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Factors affecting the measurement of formaldehyde produced by enzymatic demethylation*

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VARIOUS pharmacologically active compounds undergo demethylation.[1–5] The liver is thought to be the principal site of this dealkylation reaction since this organ, in contrast to several other tissues, possesses an active demethylating enzyme system.[6] Balance studies indicate that incubation of liver fractions with appropriate substrates results in the formation of 1 mole formaldehyde for every mole of demethylated product formed.[2, 3] A convenient means of determining the rate and extent of demethylation is to measure the formaldehyde production.

Most methods used in the measurement of formaldehyde are based upon procedures introduced by MacFayden[7] or Nash[8] and involve the formation of a colored compound whose light absorption can be measured by a standard laboratory spectrophotometer. In recent years the Nash procedure has gained wider acceptance, since this method offers a simpler and more rapid approach to the study of enzymatic demethylation.

Deproteinization of tissue homogenates is a preliminary step in the measurement of formaldehyde production. The present investigation was undertaken to evaluate this step, since findings in our laboratory had indicated that the choice of protein precipitant and the conditions under which the color is developed affect the sensitivity and accuracy of the Nash procedure.

Rabbits weighing 2–4 kg were stunned by a blow on the head. The liver was removed, rinsed in cold 1·15% KCl and homogenized in approximately two volumes of KCl. Homogenization at 4° was carried out in a Waring Blendor, and microsomal and 9000 *g* fractions were prepared by subjecting the homogenate to differential centrifugation.[9] Each fraction was resuspended in 1·15% KCl so that 1 ml contained the equivalent of 250 mg fresh liver. All incubation mixtures contained 1 ml of either the 9000 *g* or microsomal fraction and were fortified as described by Rubin *et al.*[10] These incubation mixtures were used for recovery studies and for experiments measuring demethylation of ethylmorphine. In the latter experiments incubations were carried out in 20-ml beakers for 15 min in an atmosphere of air in a Dubnoff metabolic shaker at 37°.

Formaldehyde standards were prepared daily by diluting Mallinckrodt formaldehyde solution. Values obtained by this method are consistent with those found when the hydrolysis of hexamethylenetetramine is used as a source of formaldehyde.[11] Either 1 ml of 1 N perchloric acid (PCA), 1 ml of 30% trichloroacetic acid (TCA), or 2 ml each of a $ZnSO_4$ and a saturated solution of $Ba(OH)_2$ (Zn-Ba) were added to the reaction mixtures as protein precipitants. The contents of the flasks were then centrifuged and either 3 ml (PCA and TCA) or 5 ml (Zn-Ba) of the supernatant was removed for determination of formaldehyde based on the method of Nash.[8] Two ml of double-strength Nash reagent was mixed with each sample, since preliminary studies indicated that color production was incomplete if less than 2 ml was used. The resulting solutions were incubated for varying times and at varying temperatures.

In all instances the inclusion of semicarbazide HCl, as a formaldehyde trapping agent, in the incubation mixture resulted in a lengthening of the time necessary for maximal color formation. If TCA was used to deproteinize either the 9000 *g* or microsomal fraction, incubation of the resulting supernatant and Nash reagent at 60° resulted in the formation of an unstable complex. When TCA was used, the optical density obtained at a given concentration of formaldehyde varied with the length of time of incubation (Fig. 1). We have found that the color complex formed in the presence of TCA could be made more stable if the color is allowed to develop at 37° rather than at 60°. Under these conditions the maximal intensity is reached in about 60 min and remains stable for at least 20 min. However, the initial part of a standard curve when TCA was used is not linear. A more stable complex was produced when PCA was used, although incubation times of longer than 27 min resulted in a gradual decline in optical density from the peak value. Blank values with PCA were invariably 2 to 3 times those obtained when other protein precipitants were used. The use of a $ZnSO_4$ solution as a

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protein precipitant resulted in the production of a relatively stable color complex. A 20% $ZnSO_4$ (35·6% $ZnSO_4 \cdot 7H_2O$) solution resulted in a more rapidly developed and slightly more intense color than did a 5% solution. Concentrations of $ZnSO_4$ of less than 5% gave a less intense color and occasionally an incomplete precipitation of the 9000 *g* fraction.

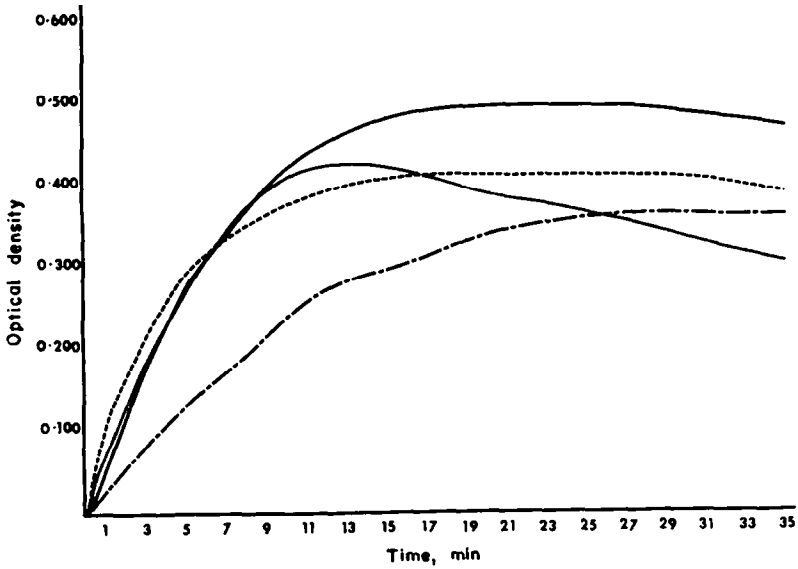

FIG. 1. Effect of protein precipitants on the time course of color development of the formaldehyde–Nash reagent chromagen. Color was developed at 60° in a protein-free solution with 10 g formaldehyde. Identical results were obtained with water or a protein-free filtrate derived from the 9000 *g* or microsomal fraction. Values are corrected for blank determination.

——— Perchloric acid; - - - - - - 20% zinc sulfate;
····· trichloroacetic acid; —·—·—·— 5% zinc sulfate.

Color formation was terminated by removing all samples from the water bath and cooling them in a water–ice bath. Optical densities were then determined with a Bausch and Lomb spectrophotometer. The color formed was stable at room temperature for several hours. All protein precipitants used in the present study provided essentially complete recovery (92–105%) of formaldehyde added

TABLE 1. MEASUREMENT OF FORMALDEHYDE PRODUCED BY THE ENZYMATIC N-DEMETHYLATION OF ETHYLMORPHINE (92×10^{-3}M) WITH DIFFERENT PROTEIN PRECIPITANTS

Protein precipitant	Liver fraction	(μmoles produced/g/hr)*
Perchloric acid	9000 *g*	6·1
Perchloric acid	microsomes	6·3
Trichloroacetic acid	9000 *g*	5·1
Trichloroacetic acid	microsomes	4·4
5% $ZnSO_4$	9000 *g*	7·7
5% $ZnSO_4$	microsomes	6·4
20% $ZnSO_4$	9000 *g*	7·0
20% $ZnSO_4$	microsomes	6·5

* Represents the mean of 2 to 4 determinations.

to incubation mixtures containing either the 9000 *g* or microsomal fractions. Concentrations of $ZnSO_4$ of less than 5% resulted in poor recovery.

The formaldehyde produced by the enzymatic demethylation of ethylmorphine (2×10^{-3}M) using 9000 *g* or microsomal fractions was measured by using the optimal conditions for color development determined by our experiments. Formaldehyde production appeared to be equivalent when PCA and 5% or 20% $ZnSO_4$ were used, but values obtained with TCA were invariably lower (Table 1).

From these studies it appears that the choice of protein precipitant, the temperature at which the color is developed, and the duration of incubation are factors which must be considered in comparing the measurements of formaldehyde production from different laboratories. The procedure developed by Nash is a rapid and accurate one, but there should be an awareness of possible pitfalls in its application to biological systems.

The use of either a 5% or 20% solution of $ZnSO_4$ as a protein precipitant appears to offer the best combination of color stability, short interval of color development (30 min at 60°), and low blank values for the measurement of formaldehyde production by liver fractions.

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A comparison of the isomers of warfarin*

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THE anticoagulant, *Rac*-warfarin [3-α-(acetonyl-benzyl)-4-hydroxycoumarin] has been widely used throughout the world as a rodenticide and clinically for the prevention of thrombosis.[1] The resolution of this important compound in this laboratory by West *et al.*[2] has made possible a study of the relative activity of its enantimorphs. The results of that study formed the basis for this report. Since the absolute configuration has also been determined by West *et al.*[2] the results are of special significance.

MATERIALS AND METHODS

Acute prolongation of prothrombin times. For 5 days preceding the experiment and throughout its duration, male Sprague-Dawley rats, 175 to 225 g, were housed 4 to 5 to a cage and maintained on a

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