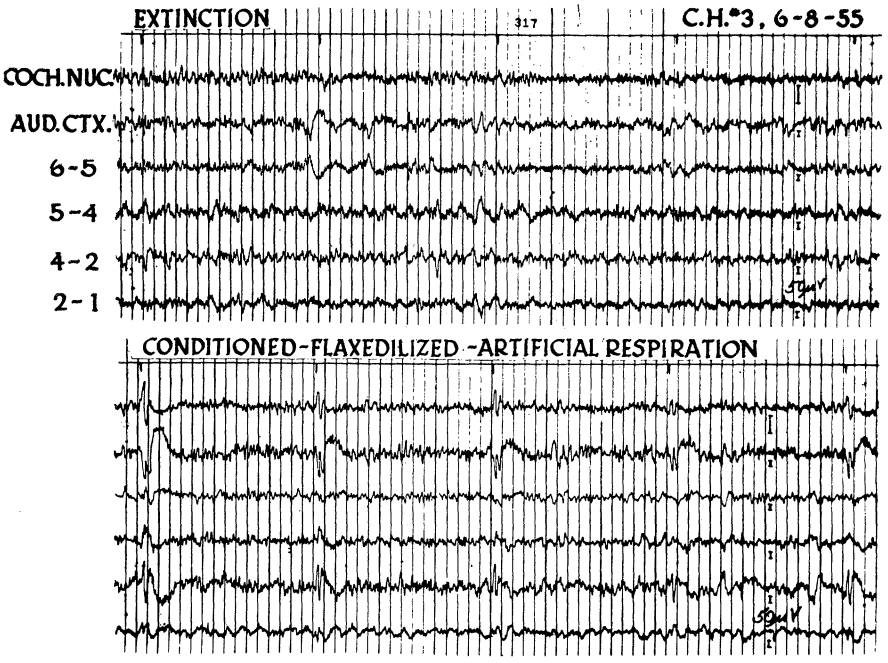

Fig. 2. EEG responses to identical clicks (indicated by artifact in upper trace) before (top records) and after a shock had been delivered for a time with each click. Bipolar recording from cochlear nucleus (second trace), monopolar from auditory cortex. The bottom four traces are derived from a hippocampal array (as yet not histologically verified) inserted in a dorsoventral line, the active tips being 2 mm apart with No. 1 the deepest. The animal had received Flaxedil prior to the bottom recording and thus exhibited no motor activity whatever.

such an animal are shown in the right column of Fig. 1. As the click continued without shock reinforcement, both motor and electric responses tended to disappear (motor long before electric), and the "extinguished" condition invariably returned after hours or days. In most of our animals, the cycle of conditioning and extinction thus defined has been repeated many times.

In view of the motor behavior accompanying the conditioned state, the possibility that the responses recorded are somehow generated by muscle activity or its consequences must obviously be ruled out. Although such considerations can hardly be held to apply to the phenomenon shown in Fig. 1, where the entire event is substantially completed within some 15 msec after the application of the stimulus, it must seriously be considered as a possibility for responses that have longer latency and duration.

Of all our attempts to settle this point, the experiments of the sort illustrated in Fig. 2 are perhaps the most conclusive. The upper record shows the extinguished record from an animal that had previously been conditioned and extinguished five times. The lower record, made about 1 hour after the upper one, shows evoked responses regularly in the stations along the classical auditory pathway (cochlear nucleus and auditory cortex) as well as in at least two of the electrodes in the hippocampus. Between the times at which the upper and lower records were made, the animal had received conditioning shocks and, additionally, gallamine triethiodide (Flaxedil, Lederle) 4 mg /kg intravenously in two divided doses within 1 minute. This dosage was sufficient to cause apparent complete muscular paralysis, and artificial respiration via an endotracheal tube was required. The only muscle activity noted was constriction of the iris as an object was brought close to the eye of the animal. Pupillary dilatation was also observed coincident with the conditioning shocks or with clicks alone immediately after shock. Certainly there was no movement of the animal that could account for the responses shown in the lower half of Fig. 2, during the recording of which, of course, only clicks were being applied at the times indicated in the top trace. It may parenthetically be stated here that, in extinguished animals that have been immobilized by Flaxedil, applied shocks promptly change the records of responses to those typical of the conditioned state.

From the data, only some of which have been presented here, the following general statements appear to be justified. When cats with indwelling electrodes are subjected to a relatively simple auditory conditioning technique, changes in electric activity of the brain apparently related to conditioning and extinction can be reliably recorded. Evoked auditory responses are larger and are seen more frequently and in more numerous locations when a given animal is in the conditioned as opposed to the extinguished state. Such changes occur near the origin of the classical auditory pathway (cochlear nucleus) as well as at its termination (auditory cortex), in portions of such limbic system structures as the hippocampus and septal area and in the head of the caudate nucleus.

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Reference

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29 August 1955

Enzymatic Synthesis of Cholyl CoA and Taurocholic Acid

It is well known that under normal conditions bile acids are excreted in bile only after they have been first conjugated with amino acids. Taurine is the amino acid most commonly involved in this conjugation reaction. No information has been published on the mechanism of formation of the amide bond between bile acids and taurine (*1*). We should like to report the presence of an enzyme, found only in the microsomes of liver, that will activate cholic acid according to the following reaction:

$$\text{Cholic acid} + \text{coA} \xrightarrow{\text{ATP, Mg}^{++}} \text{cholyl coA} \quad (1)$$

The isolation of cholyl coenzyme A (co-A) (Fig. 1) reported here represents, we believe, the first identification of an activated steroid in a biological system.

A second enzyme has been found in the supernatant fraction of liver that will carry out the reaction:

$$\text{Cholyl coA} + \text{taurine} \longrightarrow \text{taurocholic acid} + \text{coA} \quad (2)$$

Both microsomal and supernatant fractions were isolated from a phosphate

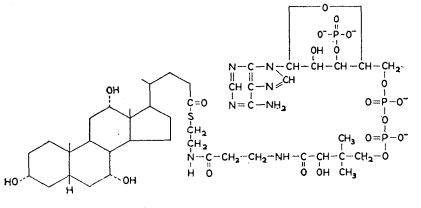

Fig. 1. Cholyl coA.

buffer homogenate by the differential centrifugation method of Schneider and Hogeboom (*2*). The microsomes were washed once each with isotonic KCl and distilled water. The supernatant was dialyzed for 24 hours against distilled water and then lyophilized. Mitochondria were isolated from an 0.88*M* sucrose homogenate (*2*). Taurocholic acid was routinely determined with the use of the furfural-H_2SO_4 reaction of Pettenkofer (*3*) after free cholic acid had been removed by extraction with acetone and ethyl ether in the proportion of 1 to 2 and ethyl ether. The identity of the product as taurocholic acid was confirmed by paper chromatography.

Incubation of 50 mg of washed, lyophilized guinea pig liver microsomes with 4 μmoles of coA, 10 μmoles of cholic acid, 44 μmoles of adenosine triphosphate (ATP), and 30 μmoles of $MgCl_2$ in 1.0 ml of 0.1*M* K_2HPO_4 buffer at *p*H 7.6 for 60 min resulted in the formation of 2.2 μmoles of a cholic acid derivative. When the compound was chromatographed on Whatman No. 1 filter paper, an ultraviolet absorbing band having the $\lambda_{max.}$ of adenine was noted at an R_f of 0.62 (60-percent propanol in H_2O) or at an R_f of 0.81 (50-percent ethanol in H_2O). Spraying a portion of the paper with the $SbCl_3$-acetic acid spray, that has been described previously (*4*) revealed a single rose-colored band that is characteristic of cholic acid. This band coincided exactly in position and shape in both solvent systems with the position and shape of the 260-mμ absorbing band; the compound was further identified as cholyl coA by means of color reactions listed in Table 1. Finally, hydrolysis of the compound resulted in the appearance of two bands on paper chromatograms, one having the R_f value and color reaction of cholic acid, the other having the R_f value and color reaction of coA.

Table 1. Identification of cholyl coA.

Test	Result
Cholic acid ($SbCl_3HAc$) (*4*)	+
Adenine ($\lambda_{max.}$ 260 mμ)	+
RSH (nitroprusside) (*8*)	–
RS—C—R′ (NaOH-nitroprusside) (*8*)	+
RS—C—R′ (hydroxylamine-$FeCl_3$) (*9*)	+
Ester phosphate (ammonium molybdate-$HClO_4$) (*10*)	+

Table 2. Role of cholyl coA in taurocholic acid synthesis. The figures in parentheses in column 1 are (i) amount, in millimicromoles, of cholyl coA and cholic acid and (ii) amount, in micromoles, of taurine added to the supernatant. The final volume was 0.6 ml in 0.03*M* K_2HPO_4 buffer at *p*H 7.6.

Compound(s) added to 20 mg of dialyzed liver supernatant	Taurocholic acid synthesized (mμ mole)
Cholyl coA (118)	0.0
Cholyl coA (118) pretreated with base* + taurine (24)	0.0
Cholic acid (166) + taurine (24)	0.0
Cholyl coA (118) + taurine (24)	85.3

* Cholyl coA in 0.1 ml water, hydrolyzed by the addition of 0.1 ml 4*N* NaOH and incubation for 2 min at 37°C. The mixture was neutralized with H_2SO_4 before addition of the liver supernatant.

If chromatographically pure cholyl coA is incubated together with taurine in the presence of dialyzed guinea pig liver supernatant, taurocholic acid is produced (Table 2); this demonstrates that cholyl coA is an intermediate in the synthesis of the conjugated bile salt.

Taurocholic acid can also be readily synthesized by combining the reactions given in Eqs. 1 and 2. Microsomes, dialyzed supernatant, cholic acid, coA, ATP, and taurine are all required for taurocholic acid production in this overall reaction. Either microsomal or supernatant fraction alone is inactive.

The enzyme responsible for cholic acid activation would appear to be distinct from those that catalyze the formation of the coA derivatives of fatty acids (*5*, *6*), benzoic acid (*6*) and *p*-aminobenzoic acid (*7*) in that the intracellular distribution of the other activating enzymes and, in one case, the tissue localization as well, are quite different from that of the cholyl coA-forming enzyme. The bile acid activating enzyme is found only in the microsomes of liver. Liver mitochondria are inactive and no kidney fraction will carry out the reaction. The enzyme that activates short-chain fatty acids, benzoic acid, and *p*-aminobenzoic acid, on the other hand, is known to be localized in the mitochondria of both liver and kidney (*6*, *7*). Likewise, the long-chain fatty acid-activating enzyme of liver supernatant (*5*), which in our studies readily synthesized palmityl coA, is incapable of so activating cholic acid.

The activating system was also found to be capable of catalyzing the formation of other bile acid-coA derivatives. Desoxycholyl coA and C^{14}-labeled lithocholyl coA have been prepared in micromole quantities. Their roles in the scheme of bile acid metabolism, with regard to both conjugation and interconversions, are now under study.

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References and Notes

1. Since completion of this paper, Elliott [*Biochim. et Biophys. Acta* 17, 440 (1955)] has reported that optimal formation of cholyl hydroxamic acid by liver microsomes required the presence of ATP, coA, and cysteine. This finding is consistent with the observation, reported here, that cholyl coA is produced by such a system.
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Correction

The names of the compounds in the last three lines of Table 1 in "Inhibition of the effect of some carcinogens by their partially hydrogenated derivatives" by Kotin, Falk, Lijinsky, and Zechmeister [*Science* **123**, 102 (20 Jan. 1956)] should read dihydrodibenzanthracene, decahydrodibenzanthracene, and perhydrodibenzanthracene.

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3 February 1956

The beauty of electricity, or any other force, is not that the power is mysterious and unexpected . . . but that it is under law and that the taught intellect can even now govern it largely.—M. FARADAY.