. TAPLEY, *J. biol. Chem.* **222**, 325 (1956).
10. T. S. SULKOWSKI and R. KARLER, *Pharmacologist* **5**, (2), 273 (1963).
11. R. W. RAWSON, H. KOCH and F. F. FLACH, in *Hormones, Brain Function and Behavior*, H. HOAGLAND, Ed., pp. 234–39. Academic Press, New York (1957).
12. M. GOLDBERG, R. HEHIR and M. HUROWITZ, *New Engl. J. Med.* **263**, 1336 (1960).

13. S. Von Hagen and L. Hurwitz, *Fed. Proc.* **23** (2), Part 1, 544 (1964).
14. E. M. Knights, Jr., R. P. MacDonald and J. Ploompuu, *Ultramicro Methods for Clinical Laboratories.* Grune & Stratton, New York (1962).
15. J. T. Miyahara and R. Karler, *Fed. Proc.* **23** (2), 178 (1964).
16. F. E. Hunter, jr., J. Davis and L. Carlat, *Biochim. biophys. Acta* **20**, 237 (1956).
17. A. L. Lehninger and B. L. Ray, *Biochim. biophys. Acta* **26**, 643 (1957).
18. C. T. Beer and J. H. Quastel, *Canad. J. Biochem.* **36**, 543 (1958).
19. F. E. Hunter, jr. and L. Ford, *J. biol. Chem.* **216**, 357 (1955).
20. J. Raaflaub, *Helv. chim. Acta* **38**, 27 (1955).
21. A. L. Lehninger, B. L. Ray and M. Schneider, *J. biophys. biochem. Cytol.* **5**, 97 (1959).
22. H. Tedeschi, *Biochim. biophys. Acta* **46**, 159 (1961).
23. K. L. Jackson and N. Pace, *J. gen. Physiol.* **40**, 47 (1956).
24. H. Kalant, G. Sereny and R. Charlebois, *New Engl. J. Med.* **267**, 1 (1962).
25. D. E. Smith, N. E. Fallis and L. Tetreault, *New Engl. J. Med.* **268**, 91 (1963).
26. A. Martonosi and R. Feretos, *J. biol. Chem.* **239**, 659 (1964).
27. J. Järnefelt, *Biochim. biophys. Acta* **48**, 104 (1961).
28. A. Schwartz and A. H. Laseter, *Biochem. Pharmacol.* **13**, 337 (1964).