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ADENOSINE TRIPHOSPHATE-DERIVED NUCLEOTIDE FORMATION IN THE PRESENCE OF ETHANOL*†

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Abstract—Formation of an unidentified nucleotide in rat liver particulate preparations incubated in the presence of ethanol or other alcohols was found. Although the nucleotide was eluted from cation exchange resin in phase with cyclic AMP and was not precipitated by nascent barium sulfate, it could be separated from known cyclic AMP by selective adsorption onto water-equilibrated alumina. The rate of nucleotide formation was dependent upon the concentration of both ATP and ethanol; however, graphical determination of bimolecular enzyme constants was not possible. Nucleotide synthesis *in vitro* was stimulated by ethanol, methanol and glycerol, but not by 1-propanol, 2-propanol, *tert*-butanol or acetaldehyde. It was hypothesized that the nucleotide was a condensation product of ATP and ethanol, and ethyl adenylate was proposed as the structure.

Of all the pharmacological and toxicological actions of ethanol, perhaps none is more insidious than ethanol-induced fatty infiltration of the liver. In a recent study, 90 per cent of a sample of alcoholics showed evidence of fatty liver at biopsy [1]. Rat hepatic triglyceride levels have been shown to be significantly elevated only 6 hr after oral administration of 6.0 g/kg of ethanol [2]. Total lipid levels in the livers of rats fed a nutritionally adequate diet were increased when 36 per cent of the total calories as carbohydrate was replaced by ethanol [3]. This ethanol-induced increase was observed even when the total lipid intake was reduced to as low as 2 per cent of the total calories. Although the role of ethanol in the etiology of fatty liver seems well-established, the mechanism by which ethanol increases hepatic lipid levels is as yet unknown.

Concomitant with the increase in hepatic triglycerides seen after both acute [2, 4, 5] and chronic [3, 6] ethanol treatment, liver ATP levels have been shown to decrease significantly [4, 5, 7]. Griffaton *et al.* [7] concluded that ethanol elicited a nucleotide catabolism but did not change the ATP/ADP ratio. This suggests that the catabolism of ATP proceeded via a pathway other than one in which an ATPase participates. Although the negative correlation between hepatic lipid and ATP levels is not necessarily proof that decreased ATP levels cause lipid accumulation, it has also been shown that administration of high doses of ATP with a single dose of ethanol not only increased hepatic ATP levels, but also decreased hepatic lipid levels [4, 5].

While studying the ability of glycerol to stabilize adenylate cyclase, Petrack *et al.* [8] found that glycerol apparently stimulated cyclic AMP formation in particulate fat cell preparations. Further investigation showed that the compound was not cyclic AMP, but rather a nucleotide which behaved like cyclic AMP in several separation procedures. This novel compound formed from ATP in the presence of glycerol appeared to be composed of an adenosine moiety and the α-phosphate, but not the β-phosphate or γ-phosphate group of ATP. When incubation was carried out with alcohols other than glycerol, a family of compounds was found the R_f values of which decreased in a paper chromatographic system as the molecular weight of the alcohol increased. From this indirect evidence it was postulated that the nucleotide formed also incorporated the mono-, di-, or trihydroxy alcohol with which the fat cells were incubated. It was mentioned that liver particulate preparations had a capacity similar to fat cells to synthesize the novel nucleotide from ATP and glycerol. In light of these observations, this study was undertaken to determine whether such a nucleotide was formed by liver from ATP and ethanol.

METHODS

Tissue preparation. Tissue particulate fractions were prepared fresh daily. Male outbred albino rats (Charles River Laboratories) were sacrificed by decapitation and the livers rapidly removed, placed on ice-cold aluminum foil and minced with scissors. Approximately 3 g liver was hand homogenized in 5.0 ml of ice-cold Tris–HCl (125 mM, pH 9.0, unless otherwise stated) using ten strokes of a 7.0-ml Ten Broeck all-glass homogenizer (Pyrex) immersed in ice. The homogenates were diluted to 100 ml with the same buffer, divided into two equal fractions and centrifuged at 900 *g* for 5 min at 4° in a Sorvall RC-3 centrifuge. The resulting supernatants were aspirated, pooled and redivided into two fractions and centrifuged at 17,000 *g* for 60 min at 4° in a Sorvall RC-2B centrifuge. The supernatants were aspirated and discarded. Each pellet was resuspended by vortexing in

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15 ml Tris–HCl (125 mM, pH 9.0, unless otherwise stated). The suspensions were pooled and stored on ice until used. Other tissues were prepared similarly with initial homogenization in 1 vol. Tris–HCl (as above), dilution to 15 vol. with the same buffer, and centrifugation as above. The 17,000 *g* pellets were resuspended in 4 vol. (times initial weight) of the same buffer. Skeletal muscle (gastrocnemius) was first homogenized using a model 182 Super Dispax Tissuemizer (Tekmar Co.) and then rehomogenized in a Ten Broeck all-glass homogenizer.

Incubation in vitro. The incubations were carried out according to a modification of the method of Petrack *et al.* [8]. An aliquot of [2,8-^{3}H]ATP (26.0 Ci/m-mole, New England Nuclear), shipped in 50% (v/v) ethanol, was evaporated to dryness in a Büchi rotary evaporator and reconstituted in aqueous 2.5 10^{-4} M ATP containing 10 mM $MgCl_2$. The following were pipetted in the indicated order into 13 100 mm disposable incubation tubes kept on ice: (1) 20 μl of aqueous 2.5 10^{-4} M ATP (disodium) containing 2.0 μCi [2,8-^{3}H]ATP and 10 mM $MgCl_2$; (2) 20 μl of distilled water or 0.78 to 100% (v/v) ethanol; (3) 20 μl of 100 mM phosphocreatine in distilled water; (4) 20 μl creatine phosphokinase (5 mg/ml) in distilled water; and (5) 20 μl of tissue preparation containing 50–100 μg protein in Tris–HCl (125 mM, pH 9.0, unless otherwise stated). Immediately after adding tissue protein, the tubes were gently swirled and placed into a Dubnoff metabolic shaker for 10 min at 37°. The reaction was halted by immersion of each tube into boiling water for 1 min. Each sample was cooled on ice and 0.9 ml of aqueous carrier cyclic AMP added.

Initial nucleotide separation was a modification of the method of Krishna *et al.* [9] for the separation of cyclic AMP. Each incubation tube was vortexed and the contents were applied to cation exchange columns made from 7-in. transfer pipettes (Rochester Scientific) plugged with glass wool and containing 2 ml of a 1:1 settled volume slurry of AG 50W-X4 200–400 mesh resin (Biorad Laboratories). The columns were eluted with distilled water and the unidentified nucleotide (unknown) was collected in a 3-ml fraction previously shown to contain cyclic AMP. These fractions were precipitated by the addition of 0.3 ml of 0.25 M $ZnSO_4$ followed by sufficient 0.25 M $Ba(OH)_2$ to neutralize the samples. The samples were vortexed and centrifuged for 6 min at 2000 *g*. Each sample was precipitated a second time with nascent $BaSO_4$, mixed with a wooden applicator stick so as not to disturb the first pellet, and recentrifuged. The supernatants were aspirated and the pellets discarded. A 1-ml aliquot of each $BaSO_4$ supernatant was applied to an alumina column containing 500 mg aluminum oxide (Brockman activity II, BDH Chemicals, Ltd.) equilibrated with distilled water. Two fractions were collected from each column. The first fraction was eluted with 4.0 ml of distilled water and collected in scintillation vials. The second fraction was eluted with 2.0 ml of 2.5 N NaOH and collected in a second set of scintillation vials; 0.6 ml of 60% $HClO_4$ was added to the second eluate to neutralize the NaOH. Complete counting mixture 3A70b (Research Products International) (10 ml) was added to each vial and the radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency was estimated by the addition of [^{3}H]toluene (New England Nuclear) to a representative sample.

Assay blanks were determined in duplicate for each experiment. Twenty μl of tissue preparation was placed into 13 100-mm incubation tubes, which were then immersed in boiling water for 1 min. All additions identical to an experimental incubation were made; the sample was diluted with carrier cyclic AMP, and all steps in product purification were carried out. Experimental samples were corrected for this assay blank, which never exceeded 60 cpm.

The product yield (pmoles/mg of protein/10 min) was calculated from the radioactivity of the product (corrected for assay blank and dilution, but not for column recovery) and the specific activity of the substrate.

In all experiments, protein was estimated by the method of Lowry *et al.* [10] using standards of bovine serum albumin (Sigma) in Tris–HCl at a concentration and pH equal to the buffer in which the tissue was homogenized.

RESULTS

Rat liver homogenates were incubated with [2,8-^{3}H]ATP, 2.6 M ethanol, and at an ATP-regenerating system (creatine phosphate and creatine phosphokinase), the assay was halted as described in Methods, and the cyclic AMP fractions were eluted from AG 50W resin. Approximately 1000 cpm of [8-^{14}C]cyclic AMP (20–30 Ci/m-mole, New England Nuclear) was added to this fraction, and precipitation with nascent $BaSO_4$ was carried out. A 1-ml aliquot of this supernatant, containing ^{14}C as known cyclic AMP and ^{3}H label on a non-$BaSO_4$-precipitable compound, was applied to a water-equilibrated alumina column. The elution profile is shown in Fig. 1. Ninety-six per cent of the ^{14}C label, but only 15 per cent of the applied ^{3}H, was eluted with water in the first 5 ml. The ^{3}H retained on the column was eluted with 2.5 N NaOH. Similar label separation with water-equilibrated alumina was achieved when [α-^{32}P]ATP was supplied as substrate and [^{3}H]cyclic AMP added to the cyclic AMP fraction eluted from AG 50W resin.

In an effort to both increase yield and decrease variability between experiments, the subcellular distri-

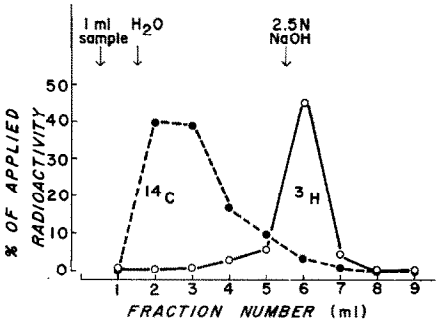

Fig. 1. Water-equilibrated alumina elution profile of $BaSO_4$ supernatant from rat liver preparation *in vitro* incubated in the presence of ethanol and [2,3-^{3}H]ATP. [^{14}C]cyclic AMP was added to the sample before precipitation with nascent $BaSO_4$.

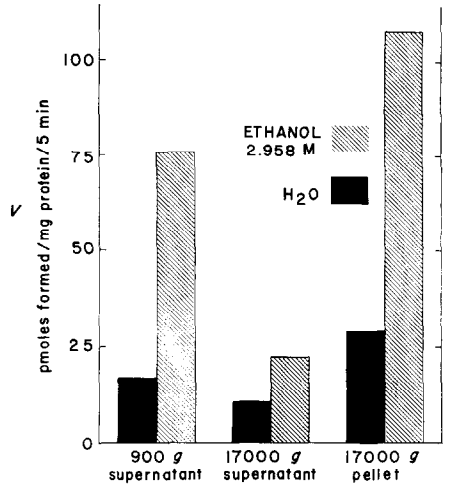

Fig. 2. Effect of ethanol on unidentified nucleotide accumulation in three subcellular fractions of rat liver incubated *in vitro* in the presence of 0.4 mM ATP and 10 mM Mg^{2+} at pH 8.0. Values are the means of triplicate incubations from a single experiment.

bution of activity was examined. Liver homogenates were prepared as described and aliquots taken of the 900 *g* supernatant, the 17,000 *g* supernatant, and the 17,000 *g* pellet resuspension. Incubations of these fractions with distilled water or 2.6 M ethanol, separation, and purification of the product were carried out as described in Methods, and the results are shown in Fig. 2. Ethanol markedly stimulated unknown formation in the 900 *g* supernatant fraction, and much less in the 17,000 *g* supernatant fraction. The greatest activity among the fractions studied was obtained with the 17,000 *g* pellet resuspension. Deletion of the ATP-regenerating system resulted in a 50 per cent loss in this activity. Unknown formation was increased in the presence of 10 mM magnesium, manganese and calcium, but not in the presence of zinc.

Samples of liver were homogenized and 17,000 *g* pellets prepared in 125 mM Tris–HCl, pH 6.5 to 9.5; incubations were carried out at 0.5 pH unit increments. Figure 3 shows that activity in the presence of ethanol increased directly with pH between 6.5 and 9.0. Activity was almost halved upon further increase to pH 9.5. Product yield without ethanol changed very little throughout the entire pH range studied.

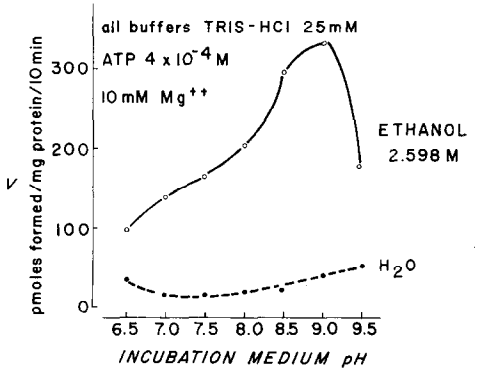

Fig. 3. Effect of incubation medium pH on unidentified nucleotide accumulation in rat liver *in vitro*. Values are the means of duplicate incubations from two experiments.

In the presence of 2.6 M ethanol and the ATP-regenerating system, ATP concentration was varied and the results are shown in Fig. 4. Reaction velocity increased with increasing ATP concentration throughout the wide range, 1.0 μM to 1.0 mM, of ATP concentrations examined (inset). Reciprocals of the intercepts of the Lineweaver–Burk transformation give estimates of 800 pmoles of unknown formed/mg of protein/10 min maximum velocity and half-maximum velocity at 250 μM ATP in the presence of 2.6 M ethanol.

In another series of experiments, ATP concentration was kept constant at 50 μM and ethanol concentration varied from 0.027 to 3.464 M (0.156–20.0%, v/v). Results are shown in Fig. 5. Velocity was proportional to ethanol concentration and approached an apparent maximum at 3.464 M. When basal activity (activity in the absence of ethanol) was subtracted from velocities in the presence of ethanol and the data were transformed to their reciprocals (inset), the resulting curve was distinctly not a linear plot. At no time was cyclic AMP formation in these liver preparations (radioactivity eluted from alumina with water) affected by ethanol.

It was of interest to gain some insight into the relationship between alcohol structure and unknown formation in this liver preparation. Results obtained with a number of alcohols are shown in Fig. 6. In the simple homologous series of primary aliphatic alcohols, methanol–ethanol–propanol, product formation decreased as chain length increased. The trihydroxy alcohol, glycerol, was about 5-fold more active than the most active primary alcohol, methanol. Product formation was not increased at any concentration of the simplest secondary alcohol tested, 2-propanol. *Tert*-butanol (not shown) was inactive at the concentrations tested (0.26 to 2.1 M). Product formation was not increased in the presence of acetaldehyde (not shown), the major metabolite of ethanol, at any concentration tested (0.011 to 0.27 M).

Results displayed in Fig. 7 show the unknown formation by particulate preparations from several

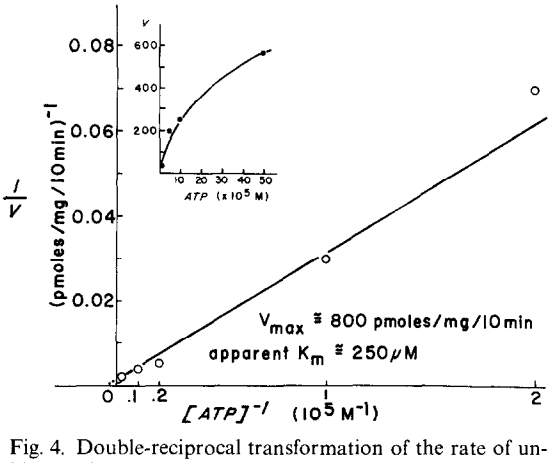

Fig. 4. Double-reciprocal transformation of the rate of unidentified nucleotide formation as a function of ATP concentration in the presence of 2.6 M ethanol in rat liver *in vitro*. Inset: arithmetic plot of unidentified nucleotide formation as a function of ATP concentration. Values are the means of triplicate incubations from a single experiment.

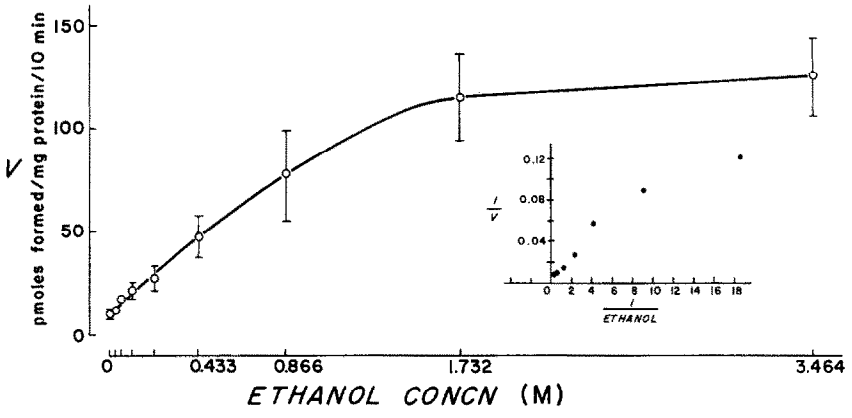

Fig. 5. Effect of ethanol on the rate of unidentified nucleotide accumulation in rat liver preparation *in vitro* in the presence of 50 μM ATP. Values are the means ± S.E.M. of triplicate incubations from three to twelve experiments. Velocities at ethanol concentrations greater than 0.433 M are significantly different from the basal velocity when analyzed by the Dunnet [11] multiple comparison test. Inset: double-reciprocal transformation of the same data after subtraction of basal velocity from velocities in the presence of ethanol.

organs. Of the tissues studied, unknown formation was greatest with preparations from liver, and much less with heart, lung and brain (not shown). There was no activity in preparations of skeletal muscle. The low activity found in heart and brain is in agreement with Petrack *et al.* [8], who also found that activity in liver was approximately equal to that in fat cell ghosts.

DISCUSSION

When rat liver particulate preparations were incubated with ATP in the presence of ethanol, a compound was formed which behaved similarly to cyclic AMP on cation exchange resin and, like cyclic AMP, was not precipitated by nascent $BaSO_4$. The established method for purifying cyclic AMP from incubates [9] selectively and quantitatively precipitates all adenine nucleotides, adenine and inorganic phosphate, while cyclic AMP remains in the supernatant. Petrack *et al.* [8], however, have shown that when fat cell preparations were incubated with ATP and glycerol or other alcohols and the incubates purified by the method of Krishna *et al.* [9], the product found in the $BaSO_4$ supernatant could be separated from known cyclic AMP by paper chromatography. Similarly, the product formed from ATP in the presence of ethanol in the experiments reported here was shown to be a compound other than cyclic AMP by selective adsorption of the product onto water-equilibrated alumina. This method was shown to be reliable for quantitative separation of the unknown from cyclic AMP.

There is much evidence that the synthesis of the unknown was enzyme-catalyzed. The reaction did not proceed when all reactants were incubated in the absence of tissue protein or in the presence of heat-denatured tissue. The rate of unknown formation increased linearly with concentrations of tissue protein up to approximately 100 μg/sample and up to 15 min

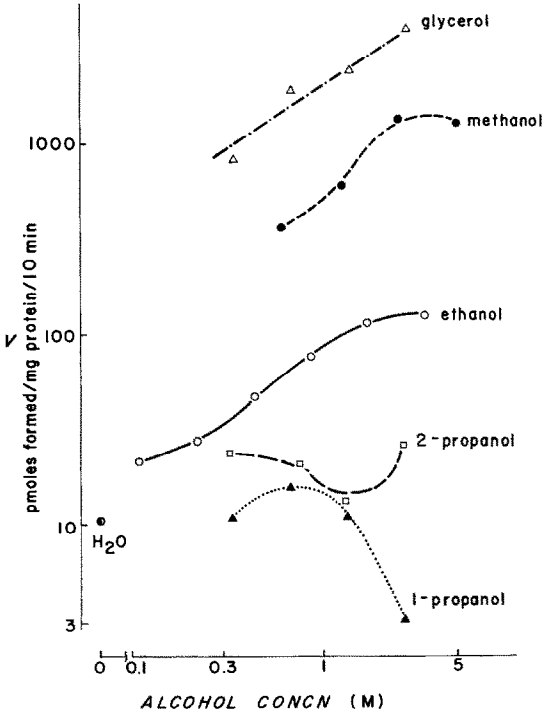

Fig. 6. Effect of several different alcohols on unidentified nucleotide formation in rat liver preparation *in vitro*. Values are the means of at least triplicate incubations.

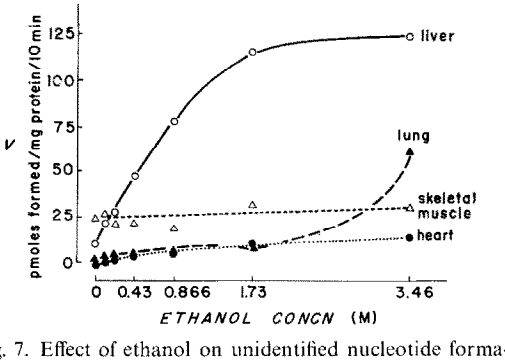

Fig. 7. Effect of ethanol on unidentified nucleotide formation in several tissues *in vitro*. Values are the means of triplicate incubations (liver data from Fig. 4).

of incubation time. In addition to having a specific tissue distribution, the enzyme also exhibited a specific subcellular distribution. The greatest specific activity was found in the 17,000 *g* pellet prepared from liver. Although in the absence of fine structure evidence it is difficult to state specifically which subcellular organelle predominates in this fraction. Gray and Whittaker [12] conclude that the 17,000 *g* fraction from brain tissue homogenized in isosmotic sucrose is a "crude mitochondrial" fraction.

The kinetics of unknown formation are complex. The observation that velocity is dependent upon both ethanol concentration and ATP concentration suggests a bimolecular reaction involving stepwise binding of the two substrates to the enzyme. The alinearity of the double-reciprocal plot of ethanol concentration vs velocity, however, does not allow graphical determination of the dissociation constants of a two-substrate enzyme [13, 14].

Although adenylate cyclase and the enzyme described here are both particulate, both use ATP as a substrate, and the two products are not $BaSO_4$-precipitable; unknown formation is catalyzed by an enzyme different from adenylate cyclase. Whereas the optimal pH for unknown formation *in vitro* was pH 9.0, the pH optimum for adenylate cyclase is 7.2 to 8.2 [15]. In addition, unknown formation had a relative requirement for 10 mM magnesium, calcium or manganese. Adenylate cyclase is inhibited by calcium concentrations greater than 0.2 mM [14].

As unknown formation did not proceed in the presence of acetaldehyde, the enzyme must bind ethanol *per se* and not an ethanol metabolite formed by the action of alcohol- or acetaldehyde-dehydrogenases. The 50 per cent decrease in unknown formation in the absence of the ATP-regenerating system suggests that the enzyme requires ATP, and not an ATP metabolite, as a substrate. In the absence of an ATP-regenerating system, ATP metabolite concentrations would greatly increase due to the action of competing ATPases and nucleotidases present in a crude mitochondrial preparation. Furthermore, if the enzyme required an ATP metabolite as substrate, the rate of unknown formation would increase, or at least not decrease, in the absence of an ATP-regenerating system. Thus, nucleotide formation is catalyzed by a single enzyme or enzyme system which binds unmetabolized ATP and ethanol.

The structure activity relationship observed in the experiments described here agrees with the preliminary results described by Petrack *et al.* [8], who also observed that ethylene glycol was the most active of those studied, and that propylene glycol was slightly more active than ethanol. The pooled activity series is: ethylene glycol > glycerol > methanol > propylene glycol > ethanol. Simple secondary or tertiary alcohols were not active in our experiments. The relationship between structure and activity for primary alcohols is increasing activity with decreasing chain length. The greater activity of polyhydroxy alcohols may be explained by an increased frequency of successful primary hydroxyl enzyme collisions which would occur when a greater number of primary hydroxyl groups are present on a molecule. The highly reproducible observation that some unknown synthesis takes place in the presence of water can best be explained by the possibility that ATP complexes with an endogenous primary alcohol present in the crude liver preparation. It is possible that this endogenous substrate could be eliminated by further enzyme purification.

In the absence of physical and chemical analysis of the product, its structure is subject to speculation. Labeled product formation from either [2,8-^{3}H]ATP or [α-^{32}P]ATP, supplied as substrate tracers in separate experiments, indicates that the product contains an adenine ring and a ribose moiety with at least an α-phosphate. This satisfies the definition of a nucleotide. In addition to the conclusion that the product is an adenine nucleotide, Petrack *et al.* [8] conclude from their studies with fat cells and different alcohols that the product does not contain the γ-phosphate and probably not the β-phosphate from ATP. In their experiments, no radioactive product was obtained when [γ-^{32}P]ATP was supplied as substrate or when [β-^{32}P]ATP was generated *in situ* with combined action of myokinase and pyruvate kinase. In addition, they conclude that the product may incorporate the alcohol into the nucleotide product in an unknown linkage. The structure of the product formed, therefore, would depend upon the alcohol supplied as substrate. The products formed from the condensation of ATP with several different alcohols would be a series of congeners, each composed of an adenosine monophosphate nucleus and a side chain, possibly in a phosphate ester linkage, of the respective alcohol.

Further research is necessary to determine if a condensation product of ethanol and ATP is involved in the effects of ethanol on the liver either by depletion of available intracellular levels of ATP or by interference with the hepatic cyclic AMP system.

In conclusion, when rat liver particulate preparations were incubated in the presence of ethanol and ATP, an adenine nucleotide was formed which, although it could be eluted from cation-exchange resin in phase with cyclic AMP and was not $BaSO_4$-precipitable, could be shown not to be cyclic AMP by selective adsorption onto aluminum oxide. In addition, the formation of this nucleotide was catalyzed by an enzyme distinct from adenylate cyclase.

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