which food is supplied is not necessarily related to the time at which it is ingested since anorexia is often one of the first symptoms of toxicity.

The availability of sulphate will also influence the ethereal sulphate conjugation of phenols. If certain sulphur compounds are administered sulphate conjugation can be made to follow first-order reaction kinetics, but the rate of production of sulphate from foodstuffs is so small (see Table 1, Bray *et al.* 1952*c*) that, except at very low dose levels of a phenol, the reaction follows zero-order kinetics, the zero-order velocity constant (v_s) varying with the nature of the food and the time of its ingestion relative to that of the dose.

SUMMARY

1. A mathematical model is derived for metabolic systems in which a precursor is converted into an intermediate by a first-order reaction, the intermediate then being conjugated by two processes, one of which is first order, and the other first order below a certain body level and zero order above that level.

2. This model is shown to apply to the metabolic fate of certain benzoic acids and phenols and certain of their precursors.

3. The effects of variation in dose level, nuclear substitution of aromatic compounds, and the composition of the diet are discussed.

4. Various data obtained from 24 hr. studies are discussed in terms of the mathematical model.

The authors wish to express their thanks to Miss B. G. Humphris and Mr P. B. Wood for their assistance with the experimental work described in this paper, and to Mr J. L. Lawson and Mr J. M. Shephard for their co-operation in the human studies.

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Liver Alcohol Dehydrogenase and Ester Formation

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(*Received* 24 *January* 1952)

As a result of his observation of clinical cases of methanol poisoning, Røe (1943) formed the opinion that the toxic effects of methanol in man were minimized by the simultaneous ingestion of ethanol. He suggested that ethanol might act as an antidote by virtue of 'the capacity of ethyl alcohol to displace methyl alcohol from the inner surfaces of cells'. The characteristic toxic effects of methanol have, however, generally been attributed to the action of its oxidation products rather than to methanol itself, and it seemed possible that the beneficial effect of ethanol might be the result of competitive inhibition of an early enzymic stage in the metabolism of methanol. Experiments carried out on human subjects have shown clearly that the metabolism of methanol can be strongly impeded at its earliest stage by the administration of ethanol, and *in vitro* tests have provided evidence of competitive inhibition by ethanol of the oxidation of methanol to formaldehyde by liver alcohol dehydrogenase (Zatman, 1946; Leaf & Zatman, 1952). The *in vitro* tests referred to were carried out with horse-

liver alcohol dehydrogenase, prepared according to Lutwak-Mann (1938), and the extent of methanol oxidation in the mixed substrate (methanol + ethanol) system was assessed by colorimetric estimation of the accumulated formaldehyde. The observation of Lutwak-Mann that alcohol dehydrogenase preparations of this type always retain appreciable aldehyde mutase activity has been confirmed. It thus became evident that further reaction of formaldehyde under the influence of mutase might obscure the issue when a more detailed examination of substrate competition in the alcohol dehydrogenase system was planned. Although Lutwak-Mann refers to formaldehyde as susceptible to dismutation under the influence of liver aldehyde mutase, no quantitative study of the reaction of this substrate appears to have been recorded. It was therefore decided to examine the behaviour of formaldehyde, alone and in the presence of methanol and ethanol, in these mutase-containing alcohol dehydrogenase preparations. This led to the recognition of a new and unexpected reaction, with which the present paper is chiefly concerned.

A preliminary account of some of the work has already been published (Kendal & Ramanathan, 1951).

METHODS

Manometric estimation of acid production. Dixon & Lutwak-Mann (1937) found that the mutase reaction was most conveniently studied manometrically in bicarbonate buffer, since under their conditions 2 mol. acetaldehyde gave a theoretical yield of 1 mol. ethanol and 1 mol. acetic acid. In the present work, formic acid production was followed manometrically in the Warburg apparatus at pH 7·4. The main vessel contained coenzyme I (CoI), enzyme, the alcohol when desired and 0·3 ml. 0·15 M-$NaHCO_3$; aqueous formaldehyde was placed in the side arm. Total liquid volume, 2·0 ml. Equilibration took place in a bath at 37° and gassing was with N_2 containing 5 % (v/v) CO_2 in the usual way for 15 min. Dixon & Lutwak-Mann, in similar experiments with acetaldehyde, found that about 20 % of the volatile aldehyde was lost during the passage of the gas stream, and made an allowance for this. It was found under our conditions, by chemical estimation of the formaldehyde present at the end of the gassing and equilibration, that the loss of formaldehyde was rather variable, but was not more than about 5 %. The variability of this loss as between different manometers is presumed to be the result of (*a*) differences in rates of gas flow, and (*b*) inequalities in surface area of formaldehyde solution exposed, due to considerable differences in shape of side arms in the series of flasks. Dixon & Lutwak-Mann found it necessary to correct for the CO_2-retention caused by the rather high protein content of their media (up to 30 mg. of enzyme preparation/ml.). At the maximum enzyme concentrations used in this work (2 mg./ml.), tests showed CO_2-retention to be negligible. None of the reaction mixtures gave a measurable O_2 uptake under aerobic conditions.

Estimations of formaldehyde and formate. Residual formaldehyde and accumulated formate were estimated at the end of the manometric period. When formaldehyde only was to be estimated, the reaction was stopped by the addition of 1 ml. 4 % (w/v) trichloroacetic acid (TCA) to 2 ml. of reaction mixture. After waiting 5 min. for completion of flocculation of the precipitated protein, 3 ml. water were added and the suspension centrifuged. An appropriate volume of the clear supernatant was then taken for colorimetric estimation of formaldehyde using the method of Leaf & Zatman (1952). Test estimations with known amounts of formaldehyde, in which the TCA was added before the formaldehyde, or in which CoI was omitted from the system, showed that the recovery was quantitative.

Formate was determined according to Bastrup (1947), and it was found necessary, when formate estimation was desired after manometric observation, to run manometer flasks with identical contents in triplicate to provide sufficient pooled reaction mixture for satisfactory estimation. Preliminary experiments showed that the formate estimation could not be reliably carried out on samples containing TCA. The enzyme reaction was therefore stopped by the addition of 0·2 ml. 6 N-HCl to each Warburg flask, and the contents of three identical flasks were pooled and made up to 20 ml. with water. Of this, 1 ml. was then treated with TCA and used for formaldehyde estimation as already described. The remainder was transferred to the Bastrup distillation apparatus and formate determined. Tests with known amounts of formate showed a 97 % recovery in estimations on samples containing 0·5–8·0 mg. formic acid, and the correction suggested by this finding was applied. The presence of formaldehyde in the sample did not influence the formate determination.

Colorimetric estimation of formic ester. The method developed by Keenan (1945) for the estimation of ethyl acetate in methanolic solution, as modified by Hestrin (1949) and Peel (1951) for use with aqueous solutions, was found to be applicable to the estimation of formic ester. It depends upon the quantitative conversion of the ester into the corresponding hydroxamic acid by alkaline hydroxylamine, followed by measurement of the colour intensity of the ferric complex of the hydroxamic acid in acid solution. The procedure of Peel was adopted, after modification necessitated by the presence of varying amounts of formaldehyde in the samples to be assayed. Peel, who estimated ethyl acetate in the presence of acetaldehyde, found that the latter in high concentration (0·1 M) caused a slow increase in colour intensity for some minutes after the completion of the addition of the reagents. By taking successive colorimeter readings at timed intervals he was able to deduce the zero-time value by extrapolation. With formaldehyde, this effect is very much greater, even in much lower concentration (0·01 M), and a simple extrapolation to zero time is not possible. It was found, however, that the colour-producing effects of methyl formate and formaldehyde were strictly additive and that, if the order of addition of reagents were so altered as to prevent coincidence of ester and hydroxylamine in alkaline solution, the result was a complete suppression of colour formation by the ester without any effect on colour formation due to formaldehyde. These findings made it possible to develop a satisfactory procedure for the estimation of formic ester in reaction mixtures containing unknown amounts of formaldehyde, as follows.

Two identical 2 ml. samples are required for each estimation. Into each of two tubes 4·0 ml. M-$NH_2OH.HCl$ is measured. To one of these tubes are then added in the order given, mixing after each addition, (*a*) 2·0 ml. sample, (*b*)

2·0 ml. 2·5 N-NaOH; after 5–10 min., (*c*) 1·0 ml. 5·6 N-HCl and (*d*) 1·0 ml. 15% (w/v) $FeCl_3$ in 0·2 N-HCl. To the other tube the same additions are made, but in the order (*b*), (*c*), (*a*), (*d*). In the first tube, colour production is due to ester and formaldehyde additively, in the second tube to formaldehyde alone. Enzyme protein gives a turbidity in the coloured solutions which must be removed by centrifuging; 5 min. at 3200 rev./min. was found to suffice. Exactly 15 min. after the addition of the $FeCl_3$ reagent, readings are taken in a photoelectric colorimeter (Evans Electroselenium Ltd.) with Ilford filter 624. The two readings are converted into methyl formate equivalents by the use of a calibration curve, and the difference between them gives the amount of methyl formate in the sample. The calibration curve is prepared by the use of the above procedure with freshly prepared aqueous standard solutions of methyl formate. The curve was linear in the range used, which was up to 15 μmoles ester/2 ml. sample. The presence of phosphate in the samples has a significant effect in diminishing the slope of the curve. The samples analysed in the present work were either phosphate-free or 0·1 M with respect to phosphate, and in the latter case a calibration curve prepared with standards also containing 0·1 M-phosphate was used. Ethyl formate is not distinguished in any way from methyl formate in this method.

Enzyme preparations. The enzyme source was horse liver. The alcohol dehydrogenase was obtained according to the instructions given by Lutwak-Mann (1938) for her 'acetone preparation'. Most of the experiments were carried out with this material, but similar results were also obtained with an aldehyde mutase preparation made according to Dixon & Lutwak-Mann (1937), with the omission of the final $Ca_3(PO_4)_2$ adsorption stage in the procedure of these authors. Neither preparation exhibited any alcohol dehydrogenase or mutase activity in the absence of added Co I. Formaldehyde in the concentrations used (usually 1 mg./ml.) did not appear to have any inactivating effect upon the enzyme. Marked inactivation was, however, noted on an occasion when the enzyme was incubated with the formaldehyde for a short period before the addition of Co I, and it seems probable that the latter has a protective effect.

Coenzyme I preparations. The instructions of Williamson & Green (1940) were followed. Spectrophotometric assay, as described by Slater (1950), indicated a purity of about 35%.

RESULTS

In preliminary work suitable conditions for manometric experiments, as regards enzyme and coenzyme concentration, were determined. The curves of Fig. 1 show the course of acid production from formaldehyde in the presence of enzyme, and the effect upon this of variation in Co I concentration. In a series of experiments of this kind it was found that whilst activity in the absence of added Co I was negligible, the introduction into the medium of 0·125 mg./ml. of the coenzyme preparation exerted nearly a maximal effect. In all subsequent experiments, therefore, the concentration of the coenzyme preparation was kept constant at 0·25 mg./ml.

Effect of methanol and ethanol on acid formation and formaldehyde disappearance. Experiments limited to manometric observation, in which mutase activity was assessed only by acid formation, showed the latter to be markedly inhibited by ethanol and but little affected by methanol, when the alcohols were present in 0·25 M concentration. A possible explanation of this result suggested itself, based on the view that the mutase is simply a

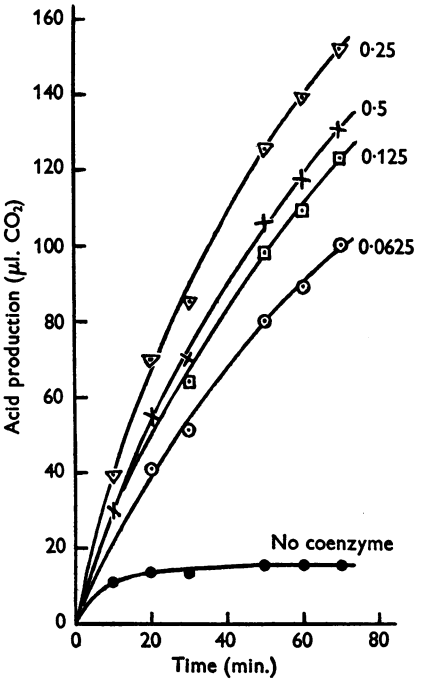


Fig. 1. Relation of mutase activity to Co I concentration. Manometric observation of acid production from formaldehyde. The reaction mixtures contained 1 mg./ml. of enzyme preparation and 0·5 mg./ml. formaldehyde. Co I concentrations (mg. preparation/ml.) as indicated by the curves.

coenzyme-linked system of alcohol and aldehyde dehydrogenases. In this view, the component reactions are

$$H.CHO + H_2O + Co\,I \rightarrow H.COOH + Co\,I\,H_2 \quad (1)$$

$$H.CHO + Co\,I\,H_2 \rightleftharpoons H.CH_2OH + Co\,I \quad (2)$$

catalysed by aldehyde dehydrogenase and alcohol dehydrogenase respectively. The simultaneous presence of ethanol might permit the alcohol dehydrogenase-catalysed reaction

$$C_2H_5OH + Co\,I \rightleftharpoons CH_3CHO + Co\,I\,H_2 \quad (3)$$

to compete with reaction (1) for the available Co I and, to a degree dependent upon the success of this competition, to diminish the rate of acid production. In such a case, the effect of ethanol on the rate of formaldehyde disappearance would be determined

Table 1. *Effect of alcohols on formaldehyde disappearance and acid production under the influence of liver enzyme*

(Manometric measurement of acid formation, with colorimetric estimation of residual formaldehyde at the end of the period of manometric observation. Enzyme concentration 1 mg./ml. Reaction time 65 min. Each flask contained initially 1 mg., i.e. 33·3 μmoles formaldehyde.)

	Methanol			Ethanol		
Alcohol concentration (M)	(a) Formaldehyde disappearance (μmoles)	(b) Acid formation (μmoles)	(a)/(b)	(a) Formaldehyde disappearance (μmoles)	(b) Acid formation (μmoles)	(a)/(b)
Nil	13·6	6·4	2·1	12·6	6·1	2·1
0·01	16·6	6·8	2·4	15·0	5·0	3·0
0·05	21·6	6·6	3·3	16·0	3·4	4·7
0·25	27·6	6·3	4·4	13·0	2·2	5·8

by the magnitudes of the reaction-velocity constants involved. If, in the simple formaldehyde-enzyme-Co I system, reaction (1) were the essentially slower reaction limiting the overall rate, its replacement by reaction (2) might increase the rate of formaldehyde disappearance. With methanol, on the other hand, the reaction corresponding to (3) is simply (2); no new reaction possibility would appear to have been introduced, and the rate of formaldehyde disappearance, if it is affected at all, should only be diminished.

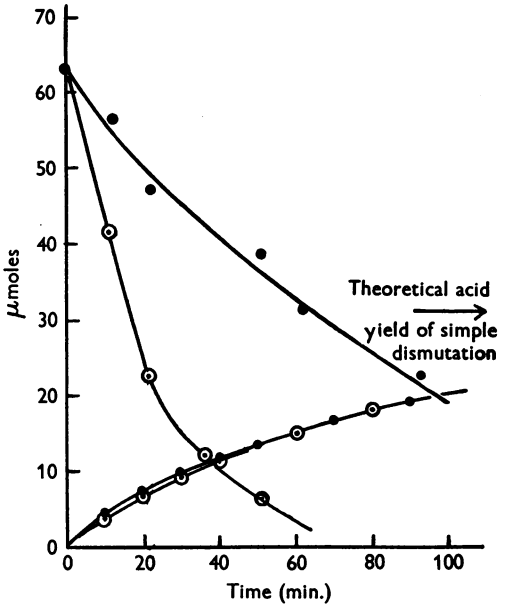


Fig. 2. Acid production (ascending curves) and formaldehyde content of the medium (descending curves) during the reaction of formaldehyde alone (●—●) and formaldehyde plus 0·25 M-methanol (⊙—⊙). Concentration of enzyme preparation, 1 mg./ml.

In further experiments the formaldehyde remaining in the medium after a period of manometric observation of acid formation was estimated. It became clear that the suggestion made above was quite inadequate to explain the results. Table 1 shows the formaldehyde disappearance and acid formation from formaldehyde alone, and from formaldehyde plus methanol or ethanol, under comparable conditions. In the absence of alcohol the molar ratio of formaldehyde disappearance to acid formation is not significantly different from the value of 2·0 expected for a simple mutase reaction. The presence of either alcohol in 0·01 M concentration has raised this ratio above the theoretical value, and at 0·25 M has more than doubled it. But the reason for the change is quite different in the two cases. With ethanol, it results from a depression of acid formation, with little change in formaldehyde disappearance. With methanol, there is no significant change in acid formation, but greatly increased formaldehyde disappearance. A possible explanation of the ethanol effect has already been outlined. Since the effect of methanol brought to light by the formaldehyde estimations fell outside the scope of that explanation, attention was now concentrated upon it.

Fig. 2 indicates the time course of the reaction at a particular enzyme concentration in systems containing formaldehyde alone and (formaldehyde plus methanol). The striking effect of methanol in accelerating formaldehyde disappearance is well shown, whilst the effect on acid formation is negligible. It is instructive to consider the magnitude of the ratio of formaldehyde disappearance to acid formation at different times during the reaction, and the change of this ratio with time is shown in Fig. 3. In the absence of methanol, the ratio remains constant near the value of 2·0 expected in a simple mutase reaction. In the presence of methanol, the ratio is more than 6 in the earliest stages; but it progressively falls, and if the incubation is sufficiently prolonged it, too, approaches a value of 2·0. The conclusion was unavoidable that a large fraction of the acid which ultimately appeared after prolonged incubation must have originated not

directly from formaldehyde, but by the rather slow degradation of some non-acidic intermediate product. The probable nature of this intermediate suggested itself when quantitative estimation of formate accumulating in the media had been carried out.

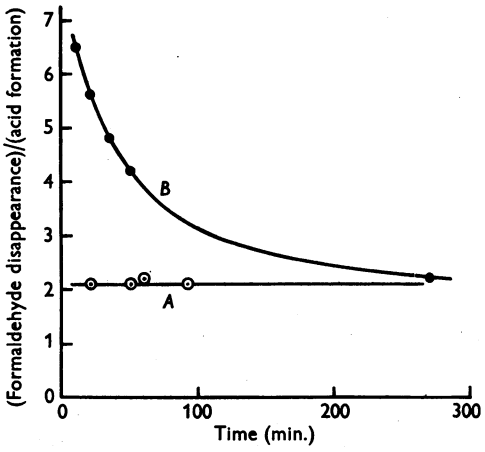


Fig. 3. Variation with time of the mol. ratio (formaldehyde disappearance)/(acid formation) during the reaction of *A*, formaldehyde alone; and *B*, formaldehyde plus methanol (data of Fig. 2).

Discrepancy between acid formation (manometric estimation) and formate production (chemical estimation). When data of the kind referred to in the preceding section were supplemented by chemical estimation of the formate present in the media at the end of the manometric period, it was found that the inclusion of the alcohols in the system resulted in a wide discrepancy between acid formation and formate production. The results set out in Table 2 are typical. As in the earlier experiments, acid production in the presence of either alcohol was much too small to accord with a simple dismutation of the aldehyde which had disappeared. In contrast, the chemical estimation of formate in the formaldehyde plus methanol system gave a figure greatly in excess of the acid formation, and a ratio of formaldehyde disappearance to formate production which could not be regarded as significantly different from the value of 2·0 expected in a simple dismutation. With formaldehyde plus ethanol, although the formate estimated chemically was greater than the acid production, it still fell far short of the requirement for dismutation of the formaldehyde disappearing.

These results made it necessary to postulate a conversion of formaldehyde into some non-acid substance which was nevertheless estimated quantitatively as formate by the Bastrup (1947) method. The first stage of the Bastrup procedure is a distillation of the sample with methanol and hydrochloric acid under conditions such that formic acid in the sample is converted into methyl formate, and passes over into an alkali trap. Such a procedure will clearly not distinguish between formate ions and volatile formate esters, and this consideration led to the formulation of a hypothesis that, in the presence of methanol, a considerable fraction of the oxidized product in the dismutation of formaldehyde was appearing as methyl formate and not as formic acid.

Appearance of volatile formate during the dismutation of formaldehyde in the presence of methanol or ethanol. The boiling points of the methyl and ethyl esters of formic acid are 31·5 and 54·3° respectively, and it was found that methyl formate in 0·1 M aqueous solution could be completely removed by aeration at room temperature in less than 30 min. As Bastrup (1947) has shown, methyl formate in such an air stream can be quantitatively trapped, with saponification, in sodium hydroxide solution and then determined according to his directions. It appeared, therefore, that aeration through media in which methyl formate accumulation was suspected into a suitable alkali trap might permit the demonstration of the presence of volatile formate. Reaction mixtures buffered with carbon dioxide-sodium bicarbonate were clearly unsuitable for such experiments, because of the necessity then of aerating with a gas mixture containing carbon dioxide which would rapidly exhaust the alkali trap. The following procedure was adopted.

Table 2. *Effect of alcohols on formaldehyde disappearance, acid formation and formate production under the influence of liver enzyme*

(Estimation of formate and residual formaldehyde after a period of manometric measurement of acid production. Enzyme concentration 1 mg./ml.; alcohol, when present, 0·25 M. Reaction time, 70 min. The figures refer to a volume of 6 ml., obtained by pooling the reaction mixtures from manometers run in triplicate in each series. The amount of formaldehyde initially present in this volume, corrected for a presumed 5% loss during gassing, was 95 μmoles.)

	Formaldehyde alone	Formaldehyde + methanol	Formaldehyde + ethanol
Acid formation (μmoles)	20·2	19·6	6·9
Formate production (μmoles)	23·8	36·2	12·4
Formaldehyde disappearance (μmoles)	53·3	81·7	61·7
Ratio: $\frac{\text{formaldehyde disappearance}}{\text{formate production}}$	2·2	2·3	5·0

The reaction vessel was a beaker fitted with a rubber stopper carrying gas inlet and outlet tubes, glass electrode and KCl-agar bridge, inlet tube for introduction of substrate and burette containing 0·01 N-NaOH. Enzyme and co-enzyme solutions, and alcohol if desired, in an appropriate volume of water, were placed in the beaker and the stopper was fitted to it. A current of CO_2-free N_2 was drawn through this, and passed thence through two absorption tubes in series, each containing 10 ml. 0·2 N-NaOH. With a slow gas stream stirring the medium, 0·01 N-NaOH was added from the burette until the pH was 7·4, as indicated by the glass electrode. The formaldehyde was added and the passage of gas increased to the limit imposed by the tendency to frothing. The latter was minimized by a smear of silicone (of unknown source) applied to the glass wall of the reaction vessel just above the fluid surface. During the reaction the pH was kept constant at 7·4 by addition of 0·01 N-NaOH from the burette as required, so that the amount added gave a measure of acid production. At the end of the experiment formate was estimated in the absorption tubes (the second of these never containing more than traces), as well as formate and formaldehyde remaining in the reaction medium. Controls showed that in the absence of enzyme no formate appeared in the traps.

The average results of two experiments are summarized in Table 3. Acid production was in reasonable agreement with the amounts of formate still present in the reaction media at the end of the experiment. The significant feature is the very large fraction (about 85 %) of the total formate production which was volatile at pH 7·4 and therefore carried over into the alkali traps, when methanol or ethanol was present. The more striking effect of methanol in accelerating formaldehyde disappearance is again manifest. The ratio of formaldehyde disappearance to total formate production is very near the value of 2·0, appropriate to a simple dismutation, in the formaldehyde alone and formaldehyde plus methanol systems, but in the formaldehyde plus ethanol system is significantly greater than this.

Effect of iodoacetate (IAA). Dixon & Lutwak-Mann (1937) found that their aldehyde mutase of liver was completely inactivated in a few minutes by 0·01 M-IAA, but Lutwak-Mann (1938) reported that liver alcohol dehydrogenase was scarcely affected by IAA at this concentration. This suggested that a study of the effect of IAA on the systems with which this paper is concerned might throw some light upon the reaction mechanisms involved. Fig. 4 shows the course of formaldehyde disappearance from reaction mixtures containing formaldehyde alone and

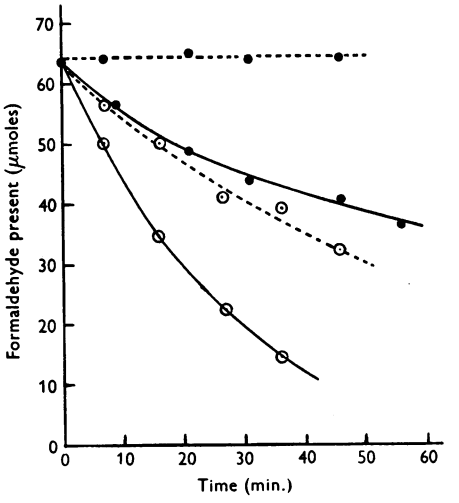


Fig. 4. Effect of iodoacetate on disappearance of formaldehyde. Bicarbonate buffer, pH 7·4, at 37°. Enzyme concentration, 2 mg./ml. ●, formaldehyde alone; ⊙, formaldehyde plus 0·25 M-methanol. Continuous lines, without IAA; broken lines, with 0·01 M-IAA.

(formaldehyde plus methanol), in each case both with and without 0·01 M-IAA. When formaldehyde alone was the substrate, inhibition by IAA was as complete as Dixon & Lutwak-Mann had found it to be. In the presence of methanol, on the other hand, the effectiveness of the IAA inhibition was less than 50 %, and the rate of formaldehyde disappearance in 0·01 M-IAA was greater than when formaldehyde alone was the substrate, without IAA. The effect of IAA on the removal of formaldehyde was found to be least of all in the presence of ethanol (Table 4).

Table 3. *Appearance of volatile formate during the dismutation reaction*

(Volume of reaction mixture 20 ml., containing enzyme preparation (2 mg./ml.), formaldehyde (1 mg./ml.), alcohol, when present (0·25 M). Temp., 22°; pH maintained constant at 7·4. Vigorous bubbling of N_2 during the reaction, passing gas into absorption tubes containing 0·2 N-NaOH. Reaction time, 2 hr. The quantities, in μmoles, are the average of closely agreeing duplicates.)

	Formaldehyde alone	Formaldehyde + methanol	Formaldehyde + ethanol
Alkali required to keep pH at 7·4	47	29	15
Formate found in reaction medium	58	35	19
Formate found in absorption tubes	42	249	102
Total formate production	100	284	121
Formaldehyde disappearance	194	551	213
Ratio: $\frac{\text{formaldehyde disappearance}}{\text{formate production}}$	1·9	1·9	2·6

Table 4. *Effect of iodoacetate on formaldehyde disappearance*

(Bicarbonate buffer at pH 7·4 and 37°, for 34 min. Enzyme preparation (2 mg./ml.), alcohol, when present (0·25 M). The figures refer to a reaction mixture volume of 2 ml. which contained initially 63 μmoles formaldehyde. Averages of closely agreeing duplicates or triplicates.)

	Formaldehyde alone		Formaldehyde + methanol		Formaldehyde + ethanol	
IAA (0·01 M)	−	+	−	+	−	+
Formaldehyde disappearance (μmoles)	20·4	1·2	46·8	23·1	23·1	18·7

The persistent disappearance of formaldehyde in the alcohol-containing media, in spite of the presence of a concentration of IAA sufficient completely to inhibit simple mutase activity in the absence of alcohol, raised the question as to whether volatile-formate production and the acceleration by alcohol of formaldehyde disappearance, were not solely determined by the activity of the alcohol dehydrogenase in the enzyme preparation (see Discussion section).

Colorimetric evidence of ester formation. The application of the hydroxamate method of ester determination finally proved to be the simplest way of demonstrating ester accumulation in reaction mixtures. This method has so far been used only in a small number of experiments, but some results which give an indication of the relationship between alcohol concentration and ester formation are shown in Table 5. The accumulation of ester appears to be maximal when the alcohol concentration is about 0·1 M and, in the case of ethanol, higher concentrations have an inhibitory effect. This last may be due to the operation of reaction (3) as suggested earlier. Ester formation occurred to an appreciable extent in 0·01 M-alcohol. Small amounts of ester are recorded as finally present when the media contained initially no alcohol; but such quantities are near the limit of accuracy of the analytical method and cannot at present be accepted as significant. Even in the absence of added alcohol, some ester formation might occur in the later stages of the reaction, through the influence of methanol formed by the dismutation.

Table 5. *Ester formation in formaldehyde-alcohol mixtures at various alcohol concentrations*

(0·1 M-Na_2HPO_4-KH_2PO_4 buffer pH 7·4 and 22°. Enzyme preparation (0·8 mg./ml.), formaldehyde (0·033 M). Ester determined by the hydroxamate colorimetric method in 2 ml. of reaction mixture after a reaction period of 1 hr.)

Alcohol concentration (M)	Ester accumulation (μmoles) in presence of	
	Methanol	Ethanol
Nil	0·5	0·5
0·01	1·8	1·5
0·10	6·0	2·1
1·00	5·7	0·9

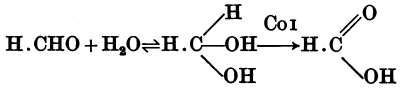
Stability of methyl formate in the reaction mixtures. The largest yields of ester were obtained in experiments at room temperature in which the ester was removed from the medium quite rapidly after its formation, by a stream of gas. In the manometric experiments at 37°, with prolonged incubation of reaction mixtures in which formaldehyde plus methanol was the substrate, ester accumulating during the early stages was subsequently hydrolysed. In the experiment to which Figs. 2 and 3 refer, about 95% of the formaldehyde had disappeared within 1 hr., but the ratio of formaldehyde disappearance to acid formation was then 4. After 4 hr. this ratio was reduced to a value very little greater than 2, indicating an almost complete hydrolysis of the ester which had been present. The stability of methyl formate in 0·1 M-Na_2HPO_4-KH_2PO_4 buffer (pH 7·4) and at room temperature was tested, using the hydroxamate method for the estimation of unhydrolysed ester. The plot of the logarithm of ester concentration against time under these conditions was linear, and the half-life of the ester was about 5 hr. The inclusion in the system of 1 mg./ml. of enzyme preparation accelerated the hydrolysis and reduced the half-life to about 1·5 hr., presumably as a result of the presence of esterase in the liver enzyme preparation. Similar tests at 37° and in bicarbonate buffer have not been carried out, because of difficulties due to the low boiling point of methyl formate, but the results obtained at room temperature make it probable that the rate of hydrolysis of the ester in the presence of the enzyme preparation has an order of magnitude consistent with our interpretation of events in the formaldehyde-methanol system.

DISCUSSION

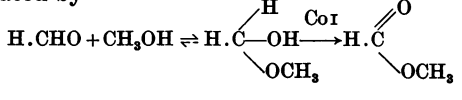
We believe that the experimental results which have been described provide ample proof of the accumulation of formic ester as a primary product of the enzymic dismutation of formaldehyde in the presence of methanol or ethanol. In the simpler case, in which the substrate is formaldehyde plus methanol, the evidence for methyl formate production may be summarized as follows: (*a*) formate, estimated chemically by a method which does not distinguish between formic ester and free formate,

is found to be produced in the proportion of 1 mol. for every 2 mol. of formaldehyde which have disappeared, which is in accord with simple dismutation theory. (*b*) Total acid production, estimated manometrically, is very much smaller than total formate production during the early stages of the reaction, but the difference becomes less as a result of prolonged incubation. (*c*) A large fraction of the formate appearing is sufficiently volatile from solution at pH 7·4 to be rapidly removed by a stream of gas at room temperature. (*d*) The application of the colorimetric hydroxamate method of ester determination gives confirmation of the presence of ester in the reaction mixtures.

While Dixon & Lutwak-Mann (1937) inclined to the view that the aldehyde mutase of liver was an enzyme distinct from the alcohol and aldehyde dehydrogenases, more recent developments, and in particular the isolations from liver of aldehyde dehydrogenase by Racker (1948) and crystalline alcohol dehydrogenase by Bonnichsen & Wassen (1948), have increased the probability that mutase is a coenzyme-linked system with aldehyde and alcohol dehydrogenase components. It is widely believed that it is the hydrated form of an aldehyde molecule which undergoes dehydrogenation to give the corresponding carboxylic acid, and in their preliminary communication Kendal & Ramanathan (1951) suggested that the appearance of methyl formate instead of formic acid in the formaldehyde plus methanol systems might be due to the presence of a sufficient amount of semi-acetal under these conditions for this to compete effectively with the hydrated form as a substrate for aldehyde dehydrogenase. The reactions



giving formic acid as the end product would then be replaced by



with methyl formate as the end product. The further supposition, that under the experimental conditions the rate of the second reaction was faster than the first, was necessary to account for the very pronounced accelerating effect of methanol on the overall rate of formaldehyde disappearance.

The results of the experiments with IAA raised a doubt as to the identity of the enzyme responsible for methyl formate production. Reference has already been made to the findings of Dixon & Lutwak-Mann (1937) that liver aldehyde mutase is completely inactivated by 0·01 M-IAA, and of Lutwak-Mann (1938) that liver alcohol dehydrogenase is not affected by IAA in this concentration. This suggests that it is only the aldehyde dehydrogenase component of the mutase which is so sensitive to IAA. In our experiments, the disappearance of formaldehyde from the simple enzyme-coenzyme-formaldehyde system was almost completely prevented by the addition of IAA, but when methanol or ethanol were also present, formaldehyde disappeared at a considerable rate in spite of the IAA. The rate in the case of the methanol system was in fact about the same as the increase in rate caused by the presence of methanol in the absence of IAA. These new facts make it likely that it is the alcohol dehydrogenase and not the aldehyde dehydrogenase which is able to dehydrogenate the semi-acetal. And indeed it may be argued that structural considerations favour this revision of opinion. The similarity between the semi-acetal $CH_3O.CH_2OH$ and the substrate *par excellence* of alcohol dehydrogenase, $CH_3.CH_2OH$, would seem to be much closer than that between the first of these molecules and the substrate $HO.CH_2OH$ of the aldehyde dehydrogenase. A rather surprising feature of the earlier experiments was the absence of any significant effect of methanol on the acid production in the early stages of the reaction, in spite of its very marked effect on formaldehyde disappearance. In terms of semi-acetal and substrate competition for the aldehyde dehydrogenase, it did not seem very likely that a diminution in direct formic acid production as a result of the substrate competition would be so exactly balanced by formic acid produced indirectly by hydrolysis of methyl formate. This difficulty is largely resolved when the semi-acetal is postulated as a substrate of the alcohol dehydrogenase only; the direct formation of formic acid by the aldehyde dehydrogenase at the beginning of the reaction may then proceed uninfluenced by the presence of methanol. Conclusive proof of the postulate that liver alcohol dehydrogenase alone, in the presence of coenzyme and methanol, brings about a 'dismutation' of formaldehyde to methanol and methyl formate awaits the results of further work with crystalline alcohol dehydrogenase devoid of aldehyde dehydrogenase activity.

One may conclude from the observations made when ethanol was present with the formaldehyde that ethyl formate is then formed, but on a smaller scale. Total formate never accounted for half the formaldehyde which disappeared, and it is presumed that in this case a significant fraction of the oxidation of $CoIH_2$ by formaldehyde to give methanol was balanced by a reduction of CoI by ethanol to give $CoIH_2$ and acetaldehyde. This is the more likely because of the much higher affinity of ethanol, compared with methanol, for alcohol dehydrogenase.

It must be admitted that there is at present no justification for an assertion that this new mechanism of ester formation is of real biochemical significance, but the phenomenon is of interest as an instance of enzymic ester formation which does not depend on the synthetic activity of an esterase. It is perhaps the kind of mechanism which might be responsible for the appearance of ethyl acetate in growing cultures of those yeasts which, as Peel (1951) has shown, produce this ester in a concentration greater than can be explained in terms of esterase-catalysed synthesis from ethanol and acetic acid. One is tempted also to speculate whether methyl formate might be a factor in determining the specific toxic effects of methanol. There seems to be fairly general agreement among students of methanol poisoning that the specific toxic effects (e.g. those on the retina) are not due to methanol itself, but to its metabolic products. They have been variously attributed to the localized action of formaldehyde or formic acid, but it has not been possible to reproduce them satisfactorily in experimental animals by the administration of these substances. Methanol, like ethanol, distributes itself rapidly and uniformly throughout the body water after it has been ingested. A dose of only 5 g., in man, produces a peak concentration of about 0·1 mg./ml., i.e. 0·003M (Leaf & Zatman, 1952). Dangerous doses, from about 10 g. upwards, may consequently be expected to give rise to concentrations which might, after primary conversion of part of the methanol into formaldehyde, lead to a secondary conversion of the latter into methyl formate by a semi-acetal dehydrogenase mechanism. Furthermore, the preferential fat-solubility of the ester is a property which might result in localization and specific effects.

SUMMARY

1. The ability of certain liver enzyme preparations to bring about the dismutation of formaldehyde has been confirmed.

2. When methanol was present in the system, the disappearance of formaldehyde was greatly accelerated, but without any corresponding acceleration of acid production. The appearance of formate was, however, increased to a degree corresponding with the acceleration of formaldehyde utilization.

3. After prolonged incubation of the reaction media, acid formation continued to increase and finally became equal to formate production.

4. The discrepancy between acid and formate production during the early stages of the reaction was due to the accumulation of a volatile ester of formic acid. The ester subsequently underwent a slow hydrolysis.

5. It is suggested that the ester formed from formaldehyde in the presence of methanol is methyl formate, and that it arises as the product of dehydrogenation of the semi-acetal formed from formaldehyde and methanol.

6. Volatile-ester formation occurs also in an enzyme-formaldehyde-ethanol system, but to a smaller extent.

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