of 24-hour rhythm. In the absence of such standardization intra-assay variability may be augmented by dissociation of susceptibility rhythms among individuals and inter-assay variability by testing in different stages of rhythm.

Control of clock-hour of testing does not necessarily suffice for reducing the contributions of rhythms to variability of bioassays; rhythms might be free-running from the local clock with periods slightly though consistently different from 24 hours(1,2). Standardization for light-synchronized periodicity analysis overcomes this difficulty: "physiologic time" in terms of stage of susceptibility rhythm can then be related to a given clock hour. Subsequent work shows further that timing of peak (or trough) in susceptibility to endotoxin can be shifted from the usual clock hour by appropriate manipulation of the lighting regimen and by observing several additional simple precautions such as avoidance of disturbances in the mouse room, *e.g.*, from presence of experimenters(1). Work carried out in reproducibly defined stages of a given physiologic rhythm is particularly critical when it allows for detection (in one stage of rhythm) of effects which might not otherwise be found (in tests done in another stage of rhythm)(3). Apart from methodologic considerations, susceptibility rhythms show how critically the stage of *periodic* changes in physiologic state can determine an organism's ability to withstand damage.

Summary. Light-synchronized periodicity analysis reveals a susceptibility rhythm in C mice to *E. coli* endotoxin. Susceptibility varies predictably and significantly along the 24-hour time scale. A dose of endotoxin which is compatible with survival of most animals when given during middle of daily dark period is highly lethal when it is given 8-12 hours earlier or later. LD_{50} also was determined for 2 samples from identical batch of endotoxin tested 12 h apart on separate groups of comparable C mice kept under conditions standardized for periodicity analysis: Differences in potency significant at 1% were seen at 2 dose levels common to both assays, the computed "potency ratio" being 3.22.

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Inhibitory Influence of Ethanol on Serotonin Metabolism. (25440)

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During a study of acute experimental intoxication using normal human subjects, it was observed that a pronounced decrease in urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) occurred during 5-hour collection period following ingestion of about 100 g of ethanol. Inasmuch as 5-HIAA is the major metabolic end-product of serotonin, (5-hydroxytryptamine) metabolism in man(1,2), this finding conjured up the possibility that during metabolism of alcohol the oxidative conversion of endogenous serotonin (and, perhaps, of other monoamines) is temporarily inhibited. Further circumstantial evidence in support of this hypothesis was adduced from preliminary pharmacological studies using mice, which demonstrated that doses of serotonin and other primary aromatic amines, *viz.*, tryptamine, dopamine, and tyramine, which *per se* were innocuous and provoked little somatic, autonomic, or behavioral effects, exerted a striking potentiation of the narcotic action and acute toxicity of alcohol. Prompted by the possibility that the neuropharmacologic

* Opinions or assertions contained herein are private ones of author and are not to be construed as official or reflecting views of Navy Department or naval service at large.

and toxicologic effects of alcohol may be related, at least in larger doses, to its influence on endogenous monoamine metabolism, the present studies were undertaken to ascertain whether a sublethal, hypnotic dose does, in fact, interfere with the metabolism of serotonin. The experimental design involved parenteral administration of serotonin to mice pretreated with alcohol, and subsequent analysis at various intervals of the whole animal, including urine and feces. The latter procedure was adopted in preference to analyses of excreta alone because even moderate doses of alcohol can induce acidosis. Accordingly, a diminution observed only in the urinary output of metabolites like 5-HIAA could be attributable merely to a decreased renal excretion rather than a suppression of their formation.† Regarding analytical methods employed, determinations were conducted of serotonin and total 5-hydroxyindole compounds as well as of 5-HIAA because, in mammalian species other than man, 5-HIAA production accounts for only a portion of the serotonin destroyed and alternate metabolic routes must be considered. In view of probable formation of unidentified conjugation products, disappearance of serotonin and formation of 5-HIAA were followed directly. Moreover, since both of these are 5-hydroxyindole compounds, it was believed that measurement of total 5-hydroxyindoles would provide added information *vis-a-vis* the influence of alcohol on alternate metabolic routes involving conjugation.

Materials and methods. Adult male albino mice, NAMRU No. 1 strain(3), weighing 25 to 30 g, were employed. The experimental ones were pretreated intraperitoneally with 4.5 g/kg of a 25% (w/v) ethanol solution in 0.9% NaCl while control animals were given an equivalent volume of 0.9% NaCl, *i.e.*, 18 ml/kg. Preliminary studies revealed that this dose of alcohol resulted in no deaths among 40 treated mice, and that the mean period of hypnosis, defined as time from loss to return of the righting reflex, was 74 minutes. Under present conditions, doses up to 5.5 g/kg produced no deaths, and LD_{50} was between 6.5 and 7.0 g/kg. Thirty minutes after alcohol or saline control injection, mice were administered 160 mg/kg of serotonin intraperitoneally, and placed directly into beakers. The serotonin solution, freshly prepared by dissolving the creatinine sulfate complex (obtained from California Corp. for Biochemical Research) in 0.9% NaCl, was carefully brought to pH 7.0 with NaOH before use. At specified times following serotonin injection, groups of 4 mice together with their excreta, thoroughly washed out of the beakers, were placed in a Waring blendor and homogenized for 4 minutes in a total volume of 0.1 N HCl 4-fold their combined weight. Thus, each 5-ml aliquot represented 1 g of body weight and theoretically would contain 160 μg serotonin if obtained from mice homogenized immediately following injection. After straining through several layers of gauze, 25-ml portions of these homogenates were mixed with 5 ml of 10% $ZnSO_4 \cdot 7H_2O$ and 2.5 ml of 1N NaOH to effect a neutral protein precipitation. Clear supernates were obtained upon centrifugation for 10 minutes at high speed. Methods employed to determine serotonin, 5-HIAA, and total 5-hydroxyindoles in duplicate aliquots of these supernates are based on the Gerngross reaction with 1-nitroso-2-naphthol. They were modifications of those reported by Udenfriend and coworkers(1,4), who found that this reagent, in dilute HCl or H_2SO_4 containing traces of nitrite, will react almost exclusively with 5-hydroxyindoles to form a violet chromophore. Of the many phenolic and indole compounds tested, p-hydroxyacetanilide and mephenesin are the only known ones not 5-hydroxyindoles, which yield a violet color. Whereas measurement of serotonin and 5-HIAA, both of which are 5-hydroxyindoles, required preliminary separation and isolation from the supernates by means of successive extractions and partitions, colorimetric determination of total 5-hydroxyindoles was carried out directly on the filtered supernates. A blank and inclusive series of standards were concurrently run with each set

† In human intoxication study already cited, urinary excretion of uric acid was also markedly decreased. This finding is possibly related to alcohol-induced acidosis, for it is well known that in conditions of acidosis, renal excretion of uric acid is diminished.

TABLE I. Influence of Ethanol Pretreatment on Amounts of Serotonin, 5-HIAA, and Total 5-Hydroxyindoles Found in Whole Body and Excreta of Mice Administered Serotonin.* (2 exp. with 4 mice for each period).†

Time after serotonin inj., min.	μg/g found						μg/g calculated	
	Serotonin		5-HIAA		Total 5-OH indoles		Conjug. & other products§	
	Control	Alcohol	Control	Alcohol	Control	Alcohol	Control	Alcohol
0	159 162		0 0		161 160		0	
30	53 (99)‡ 70	(44.5) 113 119	33 (27)‡ 21	(9) 10 8	85 (89.5)‡ 94	(126) 124 128	72	35.5
60	38 (119.5) 44	(75.5) 79 91	36 (32) 28	(18) 22 14	75 (77.5) 80	(106.5) 101 112	87.5	57.5
90	25 (134.5) 27	(110) 44 57	35 (32) 29	(27.5) 29 26	70 (72.5) 75	(90) 83 97	102.5	82.5
120	13 (145.5) 17	(129) 31 32	30 (28) 26	(30) 32 28	61 (64.5) 68	(79) 76 82	117.5	99.0
150	11 (147) 14	(135.5) 23 27	28 (26.5) 25	(28) 30 26	56 (61) 66	(73) 69 77	120.5	107.5

* Exp. mice were pretreated with 4.5 g/kg of a 25% ethanol solution in 0.9% NaCl; control mice were given 18 ml/kg of 0.9% NaCl. Thirty min. later, all were inj. I.P. with 160 mg/kg serotonin.

† Each value represents mean of duplicate dets. conducted on pooled carcasses and excreta of 4 mice, except for those in parentheses and the last 2 columns, which represent means of 8 animals.

‡ Values in parentheses, from left to right, represent: serotonin destroyed, calculated by subtracting mean amount recovered from mean zero-time value; mean 5-HIAA found; mean total 5-OH indoles found.

§ Metabolites of serotonin other than 5-HIAA, calculated by subtracting mean amount of 5-HIAA found from mean amount of serotonin destroyed.

of determinations and carried through all extraction and color development procedures.

Results. Table I embodies analytical findings of 2 experiments on the influence of ethanol on metabolism of parenterally administered serotonin. In mice pretreated with an hypnotic, sublethal dose of alcohol, rate of destruction of exogenous serotonin was appreciably decreased. Concordant evidence for the inhibitory effect of alcohol on serotonin metabolism is provided by the following data: (1) recovery of larger amounts of unmetabolized serotonin at successive intervals following injection; (2) detection of smaller amounts of 5-HIAA, particularly in early stages of detoxification‡; (3) finding of greater quantities of total 5-hydroxyindole compounds despite the smaller amounts of contributory 5-HIAA; (4) formation of less "conjugated and other products" *i.e.*, metabolites of serotonin, other than 5-HIAA, calculated by subtracting mean amount of 5-HIAA found from mean amount of serotonin destroyed. The interrelationships of these findings are portrayed in Fig. 1.

In connection with formation of metabolites other than 5-HIAA, it is noteworthy that the amounts of 5-HIAA recovered do not account for the total quantities of serotonin destroyed. For example, after 30 minutes when 99 and 44.5 μg/g of serotonin had been metabolized by control and alcohol-treated mice, resp., only about 27 and 9 μg/g, resp., were accounted for as formed 5-HIAA. Obviously, a substantial portion of administered serotonin had been converted *via* another route(s), a probable one being conjugation of its 5-hydroxyl group. This is confirmed by total 5-hydroxyindole data which also exhibit successive declines. Theoretically, total 5-OH indole level would remain constant (around 160 μg/g) if serotonin were converted only to 5-HIAA since both are 5-hydroxyindoles. In view of its progressive fall, it may be concluded that serotonin disappears not only as a consequence of oxidation of its aliphatic side chain but also *via* conjugation of its aromatic 5-OH group, *e.g.*, glucuronide formation. Only recently, Weissbach and cowork-

‡ As indicated by data of Table I, 5-HIAA itself probably undergoes further alteration, *e.g.*, oxidation, conjugation, etc. These conversions, particularly by conjugation, may also be inhibited by alcohol.

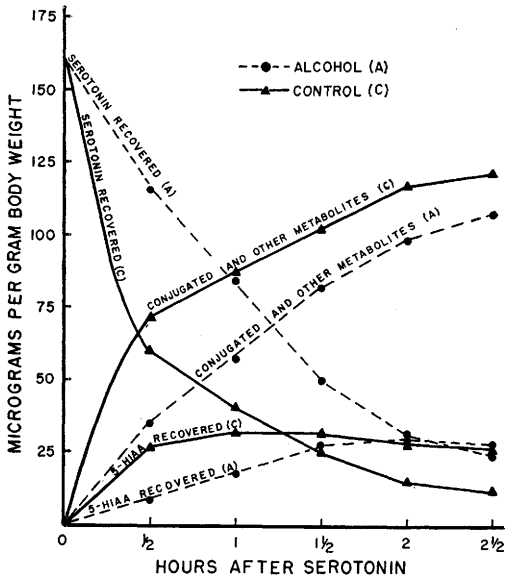

FIG. 1. Influence of ethanol pretreatment on metabolism of serotonin parenterally administered to mice. Thirty min. after I.P. inj. of 4.5 g/kg ethanol, mice were given 160 mg/kg serotonin I.P. At various times after the serotonin dose, the whole animal and its excreta were homogenized and assayed for serotonin, 5-HIAA, and total 5-OH indoles. The "conjugated and other metabolites" represent products derived from serotonin other than 5-HIAA. Each point represents mean value for 8 mice.

ers(5) reported that serotonin-o-glucuronide was, in fact, a normal, major metabolite which accounted for at least 25% of a dose of C^{14}-labeled serotonin administered to mice. Thus, data of Table I reveal that, in addition to inhibiting oxidation of serotonin to 5-HIAA, narcotic doses of alcohol depress its biotransformation *via* glucuronide conjugation.

Perhaps, one more observation should be made from data in Table I, concerning total 5-hydroxyindole compounds measured. If the total 5-OH indoles comprised only serotonin and 5-HIAA, its value should never be greater than their summation. However, one finds that it is. In fact, as detoxification of serotonin progresses, there is an increasing disparity between total 5-OH indoles found and the sum of serotonin and 5-HIAA recovered. This strongly suggests that, in addition to 5-HIAA and serotonin-o-glucuronide, there are metabolites of serotonin which possess an unconjugated 5-OH aromatic group and an oxidized (other than acetic), and/or oxidized (including acetic) plus conjugated, and/or conjugated (or methylated?) aliphatic side chain. It is possible that this group constitutes an even greater proportion of unidentified end-products than is indicated by the 5-OH indole data inasmuch as conjugation, methylation, etc. of the side chain probably interferes with intensity of the 5-OH indole color reaction.

Discussion. The present findings revealed that a sublethal, hypnotic dose of alcohol in the mouse inhibited metabolism of a parenteral dose of serotonin, and depressed its biotransformation *via* oxidation and conjugation. The oxidative conversion of serotonin involves its deamination by monoamine oxidase to an intermediate aldehyde and subsequent oxidation of the latter by means of aldehyde dehydrogenase and DPN to 5-HIAA(6). Moreover, both stages of ethanol oxidation require DPN as a coenzyme, and the second stage utilizes the same enzymatic machinery involved in oxidative conversion of the aldehyde formed from serotonin. The preceding enzymatic considerations suggest that during the initial period, when rate of alcohol metabolism was maximum, its oxidation competitively inhibited the oxidative metabolism of serotonin, and perhaps, in particular, oxidation of the aldehyde derived from it. Under such conditions, one might expect that more serotonin would be metabolized *via* an available, alternate route, *e.g.*, conjugation. However, concurrent depression of this metabolic route occurred. Perhaps, competitive inhibition also underlies the mechanism by which ethanol delays conjugation of serotonin in view of the fact that DPN is involved in glucuronic acid formation, and that small amounts of ethanol are metabolized *via* glucuronic acid conjugation(7).

Although inhibition of only serotonin metabolism was demonstrated by the present chemical data, it is reasonable to assume that detoxification of other primary aromatic monoamines would be similarly affected inasmuch as they are inactivated *via* the same metabolic route(s). Endogenous amines like dopamine, tryptamine, tyramine (and to a lesser extent, norepinephrine and epinephrine) are all good substrates for the monoamine oxidase of liver, brain, intestine, kidney and other

tissues, and are oxidatively deaminated by it to corresponding aldehydes. Other circumstantial evidence in support of this hypothesis is provided by the pharmacological studies already cited, which showed that large amounts of serotonin, tryptamine, dopamine and tyramine could be injected intraperitoneally in the mouse without provoking hypnosis or death. However, if animals were pretreated with the same hypnotic dose of alcohol as presently employed, even a moderate dose of these amines induced a substantial mortality as well as a decisive prolongation of sleeping time. In fact, extremely small doses, which *per se* provoked little somatic, autonomic, or behavioral effects, induced a marked potentiation of the narcotic action of alcohol despite the fact that neither brain alcohol content nor overall body rate of alcohol destruction were modified.

Inasmuch as the amines employed in these experiments as well as in the present ones were administered intraperitoneally, the liver was probably the main site of their metabolism, as it is of alcohol, in general. Together then, chemical and pharmacological findings strongly suggest that large doses of alcohol may impair, at least temporarily, the functional capacity of liver to detoxify serotonin and other primary aromatic monoamines. Thus, they differ from the majority of studies reported which revealed little or no demonstrable functional impairment in intoxicated men subjected to a battery of liver function tests. It is the usual custom, at present, to regard pathological alterations in the liver and other organs of chronic alcoholics as malnutritional sequelae or complications rather than ascribe them to a direct effect of alcohol. In view of the accepted role of liver as an inactivator of endogenous amines and perhaps, some exogenous ones formed in the intestinal lumen by bacterial activity, it is tempting to implicate a chronic alcohol-induced suppression of aromatic monoamine metabolism in the etiology of degenerative changes in this organ after years of excessive and habitual ingestion of alcohol.

Theoretically, the monoamine metabolism of the brain§ should also be influenced by levels of alcohol which affect liver monoamine metabolism inasmuch as it does not possess multiple routes of amine inactivation and depends mainly on oxidative conversion *via* its monoamine oxidase content. Within the last few years a number of aromatic monoamines, *viz.*, serotonin, norepinephrine, dopamine, and tryptamine, have been shown to occur naturally and selectively in the brain. Moreover, there is a growing body of evidence that some of these endogenous monoamines may act as neurohumoral agents in the regulation of central transmission in certain areas. While the possibility still cannot be precluded that narcotic properties of alcohol are derived from a primary interference with some stage of neuronal oxidative and/or high-energy bond metabolism, it appears reasonable to postulate that, at least in higher concentrations, there is a casual relationship between the neuropharmacologic action of alcohol and its influence on monoamine metabolism.

Summary. The influence of a sublethal, hypnotic dose of ethanol on metabolism of serotonin was investigated in the mouse. Thirty minutes after intraperitoneal injection of 4.5 g/kg of ethanol, mice were administered 160 mg/kg of serotonin intraperitoneally. At various intervals after the serotonin dose, the whole animal and its excreta were homogenized and analyzed for serotonin, 5-HIAA, and total 5-hydroxyindole compounds. The results clearly demonstrated that alcohol exerted an appreciable inhibitory effect on metabolism of serotonin and depressed its biotransformation *via* oxidation and conjugation. The enzymatic, toxicologic, and neuropharmacologic implications are discussed.

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§ Of all body tissues, the brain of a mouse administered iproniazid, a potent monoamine oxidase inhibitor, exhibits the most striking effects either in endogenous serotonin metabolism or after 5-hydroxytrytophan administration.

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Thyroid Hormone and Mammary Gland Growth in the Rat.* (25441)

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The beneficial effect of thyroid hormone and thyroid active substances upon lactation is well established(1), however, little is known concerning the role of this hormone in influencing growth of mammary gland. Lactation studies have shown that if hormones influencing lactation were administered in optimal amounts, increased milk yields occurred with marked reduction in variability of response among members of the population(2,3). Similarly, any hormone, if present in suboptimal amounts, may also limit mammary gland growth or, at least, contribute to variability in growth which occurs during normal pregnancy or to that of glands developed experimentally with optimal levels of estrogen and progesterone(4). In the present study we determined the effect of mild hyperthyroidism upon experimental development of rat mammary gland using desoxyribosenucleic acid (DNA) as an index of growth.

Materials and methods. Groups of ovariectomized rats of the Sprague-Dawley-Rolfsmeyer strain with an initial weight of 225-275 g were used. Body weight was recorded daily. Fifteen ovariectomized rats killed 34 days post-castration served as controls. Remaining ovariectomized rats received daily subc. injections for 19 days commencing 14 day post-castration as follows: 1) 18 received either 1 or 2 μg estradiol benzoate (EB); 2) 29 were injected with 1 μg EB and 3 or 6 mg progesterone (P); 3) 13 received 2 μg EB and 6 mg P; 4) 31 were treated with 1 μg EB, 3 mg P and 3 or 6 μg l-thyroxine; and 5) 49 received 2 μg EB, 6 mg P and 3 or 6 μg thyroxine. Rats were killed one day after last injection, skinned rapidly and abdominal-inguinal glands removed and immediately frozen for 4 days. Tissues were then thawed and fat extracted in boiling 95% ethanol for 6 hours followed by ether extraction for another 6 hours. DNA was extracted from 25 mg aliquots of finely ground, dry, fat-free tissue (DFFT) by the method of Schneider(5) with exclusion of the fat extraction and cold trichloroacetic acid (TCA) extraction steps. DNA was determined by the method of Webb and Levy(6). The product of the quantity DNA/mg DFFT and total DFFT, expressed as unit body weight, was estimated as the total DNA/posterior 6 glands. Visual observations were made for presence of lobule-alveolar development but no attempt was made to visually quantitate extent of growth.

Results. Total DNA of glands of ovariectomized rats receiving 2 μg EB for 19 days did not differ significantly from those of animals receiving 1 μg EB (Table I). Injections of 1 μg EB and 3 mg P increased mammary gland DNA above that of rats receiving EB alone, but upon increasing EB and P levels to 2 μg and 6 mg, respectively, no significant increase in growth occurred. Addition of 3 or

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