The Concentration of Apparent Endogenous Ethanol

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THE CONCENTRATIONS of endogenous ethanol reported in various tissues were the subject of recent review (1). It was concluded that the available evidence points to a normal level of ethyl alcohol in humans of between 20 and 30 mg. per liter of blood or serum. Such concentrations require the formation of ethanol in amounts equivalent to between 14 and 20 per cent of the basal metabolic rate. Because of their pertinence to a theory of the etiology of alcoholism, apart even from its intrinsic interest as a possibly important intermediary pathway, experiments have been performed in the manner suggested in the review in an attempt to clarify the issues. It has not been possible to confirm the concentrations reported. The present paper describes the techniques used and the results obtained.

Determination of Ethyl Alcohol

Ethyl alcohol was determined by means of gas–liquid chromatography, employing an Aerograph, Model A-600-B (Wilkens Instrument & Research, Inc., Walnut Creek, Calif.) equipped with a hydrogen flame ionization detector, whose electrometer amplified output was registered by a 1-mv. potentiometric recorder (Type H Leeds & Northrup). In equipment for gas–liquid chromatography, the sample for analysis is introduced onto a chromatographic column chosen for its ability to partition components of interest in such a manner as to present the detector (placed at the distal end of the column) with clearly separated compounds.

Only vapor samples were used in the present experiments. These were reproducibly introduced onto the column by means of a heated automatic gas sampling valve which was fitted with replaceable sample loops of any desired capacity. Alveolar air was obtained by a deep

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expiration into a 3-ft. long 0.5-in. diameter copper tube, suitably bent to occupy less space, which was placed within a heated space of the apparatus; a ⅛-in. copper tube was soldered 2 in. from the proximal end and led to one side of the sampling loop. A 20-ml. glass syringe was attached to a second port of the sampling loop and was used to rinse the sampling loop with successive portions of alveolar air; operation of the valve then admitted the chosen volume of vapor sample onto the column.

A 5-ft. ⅛-in. diameter column of 5-per-cent silicone SE-30 on 60/80 acid-washed Chromosorb-W was found adequate for the determination of ethyl alcohol in alveolar air if the specificity and sensitivity demanded was not too high. At 35°C, and with a flow of about 20 ml. of nitrogen per minute through the column, the ethyl alcohol in 0.25 ml. of vapor sample appears at the detector in about 65 seconds, acetone and methanol in 45 sec. and acetaldehyde in 31 sec. If more than a nontrivial quantity of methanol is present, it tails sufficiently to interfere with the ethanol response. Human alveolar air also contains a material which exhibits a peak at 50 sec. and at the full sensitivity of the instrument this interferes with the alcohol peak; this material is water-insoluble and has not yet been identified. For forensic purposes, or where alcohol has been ingested for experimental reasons, this column is adequate and the full sensitivity of the amplifier yields a peak of 1 division on the 1-mv. recorder (=0.01 mv.) for a concentration of 0.3 mg. of ethanol per liter of blood; when a 0.25-ml. sample of alveolar air is employed, 37×10^{-12} g. of ethanol reach the detector; the hydrogen flame generates a not inconsiderable amount of current at the highest gain of the amplifier, so that the usable sensitivity is 3 mg. per liter of blood. A larger sampling loop, 6.98 ml., increases the interference of the unknown constituent appearing at 50 sec., thereby not leading to a real increase in the amount of ethanol detectable. Because greater usable sensitivity was desirable, with an increase in specificity, a 5-ft. ⅛-in. diameter column of 20-per-cent Halcomid M-180L on 60/80 acid-washed Chromosorb was tried, with success. With this column, acetaldehyde reaches the detector in 60 sec., diethyl ether in 75 sec., the unknown water-insoluble material from alveolar air in 100 sec., acetone in 129 sec., methanol in 225 sec., and ethanol in 300 sec.; these time relationships are maintained, of course, with any change in the flow of nitrogen. The large sampling loop, using a 6.98-ml. alveolar air sample, now produces with ethanol a detector response at the greatest usable sensitivity ($\frac{1}{10}$ the highest sensitivity), equivalent to 0.5 mg. of ethanol per liter of blood; about 1750×10^{-12} g. of ethanol reaches the detector to give a significant detectable response. The difference in sensitivity between the 0.25-ml. sample on the silicone column and the 6.98-ml. sample on the Halcomid column is not in the ratio of the sample volumes; this relation is not maintained for peak height because of the greater sharpness of the peak with the smaller sample and column; however, the integrated peak–time appears to be so related. The partitioning of the ethanol from other materials is sharp, no other compounds from alveolar air appearing in

the region of ethanol. In this respect, using alveolar air, the present method appears to suffer from none of the interference exhibited by the alcohol dehydrogenase method for the determination of ethanol, and, of course, by the usual chemical methods. In part, this may be ascribed to the initial separation of volatile compounds that takes place in the lungs.

Calibration for ethanol and other volatile compounds is accomplished by bubbling nitrogen through dilute aqueous solutions of the compounds at a known temperature, admitting the effluent nitrogen stream containing the compounds into the ½-in. copper tube and transferring the requisite sample to the sampling loop. The values of the partition coefficients for alcohol between air and water and air and blood, as determined by Harper et al. (2), were used. Nitrogen bubbled through a solution containing 645 mg. of ethanol per liter of water at 20°C contains 0.10 mg. of ethanol per liter of nitrogen, which is the concentration of ethanol appearing in alveolar air when the concentration of ethanol in the blood is 203 mg. per liter. Distillates of liver were analyzed by the same technique as that used for calibration.

Results

Rate of Ethanol Disappearance at Low Concentrations

For a total of five sessions, two men and one woman drank between 0.26 and 0.6 g. of ethyl alcohol per kg. of body weight; the disappearance of alcohol was followed by analysis of alveolar air. Because the item of interest was the rate of alcohol disappearance in the later exponential portion of the curve, frequent analyses were made in the later stages down to levels equivalent to 2 mg. per liter of blood. From these data the constant relating the disappearance of ethanol to the concentration was computed. If the concentration is expressed as milligrams per liter of serum, and the rate of alcohol disappearance as milligrams per liter of serum per hour, then the constants (as $hour^{-1}$) found, by which the concentration is to be multiplied to obtain the rate, were 1.42, 1.56, 1.60, 1.77 and 1.80, with a mean of 1.64; this directly determined value agrees closely with the previously computed value of 1.68 (1). No relation of the value to the nutritional status or sex of the subjects was found. Visual inspection of the plotted data indicates a fair degree of linearity of the alcohol concentration versus time down to levels of 100 mg. per liter, and sometimes to 50 mg. per liter, where the trend becomes definitely exponential. Of interest is the fact that acetaldehyde was detectable, albeit at low levels, coincident with the appearance of ethanol, but the level of acetaldehyde

seemed to correspond better to the rate of disappearance of ethanol than to its concentration; in the linear portion of the curve the level of acetaldehyde remained constant, even though the alcohol declined, but then decreased in the exponential portion. The acetaldehyde peak was definite even when the alcohol level had declined to between 10 and 20 mg. per liter.

Ethanol Level in Alcoholics and Nonalcoholics

Alveolar air samples from 19 nonalcoholics (10 women, 9 men) and from 6 alcoholics (2 women, 4 men) were analyzed. No sex differences were evident. In 7 of the 19 nonalcoholics there was no detectable alcohol; in 5 the equivalent of 0.5 mg. per liter of blood was found; in 4, about 1.0 mg. per liter; and in 3, about 1.5 mg. per liter. In 3 of the alcoholics there was no detectable alcohol; 2 had 0.5 mg. per liter; and 1 had 1.0 mg. per liter. The levels bore no obvious relations to nutritional state or last previous ingestion of alcoholic beverages, and because the levels were trivial no efforts were directed to decide whether the amounts present were actually of endogenous origin. At no time was a peak of acetaldehyde discernible; such a peak might have appeared if the normal level of ethanol were 10 mg. per liter or more.[2]

Effect of Decreased Oxygen Tension

Six human subjects (two women, four men) were exposed for 1 to 2 hr. to 12.5-per-cent oxygen, equivalent to an altitude of 4200 m. The breathing mixture was administered through a plastic face mask at a total gas flow of 30 liters per min.; the desired concentration of oxygen was attained by regulating the admixture of room air with high-purity tank nitrogen, the flow of air and nitrogen being adjusted with calibrated flowmeters. In none of the subjects was there an increase over the preexposure ethanol level

[2] There was a notable difference between the two groups in the amounts of acetone present. In the nonalcoholics the calculated concentration of acetone in the blood ranged between 0.13 and 0.29 mg. per liter, with a mean of 0.19 ± 0.04. In the alcoholics the concentrations were 0.09, 0.31, 0.35, 0.55, 2.5 and 4.0 mg. per liter. All of the alcoholics appeared well nourished and all had eaten shortly before the air sample was taken for analysis. In nonalcoholics fasted for up to 24 hr. the highest concentration of acetone observed has been 0.70 mg. per liter. These observations are being extended and their import probed.

(which was less than 1 mg. per liter in every subject) nor was there any change in the chromatogram of the alveolar air.

The exposure was intensified in four albino rats who were placed in a low-pressure chamber at 307 mm. Hg, equivalent to an altitude of 7200 m., for up to 5 hr.; both fed and fasted animals were tested.

Samples equivalent to alveolar air samples were obtained from the animals in two ways: (*1*) The rat was placed in a small snugly fitted polyethylene chamber formed from two bottomless bottles, the chamber was sealed for 2½ to 3 min., and a sample of rebreathed air was taken for analysis. (2) Between 40 and 50 ml. of nitrogen were injected into the peritoneal cavity and, after 10 to 20 min. for equilibration, nitrogen was withdrawn for analysis. The rebreathed air contains odoriferous and chromatographically visible peaks of materials whose source is undoubtedly fecal; none of these, however, interfere with the alcohol peak. As far as ethanol is concerned, both methods yield the same result. At no time was there any evidence of an increase in ethanol, which was never greater than 0.5 mg. per liter, from the preexposure level.

Ethanol in Rat Liver

Equilibration of nitrogen within the peritoneal space is essentially a means of determining, in vivo, the concentration of volatile materials arising from all tissues, including liver, in contact with the nitrogen. In six of seven rats, fasted and fed, the concentration of ethanol found was either equivalent to 0.5 mg. per liter of blood or none was detected; in one animal it was 1.3 mg. per liter. If present, ethanol could, of course, be detected and measured; its concentration after ingestion or injection was easily followed by nitrogen equilibration to levels below 1 mg. per liter.

As further assurance of these concentrations, the liver itself was analyzed for ethyl alcohol. Rats were killed by cervical dislocation, the liver excised, weighed and blended with 160 ml. of N/12 sulfuric acid followed in 15 sec. by the addition of 20 ml. of 10-per-cent sodium tungstate. The mixture was centrifuged and the essentially protein-free supernatant was distilled to ⅔ volume and redistilled successively to a volume approximately the original weight of the liver. The final distillate was placed in a 30 × 200-mm. test tube fitted with a fritted glass bubbler and analyzed as described previously. Loss or gain of alcohol could have occurred only in the

time from death to the blending of the liver; recovery experiments in which liver was blended with acid containing small amounts of alcohol indicated no loss of alcohol caused by the presence of the liver. The time involved from death to the blending operation was less than 1 min.; calculations on this basis indicate that if there were any loss it would not increase the alcohol actually found by more than 10 to 20 per cent. In livers from both fed and fasted rats, 9.0 mg. of ethanol per 1,000 g., or less, was found.

Discussion

From the results in this series of experiments it is a virtual certainty that ethanol occurs in the blood of man and rat in concentrations up to 1.5 mg. per liter, and in the liver of the rat in concentrations not exceeding 9 mg. per 1,000 g. The specificity of the chromatographic technique leaves no doubt that ethanol was actually being measured, and, assuredly, that other materials were not being measured as ethanol. Whether the amounts found arise endogenously is not known from these experiments and, indeed, was not investigated; at such levels, however, were these amounts to be produced from endogenous sources, the rate of disappearance indicates that the contribution of ethanol to the energy economy would be less than 1 per cent of the basal metabolic rate.

The results presented do not bear out the tentative conclusions arrived at in the recent review (1). There is no longer any basis for assuming that a concentration of 20 to 30 mg. of ethanol per liter actually exists, nor does the lack of any difference between alcoholics and nonalcoholics offer any basis for speculation on the possible role of endogenous ethanol in the etiology of alcoholism.

Except for the unequivocal direct confirmation of the exponential character and value of the rate of disappearance of ethanol at levels less than 100 mg. per liter, there is a decided lack of agreement between the results here and those generally reported (1), although the findings of levels of 1 mg. per liter or less are supported by analyses with the enzymatic alcohol dehydrogenase method (3, 4). The higher concentrations reported were found in most cases with less specific chemical methods, albeit with some manner of preliminary separation of reducing materials. Because of the omnipresent nature of ethyl alcohol in the laboratory, it is difficult to avoid the conclusion that whether the method used is strictly chemical or enzymatic, the handling that samples undergo is more likely

to result in increases in ethanol content, rather than the reverse. The present determinations do not suffer from this disability and represent the simplest and least possible handling of samples. No previous determinations have made use of alveolar air for this present purpose, nor does any other bodily constituent seem so well adapted for avoiding the pitfalls likely to arise when measuring the small amounts of ethanol involved.

The determinations of ethyl alcohol in rat liver were performed so that they could be compared with those reported by McManus, Contag and Olson (5). These investigators found some 52 mg. of ethanol per 1000 g. of liver and somewhat less than half of this in rat plasma; the work was done with obvious care and there appears to be no way in which to reconcile results which differ by a factor of 6 in the case of liver and by a factor of about 50 in the case of plasma. The only possibility is the assumption of some formation of ethanol between the time of death and the preparation of the tissue lyophilate which was used for analysis. Some credence is lent to this possibility because McManus, Contag and Olson homogenized the liver in cold phosphate buffer at *p*H 7.4, then sometimes heated it before freezing the homogenate for the preparation of the lyophilate. The process of blending in N/12 sulfuric acid, followed in 15 sec. by heavy-metal denaturation of proteins, would seem to allow less scope and time for a possible formation of ethanol; and, indeed, the concentration found is less. Similarly, the use of equilibrated peritoneal nitrogen or rebreathed air avoids the time lag altogether and gives the lowest value.

The effect of a lowered oxygen tension in increasing blood alcohol was tested because the reports in the literature (6, 7, 8, 9) are unanimous in finding an increase; and, in the 3 investigations with human subjects, values of blood alcohol in the prehypoxic state were made. Thus, even though a chemical method, either the Nicloux or the Widmark, was used, absolute values can be disregarded and attention may be focused upon the differences between the pre- and posthypoxic conditions. Leonardi (6) found in 3 of his 10 12-hr. fasted subjects a rise of 20 to 240 mg. of ethyl alcohol per liter; Saviano and Vacca (7), in 14 of 20 subjects, found an increase of 1 to 73 mg. per liter; Zysk, Witkowski and Kaleta (8) found an average rise of 160 mg. per liter in 94 subjects; all the subjects were at equivalent altitudes of 3,000 to 4,000 m. for 1 to 2 hr. Increases of these magnitudes would have been detected

and measured with no difficulty in the experiments here, if they had occurred. Actually, not only was there no observed increase in ethanol, there was no increase in any material appearing in respired air and detectable by chromatography. Apparently, then, in most subjects, there is an increase, as the result of exposure to low oxygen tensions, of some reducing material or materials other than ethanol.

It seems not unreasonable to conclude that ethanol is present normally in man to the extent of not more than 1.5 mg. per liter; there is no proof that it arises endogenously or that, if endogenously formed, it is an important feature in the body's economy or has any relation to the etiology of alcoholism.

Summary

Experiments were conducted to clarify the question of endogenous ethanol. The sensitivity and specificity of gas–liquid chromatography has been exploited; with the use of the proper chromatographic column ethanol can be identified and its presence measured in vapor samples, such as alveolar air, at concentrations equivalent to 0.5 mg. of ethanol per liter of blood. A column with Halcomid M-108L as the active phase and a hydrogen flame ionization detector were used.

Using alveolar air for sampling, no differences in ethanol concentration were noted between 19 nonalcoholics and 6 alcoholics. In 10 subjects there was no detectable alcohol; in 7, the concentration was equivalent to 0.5 mg. per liter of blood; in 5, 1.0 mg. per liter; and in 3, 1.5 mg. per liter.

The first direct determinations in human subjects of the rate of disappearance of ethanol at concentrations below 100 mg. per liter of serum are reported. The rate of disappearance is linear until this level, or in some subjects as low as 50 mg. per liter, is reached. The rate of disappearance, in milligrams per liter per hour, may be calculated as the product of the concentration, in milligrams per liter, and the constant 1.64, the mean of five experiments in three subjects.

Exposure of six human subjects to simulated altitudes of 4000 m. for 1 to 2 hr. and four rats to 7000 m. for 5 hr. did not produce any increase in the alcohol concentration or in any other carbonaceous material appearing in the expired air.

Direct determination of the concentration of ethanol in rat liver

gave a value of 9 mg. per 1000 g., in contradistinction to one-tenth these values when peritoneally equilibrated nitrogen was used in the living animal. It is suggested that ethanol formation may take place during handling of tissues and thus account for the discrepancy.

It is concluded that ethyl alcohol may be present in humans in concentrations up to 1.5 mg. per liter of blood but whether this alcohol is of endogenous origin is unresolved; even if such formation were endogenous, its fraction of the basal metabolic rate would not exceed 1 per cent. A possible role of endogenous ethanol in a theory of the etiology of alcoholism appears excluded.

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