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CHOLESTEROLGENESIS *IN VITRO* FROM ETHANOL*

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Abstract—Ethanol is a precursor for cholesterolgenesis. The reaction route of ethanol's conversion to cholesterol is through acetate and thence to cholesterol. The rate of cholesterolgenesis from ethanol is equal to the rate of cholesterolgenesis from acetate. However, ethanol at $3{\cdot}44 \times 10^{-1}$ M is a noncompetitive inhibitor of cholesterolgenesis from acetate; but ethanol at $1{\cdot}0 \times 10^{-3}$ M decreases the specific activity of ^{14}C-acetate and thus decreases the incorporation of ^{14}C-acetate substrate into cholesterol. Injection of ethanol into rats 1 hr before the animals were sacrificed resulted in a decreased incorporation *in vitro* of ^{14}C-acetate and ^{14}C-ethanol into cholesterol, which is a resultant of decreasing the specific activities of the ^{14}C-acetate and ^{14}C-ethanol substrates used to study cholesterolgenesis *in vitro*. ^{14}C-mevalonate incorporation into cholesterol is not affected by ethanol administration *in vivo*. Disulfiram increases the rate of cholesterolgenesis *in vitro* from ^{14}C-acetate. Acetaldehyde decreases ^{14}C-acetate incorporation into cholesterol.[4]

PATIENTS with chronic alcoholism have a hypercholesterolemia[1–3] associated with a general hyperlipidemia.[1–4] Dogs chronically treated with ethanol demonstrate a typical hyperlipidemia similar to that of the alcoholic patients. These studies suggest that ethanol is converted to acetate and thence to lipids.[5] However, recently it was reported that ethanol *in vitro* decreases incorporation of ^{14}C-acetate and ^{14}C-mevalonate into cholesterol.[6] It was therefore desirable to determine if this apparent decrease in incorporation was a result of the conversion of ethanol to acetate, which decreases the specific activity of the labeled acetate and thus would be indicated as decreased cholesterolgenesis. Furthermore, it was desirable to determine if ethanol is a precursor for cholesterolgenesis in this system. These studies confirm the conversion *in vitro* of ethanol to cholesterol and demonstrate that this conversion is as rapid as is the conversion of acetate to cholesterol.

METHODS

The methods have been described earlier[6, 7] and consist of 2 ml of a combined liver homogenate from several rats containing only microsomes and the soluble cellular fraction, which was prepared with modification according to the procedure of Bucher and McGarrahan.[8] Either ^{14}C-acetate or ^{14}C-mevalonate was employed as substrate. Cofactors and pH 7·0 phosphate buffer, described by Knauss *et al.*,[9] were also added to the enzymic system. Various test substances were added to the enzymic mixtures.

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The final total volume of the system was adjusted to 5·0 ml. The system was incubated for 1 hr in a shaking water bath maintained at 37°. The *de novo* synthesized cholesterol was isolated from the hydrolyzed system by extraction with petroleum ether and precipitation with tomatine. The radiometric determination was made according to the procedure of Kabara *et al.*[10]

The disintegration rate per minute (dpm), mean, standard deviation (S.D.) and Student *t*-tests between means were computed by an IBM 1620 computer. The tabular and graphical representations are expressed as the average of 4 determinations plus or minus the standard deviation. The Student *t*-test was used for comparing means of the various experimental groups. Only P values equal to or greater than the 98 per cent level were accepted as significant.

Many of the enzyme preparations used in these studies were stored for up to 7 weeks at dry ice temperatures, which does not alter enzymic activity in this system.[6]

Lineweaver–Burk studies of alcohol's inhibition of cholesterolgenesis. Two studies were conducted using $3{\cdot}4 \times 10^{-1}$ M and $1{\cdot}0 \times 10^{-3}$ M ethanol as inhibitors of cholesterolgenesis. ^{14}C-acetate concentrations of $5{\cdot}0 \times 10^{-5}$ M, $1{\cdot}0 \times 10^{-4}$ M, $2{\cdot}5 \times 10^{-4}$ M, $7{\cdot}5 \times 10^{-4}$ M and $1{\cdot}5 \times 10^{-3}$ M were employed in the five groups of four samples respectively. The control samples contained no ethanol. The isolation and radiometric determinations of the *de novo* synthesized cholesterol from the incubation media were accomplished as previously described.[6, 7] The results are expressed as the reciprocal of the substrate concentration on the abscissa and as the reciprocal of the average dpm of ^{14}C-cholesterol formed during 1 hr of incubation along the ordinate.

Determination of maximum 2-^{14}C-ethanol substrate concentration. To four samples of each of the six groups was added, respectively, the following 2-^{14}C-ethanol concentrations: $0{\cdot}25 \times 10^{-3}$ M, $0{\cdot}50 \times 10^{-3}$ M, $0{\cdot}75 \times 10^{-3}$ M, $1{\cdot}00 \times 10^{-3}$ M, $1{\cdot}50 \times 10^{-3}$ M and $2{\cdot}00 \times 10^{-3}$ M. The specific activity of the 2-^{14}C-ethanol in the samples above was equal. The incubations, isolation and radiometric determination of the *de novo* synthesized cholesterol were accomplished as previously described.[6, 7]

Effect of administration in vivo *of ethanol on cholesterolgenesis* in vitro. Two ml of a 40 per cent (v/v) saline solution of ethanol was injected intraperitoneally into ten, 250–325 g Sprague–Dawley, white, male rats. Ten control animals received 2 ml saline only. One hr after the injection, the animals were sacrificed and their livers were removed and homogenized in the usual way. The cholesterolgenic activity of each liver homogenate obtained from the 10 saline-treated and 10 ethanol-treated rats was determined as previously described, with radioactive ethanol, acetate or mevalonate as substrate. A comparison of cholesterolgenesis between the liver homogenates from the saline-treated and ethanol-treated animals was done using the Student *t*-test.

RESULTS

The decreased incorporation of radioactive acetate and mevalonate into cholesterol produced by $1{\cdot}0 \times 10^{-1}$ M ethanol is demonstrated in Table 1. It is of interest to note that at a $1{\cdot}0 \times 10^{-3}$ M ethanol concentration only the incorporation of acetate into cholesterol is inhibited, whereas the incorporation of mevalonate into cholesterol is unaffected (see Table 1). The specific type of inhibition produced by ethanol at these two concentrations is obviously different. This difference is demonstrated in Figs. 1 and 2. Ethanol at $1{\cdot}0 \times 10^{-3}$ M is a competitive inhibitor of cholesterolgenesis with

TABLE 1. EFFECT OF ETHANOL ON CHOLESTEROLGENESIS FROM ^{14}C-ACETATE*

Group no.	Additions *in vitro* to enzymic system	Exp. 1 ^{14}C-acetate (dpm)	(± S. D.)	Exp. 2 ^{14}C-mevalonate (dpm)	(± S. D.)
1	Control	6044	1125	57,340	6756
2	$1{\cdot}0 \times 10^{-1}$ M ethanol	1216†	250	38,501†	2015
3	$1{\cdot}0 \times 10^{-3}$ M ethanol	3628†	601	64,675	6237
4	$1{\cdot}0 \times 10^{-5}$ M ethanol	4849	1168	54,234	5938
5	$1{\cdot}0 \times 10^{-6}$ M ethanol	5641	1320	49,953	1601

* The study was conducted with five groups of four samples in each of two experiments. ^{14}C-acetate was used as substrate in all samples of the first experiment and ^{14}C-mevalonate was used as substrate in all samples of the second experiment. No test material was added to the samples of the first group in each experiment. To the samples of the four groups of each remaining experiment was added enough ethanol so that the final ethanol concentrations in the enzymic system were: $1{\cdot}0 \times 10^{-1}$ M, $1{\cdot}0 \times 10^{-3}$ M, $1{\cdot}0 \times 10^{-5}$ M and $1{\cdot}0 \times 10^{-6}$ M, respectively.

† Significant.

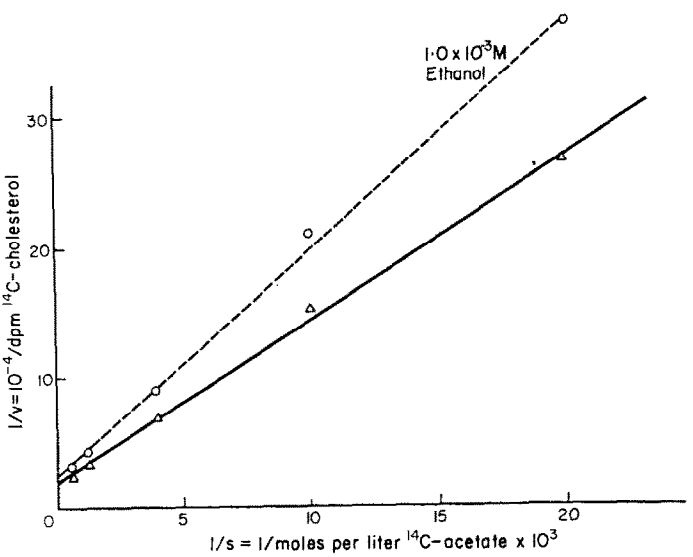

FIG. 1. Lineweaver–Burk plot of the inhibition of $1{\cdot}0 \times 10^{-3}$ M ethanol on cholesterolgenesis from acetate.

acetate substrate; at $3{\cdot}4 \times 10^{-1}$ M, ethanol is a non-competitive inhibitor of cholesterolgenesis with acetate (see Fig. 2) and with mevalonate substrates (unpublished results).

The incorporation of radioactive ethanol into cholesterol is shown in Table 2. The rate of cholesterolgenesis from ethanol is not significantly different than that from radioactive acetate (see Table 2). The maximum ethanol concentration for maximum cholesterolgenesis is similar to that of acetate and lies between $1{\cdot}0 \times 10^{-3}$ and $1{\cdot}5 \times 10^{-3}$ M (see Fig. 3).

The metabolic conversion of ethanol to acetate involves the intermediary formation of acetaldehyde. Cholesterolgenesis from mevalonate and acetate substrate decreases in

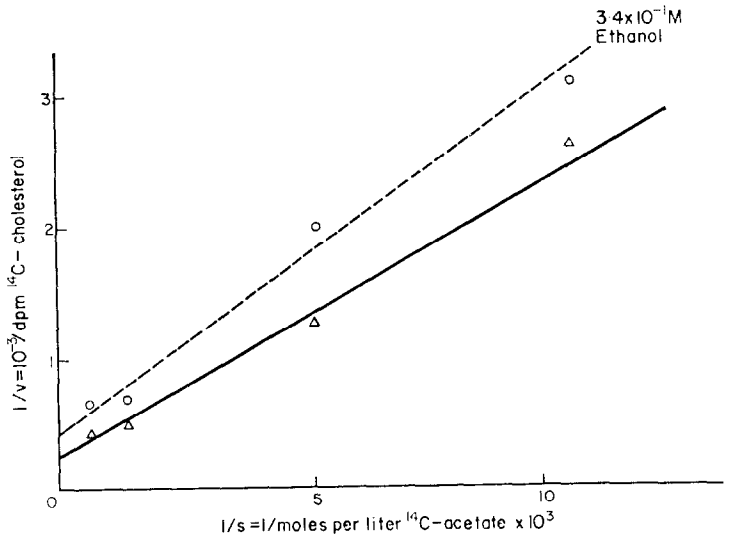

FIG. 2. Lineweaver–Burk plot of the inhibition of $3{\cdot}4 \times 10^{-1}$ M ethanol on cholesterolgenesis from acetate.

TABLE 2. CHOLESTEROLGENESIS FROM ^{14}C-ETHANOL AND ^{14}C-ACETATE WITH TIME*

Group no.	Time of incubation (min)	Exp. 1 2-^{14}C-ethanol (dpm)	(± S. D.)	Exp. 2 2-^{14}C-acetate (dpm)	(± S. D.)
1	15	118	43	216	102
2	30	297	154	839	373
3	45	916	251	1494	911
4	60	1260	222	1830	564
5	90	1763	93	2190	503

* The study was conducted with five groups of four samples in each of two experiments. ^{14}C-ethanol was used as substrate in all samples of the first experiment and ^{14}C-acetate was used as substrate in all samples of the second experiment. The same enzyme pool was used in each experiment. The first group of samples in each experiment was incubated for 15 min; the samples of the second group of each experiment were incubated for 45 min; the samples of the fourth group of each experiment were incubated for 60 min; and the samples of the fifth group of each experiment were incubated for 90 min.

TABLE 3. EFFECTS OF ACETALDEHYDE ON CHOLESTEROLGENESIS *IN VITRO**

Group no.	Additions *in vitro* to enzymic system	^{14}C-acetate (dpm)	(± S. D.)	^{14}C-mevalonate (dpm)	(± S. D.)
1	Control	975	360	12,986	2504
2	$1{\cdot}0 \times 10^{-1}$ M acetaldehyde	111	24†	4552†	783
3	$1{\cdot}0 \times 10^{-3}$ M acetaldehyde	589	281	12,265	2924
4	$1{\cdot}0 \times 10^{-5}$ M acetaldehyde	812	337	11,991	1335
5	$1{\cdot}0 \times 10^{-6}$ M acetaldehyde	702	270	12,257	479

* The study was conducted with five groups of four samples in each of two experiments. ^{14}C-acetate was used as substrate in all samples of the first experiment and ^{14}C-mevalonate was used as substrate in all samples of the second experiment. No test material was added to the samples of the first group in each experiment, which served as the control group. To the samples of groups 2–5 of each experiment was added, respectively, acetaldehyde at the following concentration: $1{\cdot}0 \times 10^{-1}$ M, $1{\cdot}0 \times 10^{-3}$ M, $1{\cdot}0 \times 10^{-5}$ M and $1{\cdot}0 \times 10^{-6}$ M.

† Significant.

the presence of $1{\cdot}0 \times 10^{-1}$ M acetaldehyde; however, no apparent inhibition is elicited at lower acetaldehyde concentrations (see Table 3).

A comparative study of the inhibition of cholesterolgenesis from ^{14}C-ethanol in the presence of nonradioactive acetate at $1{\cdot}0 \times 10^{-3}$ M is demonstrated in Table 4. When an equal concentration of "cold" acetate as ^{14}C-ethanol substrate concentration is added to the enzymic system, approximately 50 per cent of the radioactivity is incorporated into cholesterol. The same amount of acetate does not influence the incorporation of ^{14}C-mevalonate into cholesterol. When equal molar concentrations and equal specific activities (i.e. 2 μc per $1{\cdot}0 \times 10^{-3}$ M) of ^{14}C-ethanol and ^{14}C-acetate are added

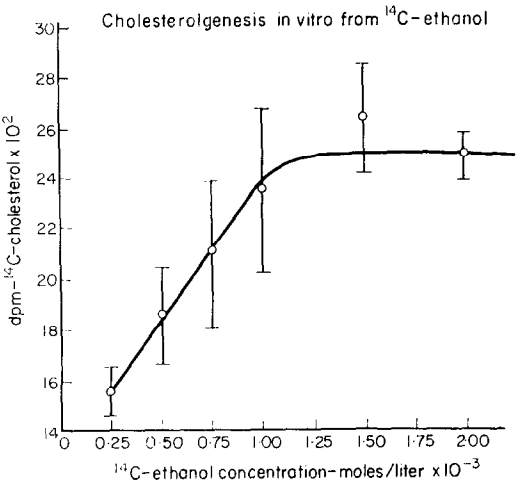

FIG. 3. Cholesterolgenesis *in vitro* from ^{14}C-ethanol.

TABLE 4. EFFECT OF ACETATE AND DMSO ON CHOLESTEROLGENESIS FROM ^{14}C-ETHANOL AND ^{14}C-MEVALONATE*

Group no.	Additions *in vitro* to enzymic system	Exp. 1 ^{14}C-ethanol (dpm)	(±S.D.)	Exp. 2 ^{14}C-acetate (dpm)	(±S.D.)	Exp. 3 ^{14}C-mevalonate (dpm)	(±S.D.)
1	Control	2366	346	2910	543	33,689	5084
2	1×10^{-3} M sodium acetate	827	198†			37,500	7592
3	DMSO	125	19†				
4	1×10^{-3} M ^{14}C-acetate	2835	858				

* The study was done in three experiments. The first experiment was conducted with four groups of four samples each; the second experiment was conducted with one group of four samples; and the third experiment was conducted with two groups of four samples each. ^{14}C-ethanol at $1{\cdot}0 \times 10^{-3}$ M, ^{14}C-acetate at $1{\cdot}0 \times 10^{-3}$ M, and ^{14}C-mevalonate at 2×10^{-3} M were used as substrate in the respective experiments. No test materials were added to the samples of the first group in each experiment. Enough nonradioactive sodium acetate was added to the samples of the second groups of experiments 1 and 3 so that the final concentration of acetate was $1{\cdot}0 \times 10^{-3}$ M. To the samples of the third group of experiment 1 was added 0·2 ml DMSO, and to the samples of the fourth group of experiment 1 was added enough radioactive acetate (of the same specific activity as that contained in experiment 2) so that the final concentration was $1{\cdot}0 \times 10^{-3}$ M.

† Significant.

simultaneously to the enzymic system, no significant difference in cholesterolgenesis occurs when compared with either $1{\cdot}0 \times 10^{-3}$ M ^{14}C-ethanol or $1{\cdot}0 \times 10^{-3}$ M ^{14}C-acetate alone (see Table 4).

Dimethylsulfoxide (DMSO), a known inhibitor of alcohol dehydrogenase,[11] inhibits cholesterolgenesis from ethanol (Table 4).

It has been demonstrated that the addition of 0·2 ml DMSO to this enzymic system increases cholesterolgenesis from acetate.[6] The addition *in vitro* of disulfiram in DMSO further increases cholesterolgenesis above the increase produced by DMSO alone. DMSO does not prevent ethanol from decreasing the incorporation of radioactive acetate into cholesterol. (Table 5) Disulfiram and DMSO do prevent the decreased incorporation of acetate into cholesterol produced by ethanol at $3{\cdot}4 \times 10^{-1}$ M, but do not return the cholesterolgenic rate to that produced in the enzymic system by the mixture of DMSO and disulfiram (Table 5). The inhibition of cholesterolgenesis from

TABLE 5. EFFECTS OF DMSO AND DISULFIRAM ON ETHANOL'S INHIBITION OF CHOLESTEROLGENESIS*

Group no.	Additions *in vitro* to enzymic system	^{14}C-acetate (dpm)	^{14}C-acetate (±S.D.)	^{14}C-mevalonate (dpm)	^{14}C-mevalonate (±S.D.)
1	0·2 ml DMSO	4746	413	15,658	1841
2	$5{\cdot}0 \times 10^{-4}$ M disulfiram + 0·2 ml DMSO	7198	219†	14,606	1675
3	$3{\cdot}4 \times 10^{-1}$ M ethanol + 0·2 ml DMSO	3315	438†	13,581	986
4	$3{\cdot}4 \times 10^{-1}$ M ethanol $5{\cdot}0 \times 10^{-4}$ M disulfiram in 0·2 ml DMSO	5227	472‡	12,407	1024†

* The study was conducted with four groups of eight samples. ^{14}C-mevalonate was used as substrate in four samples of each group and ^{14}C-acetate was used as substrate in the remaining four samples of each group. To all the samples of the first group was added 0·2 ml DMSO; to the samples of the second group was added $5{\cdot}0 \times 10^{-4}$ M disulfiram and 0·2 ml DMSO. Ethanol at $3{\cdot}4 \times 10^{-1}$ M and 0·2 ml DMSO were added to samples of the third group. The fourth group contained additions of the third group plus $5{\cdot}0 \times 10^{-4}$ M disulfiram.

† Significant.

‡ Significantly different from value for group 2.

TABLE 6. EFFECT OF ETHANOL ADMINISTRATION *IN VIVO* ON CHOLESTEROLGENESIS *IN VITRO**

Group no.	Treatment *in vivo*	^{14}C-acetate (dpm)	^{14}C-acetate (±S.D.)	^{14}C-ethanol (dpm)	^{14}C-ethanol (±S.D.)	^{14}C-mevalonate (dpm)	^{14}C-mevalonate (±S.D.)
1	Saline	26,673	22,970	24,924	14,806	39,265	11,443
2	Ethanol	4333	2586†	1584	1328†,‡	40,338	10,098

* The study was conducted by injecting two groups of ten rats with either 2 ml saline or 2 ml of a saline solution containing 0·8 ml ethanol. One hr after the injections, the rats were sacrificed. Liver homogenates from each rat were prepared and studied separately, using either ^{14}C-acetate, ^{14}C-ethanol or ^{14}C-mevalonate as substrate by the methods outlined in the text.

† Significant.

‡ Significantly different from value for ethanol-injected animals when ^{14}C-acetate was substrate.

mevalonate at high ethanol concentrations was not prevented by the inhibitors of ethanol metabolism, disulfiram or DMSO, or both.

Table 6 illustrates that ethanol was incorporated into cholesterol at a rate equal to that of acetate in individual rat liver homogenates. The acute administration of ethanol to rats 1 hr before sacrifice decreases the incorporation *in vitro* of both ^{14}C-acetate and ^{14}C-ethanol into cholesterol. The decreased incorporation of ^{14}C-ethanol into cholesterol in ethanol-treated rats is greater than the ^{14}C-acetate incorporation into cholesterol, whereas the incorporation of ^{14}C-mevalonate into cholesterol is unaffected by the administration *in vivo* of alcohol to rats.

DISCUSSION

The enzymic system used here is complex in that over 27 distinct enzymic steps have been described for the biosynthesis of cholesterol. The system is even more interesting in that some of the enzymes are contained in microsomal vesicles; therefore, substrate for microsomal enzymes must move through the microsomal membrane into the microsomes and, after the appropriate enzymic action, must move from the vesicles into the exterior milieu for continued cholesterolgenesis. The rate-determining enzyme of this series of reactions occurs in the microsomes and is concerned with the reduction of beta-hydroxy-beta-methyl-glutaryl-coenzyme A (HMG-CoA) to form mevalonic acid.[12] When mevalonic acid is used as substrate, the rate-determining cholesterolgenic reaction from acetate substrate is bypassed.

The apparent inhibition of cholesterolgenesis (see Table 1 and 6) in the presence of alcohol is a reflection of alcohol's conversion to acetate, and then to cholesterol. This conclusion is based on the following facts: Ethanol is a competitive inhibitor of cholesterolgenesis from acetate. When equal concentrations and specific activities of radioactive ethanol and acetate are added to the enzymic system, the same incorporation of radioactivity occurs in the *de novo* synthesized cholesterol as does occur when either radioactive ethanol or acetate alone serves as the cholesterolgenic precursor. Finally, when nonradioactive ethanol is added to a ^{14}C-acetate cholesterolgenic system, a decreased incorporation of radioactivity into cholesterol occurs; conversely, when nonradioactive acetate is added to a ^{14}C-ethanol cholesterolgenic system, an equal diminution of radioactivity is incorporated into cholesterol (see Tables 1 and 4).

Theoretically, it was considered that ethanol might be converted to acetyl-coenzyme A (acetyl-CoA) without intervention at the level of acetate analogous to the mammalian pyruvate oxidase system or bacterial aldehyde oxidase system. If this were the case, would enhanced cholesterolgenesis occur? This possibility exists in this system, since the rate-limiting step of cholesterolgenesis requires NADPH; also the metabolic conversion of alcohol to form acetate produces NADH, which may be converted to NADPH via action of transhydrogenases. This increased NADH produced by the conversion of ethanol to acetate obviously did not overcome the rate-limiting step of cholesterolgenesis from acetate. This is not surprising, since an NADPH generating system is incorporated into the cholesterolgenic system by the addition of glucose-1-phosphate and NADP. Finally, it was concluded that the incorporation of ethanol into cholesterol was by the same route as that of acetate and indeed these results support the concept that ethanol's conversion to cholesterol is a result of its conversion to acetate and thence to cholesterol. It is obvious that the conversion of ethanol to cholesterol is just as rapid as is acetate's conversion to cholesterol (see Table 2).

Consequently, the cholesterolgenic rate-determining biosynthetic step is slower than is alcohol's conversion to acetate. It is concluded that cholesterolgenesis occurs at a normal rate in the presence of 1×10^{-3} M ethanol and 1×10^{-3} M acetate, and that the apparent inhibition of ethanol at 1×10^{-3} M is a reflection of the conversion of ethanol to acetate, thus decreasing the specific activity of the acetate pool in the system.

To further substantiate ethanol's conversion to acetate and thence to cholesterol, DMSO was used to inhibit alcohol dehydrogenase in the enzymic system.[11] It was shown that cholesterolgenesis from ethanol was decreased when DMSO was added to the system, whereas it is known that DMSO stimulates cholesterolgenesis when acetate is substrate.[6]

When 0·1 ml ($3{\cdot}4 \times 10^{-1}$ M) ethanol is added to the system in the presence of DMSO, an inhibition of cholesterolgenesis occurs both from acetate and mevalonate substrate (Table 5), suggesting that alcohol at this concentration is a true inhibitor of cholesterolgenesis. This is substantiated when mevalonate is substrate, since in the presence of $1{\cdot}0 \times 10^{-3}$ M acetate or ethanol no inhibition of cholesterolgenesis resulted. Further evidence of this inhibition is demonstrated in studies in which ethanol decreased cholesterolgenesis in the presence of both DMSO and disulfiram (Table 5). The noncompetitive inhibition of cholesterolgenesis by ethanol both from acetate and mevalonate substrate at high concentrations of ethanol (see Fig. 2) possibly reflects an alteration of microsomal enzymes, since microsomes are necessary for cholesterolgenesis both from mevalonate and acetate substrate.

Disulfiram with DMSO increased cholesterolgenesis from acetate in this system. Since maximum substrate concentration is available (thus acetate utilization by other pathways would not be demonstrated), it is concluded that disulfiram has a catalytic activity. Disulfiram is probably affecting either directly or indirectly the well-known rate-determining reaction. This would seem so, particularly since cholesterolgenesis from mevalonate was unaffected by disulfiram. Furthermore, it is known that HMG-CoA reductase, the rate-limiting enzyme, contains sulfhydryl groups which are necessary for its activity. This enzyme is stabilized by, and the reductase activity is stimulated by thiol compounds.[13] Disulfiram, bis(diethylthiocarbamate) disulfide, may in some way activate HMG-CoA reductase in this system by inter-reacting with the sulfhydryl groups of HMG-CoA reductase.

The use of acetaldehyde in this enzymic system follows logically from the known metabolism of ethanol. Moreover, it has been observed that in some cases the effect of ethanol on enzymic systems could be attributed to that of acetaldehyde produced by the oxidation of ethanol.[14] A clear inhibition of cholesterolgenesis from acetate and mevalonate substrate was produced by acetaldehyde at $1{\cdot}0 \times 10^{-1}$M. Since this inhibition was nonspecific, i.e. both from acetate and mevalonate substrate, it was concluded that the inhibition was probably nonspecific, similar to that occurring when high concentrations of ethanol are added to the system. At lower concentrations, the high volatility of acetaldehyde and its combination with amines to form acetals preclude a firm conclusion as to its conversion into acetate and thence to cholesterol.

When ethanol is injected into animals 1 hr before they are sacrificed, a decreased ^{14}C incorporation *in vitro* into cholesterol from ^{14}C-ethanol and ^{14}C-acetate substrate occurs (Table 6). No effect was noted when mevalonate was substrate. The results of these studies suggest that ethanol *in vivo* was converted to acetate, which increased the alcohol and acetate pools of the liver homogenate, resulting in decreased specific

activity *in vitro* of the ^{14}C-acetate and ^{14}C-ethanol substrate used for cholesterolgenesis *in vitro*. As a consequence a decreased ^{14}C content of the derived ^{14}C-cholesterol *in vitro* resulted. These studies suggest that acetate pools may be an important aspect of alcohol's metabolism in the intact animal.

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