of mouse > rat > guinea pig (4), one would expect the excretion of glutathione degradation products in these three species to be mouse > rat > guinea pig. This pattern was observed; the mouse excreted 15.5% of the dose in 14 hr as glutathione degradation products (Table IV) compared to a 12-hr excretion of 5.1% in the rat (2) and 1% in the guinea pig (3). Excretion of glutathione degradation products in the mouse increased following aspirin pretreatment (Table IV). On the basis of the catabolite hypothesis (20), this result suggests that aspirin-pretreated mice are more susceptible to acetaminophen-induced hepatotoxicity than are vehicle-pretreated mice.

However, if aspirin increased the pool of cysteine or precursors of cysteine by reducing active sulfate levels, as already suggested, then the increased urinary excretion of glutathione degradation products could indicate an increase in the conjugative detoxication of the toxic metabolite or protection from acetaminophen-induced hepatotoxicity. In support of the latter hypothesis is a study where aspirin showed a protective effect against toxic doses of acetaminophen (21).

CONCLUSION

The metabolite profile of acetaminophen in the mouse was not exactly the same as that observed in humans (22, 23). However, the mouse biotransformed acetaminophen more like the human than either the rat (2) or the guinea pig (3). Since the mouse is more susceptible to acetaminophen hepatotoxicity than either the rat or the guinea pig (4) and biotransformation of acetaminophen in the mouse resembles that occurring in humans, the mouse is a better animal model than either the rat or guinea pig for toxicological studies involving acetaminophen.

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Colorimetric Determination of Aliphatic Acids

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Abstract □ A colorimetric method for the determination of carboxylic acids based on the dicyclohexylcarbodiimide-coupled reaction of 2-nitrophenylhydrazine and carboxylic acids is described. The product of the reaction is extracted into aqueous sodium hydroxide to produce a blue color. This method is suitable for the analysis of aliphatic acids, but aromatic acids do not react under these conditions.

Keyphrases □ Aliphatic carboxylic acids—colorimetric analysis in solutions □ Carboxylic acids, aliphatic—colorimetric analysis in solutions □ Colorimetry—analysis, aliphatic carboxylic acids in solutions

The recent application of the coupling agent dicyclohexylcarbodiimide in the analysis of carboxylic acids *via* hydroxamic acid formation (1) prompted investigation of this reagent to couple carboxylic acids with 2-nitrophenylhydrazine. An earlier study (2) showed that the hydrazide resulting from the reaction of 2-nitrophenylhydrazine with activated carboxylic acid derivatives such as acid anhydrides and acid chlorides gives an intense blue color in aqueous hydroxide solutions. This color formation was useful for the colorimetric analysis of these substances.

The objective of this study was to adapt the dicyclohexylcarbodiimide-coupled reaction of carboxylic acids and 2-nitrophenylhydrazine to produce a colorimetric method for the determination of carboxylic acids.

EXPERIMENTAL

Materials—Unless otherwise stated, analytical reagent grade chemicals were used. Acetonitrile was distilled from phosphorus pentoxide, with the fraction boiling at 81.5° being collected. Dichloromethane was distilled directly, with the fraction boiling at 39.5° being collected. 2-Nitrophenylhydrazine was recrystallized from water–methanol (85:15), yielding yellow-orange needles, mp 92–92.5°.

Carboxylic acid solutions were prepared by dilution of a stock solution of the acid in dichloromethane[1]. The exact concentrations of the stock solutions were determined by potentiometric titration with standard sodium hydroxide in methanol. Stock solutions of 2-nitrophenylhydrazine (in dichloromethane) and dicyclohexylcarbodiimide in acetonitrile were prepared fresh daily.

All reactions[2] were carried out at 60 ± 0.2° in 15-ml round-bottom centrifuge tubes with polytef-lined caps.

Effect of Pyridine on Color Formation—Appropriate volumes of acetic acid, dicyclohexylcarbodiimide, 2-nitrophenylhydrazine, and pyridine were added to a centrifuge tube. The amount of pyridine added was varied up to 40.5% (v/v), with dichloromethane being used to maintain a constant volume. The sealed tubes were heated for 15 min at 60° and cooled. Aliquots of 5 ml of 1 *N* NaOH were added to each tube. The tubes were then rocked for 15 min and centrifuged at 2000 rpm for 15 min.

The absorbance of the aqueous layer was measured at 535 nm in a 1-cm cell against an appropriate blank. The concentrations of the reagents in the reaction mixture were: acetic acid, 0.00014 *M*; dicyclohexylcarbodiimide, 0.0135 *M*; 2-nitrophenylhydrazine, 0.0132 *M*; and pyridine, 0–40.5% (v/v). The volume of the reaction mixture was 7.4 ml before heating and subsequent addition of 5 ml of 1 *N* NaOH.

Effect of 2-Nitrophenylhydrazine Concentration on Color Formation—Reaction mixtures were prepared as described, except that the 2-nitrophenylhydrazine concentration was varied while all other parameters were held constant. The samples were treated in the same way as described. The concentrations of reagents in the reaction mixture were: acetic acid, 0.00013 *M*; dicyclohexylcarbodiimide, 0.0119 *M*; pyridine, 16.67% (v/v)[3]; and 2-nitrophenylhydrazine, 0–0.0292 *M*. The volume of the reaction mixture was held constant at 8.4 ml before heating and subsequent addition of 5 ml of 1 *N* NaOH by addition of appropriate amounts of dichloromethane.

Effect of Dicyclohexylcarbodiimide Concentration on Color Formation—With the optimum concentrations of pyridine and 2-nitrophenylhydrazine as previously determined, reaction mixtures were prepared with varying amounts of dicyclohexylcarbodiimide (0–0.090 *M*) and a fixed acetic acid concentration. The samples were treated as before at 60°. The concentrations of reactants in the final reaction mixture were: acetic acid, 0.00018 *M*; 2-nitrophenylhydrazine, 0.0163 *M*; pyridine, 16.67% (v/v); and dicyclohexylcarbodiimide, 0–0.0417 *M*. The volume of the reaction mixture, before heating and subsequent addition of 5 ml of 1 *N* NaOH, was maintained at 8.0 ml by addition of acetonitrile and dichloromethane.

Determination of Reaction Time for Acetic Acid—Reaction mixtures were prepared containing acetic acid, 0.00017 *M*; dicyclohexylcarbodiimide, 0.042 *M*; pyridine, 16.67% (v/v); and 2-nitrophenylhydrazine, 0.0163 *M* (final volume 6.0 ml). The reaction mixture was heated at 60°, and samples were removed as a function of time. The samples were cooled, rocked for 15 min with 5.0 ml of 1 *N* NaOH, and centrifuged at 2000 rpm for 15 min. The absorbance of the aqueous layer was measured at 535 nm.

Suggested Analytical Procedure for Acetic Acid—Place 2.0 ml of an acetic acid solution in a 15-ml centrifuge tube. Add 1.0 ml of dicyclohexylcarbodiimide (0.250 *M*), 1.0 ml of pyridine, and 2.0 ml of 2-nitrophenylhydrazine (0.049 *M*). React at 60° for 30 min, cool, and add 5.0 ml of 1 *N* NaOH. Rock the tube, and centrifuge for 15 min at 2000 rpm. Measure the absorbance of the aqueous phase at 535 nm against an appropriately prepared blank.

Determination of Extent of Reaction of Other Carboxylic Acids—Reaction mixtures containing other carboxylic acids were prepared in the same manner as in the acetic acid studies. The reactions were carried out at 60°, with samples being withdrawn as a function of time and treated as previously described.

Determination of Extent of Reaction of Other Carboxylic Acid Derivatives—Reaction mixtures were prepared by using the suggested analytical procedure for acetic acid, with the samples being 10^{-3} and 10^{-2} *M* solutions of acetamide, butyrolactone, and ethyl acetate in dichloromethane. The reaction mixtures were then subjected to the suggested analytical procedure for acetic acid.

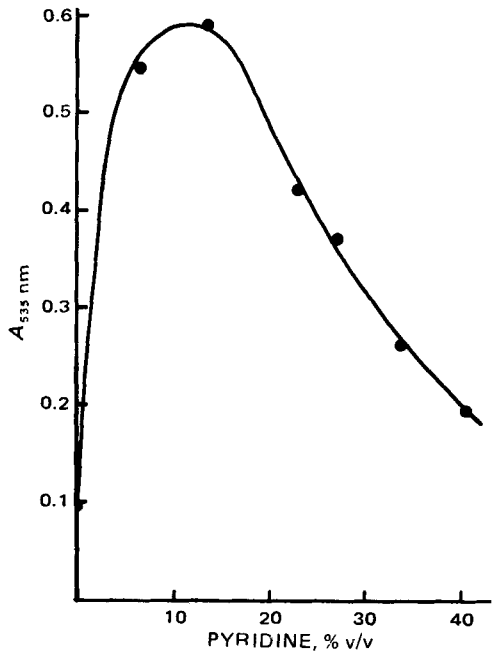

Figure 1—*Effect of pyridine concentration on color formation resulting from the reaction of acetic acid.*

RESULTS AND DISCUSSION

Preliminary experiments indicated that polar solvents facilitated the dicyclohexylcarbodiimide-coupled reaction of carboxylic acids and 2-nitrophenylhydrazine. Use of protic solvents such as methanol gave irreproducible results. This phenomenon was probably due to the condensation of the carboxylic acid and the solvent to form an unreactive ester. To find a suitable polar, aprotic solvent, a series of halogenated hydrocarbons was studied. Dichloromethane gave better results than chloroform or 1,2-dichloroethane. In an attempt to increase the polarity of the solvent by the addition of pyridine, the observation was made that pyridine catalyzed the reaction of interest. A systematic study was then undertaken to establish the optimum conditions for this reaction.

Figure 1 shows the effect of pyridine concentration on the color formation when acetic acid was used as the substrate. Absorbance measurements were made prior to the completion of the reaction. Consequently, differences in absorbances indicate differences in reaction rates. Pyridine apparently catalyzed the reaction at lower concentrations and inhibited the reaction at high concentrations. A more reasonable explanation is that the increasing pyridine concentration adversely affected the partitioning of the reaction product from the reaction mixture into the basic aqueous phase. Regardless of the cause of the observed behavior, addition of pyridine (16.7% v/v) produced an adequate increase in the reaction rate.

The effect of 2-nitrophenylhydrazine concentration was investigated under the same conditions with the optimum pyridine concentration. Color formation increased as a function of the 2-nitrophenylhydrazine concentration (Fig. 2). Since the blank absorbances were quite high at concentrations above 0.0175 *M*, a concentration of 0.0163 *M* was selected for future studies. This value gave sufficiently high yields with an acceptable blank level.

A similar study was conducted to find the optimum concentration of dicyclohexylcarbodiimide (Fig. 3). A maximum yield was obtained at a concentration of about 0.04 *M*. The decrease in absorbance at higher concentration is unexplained.

Under the conditions of optimum concentrations of pyridine, dicyclohexylcarbodiimide, and 2-nitrophenylhydrazine, acetic acid reacted completely within 30 min at 60°. Subsequently, multiple samples of acetic acid solutions of known concentration were subjected to the suggested analytical procedure. Three separate runs of seven concentrations each gave a linear regression line described by:

$$\text{absorbance} = 1527[\text{acetic acid } (M)] - 0.008 \qquad \text{(Eq. 1)}$$

The correlation coefficient for this equation was 0.994 with a standard

[1] In some instances, it was necessary to prepare stock solutions in a 50% (v/v) solution of dichloromethane and pyridine. The pyridine concentration was taken into account in later experiments.

[2] The rate of this reaction increases with temperature. However, the high volatility of the solvent (dichloromethane) prevents running these reactions at higher temperatures.

[3] Although the maximum yield was obtained at approximately 12% (v/v) pyridine, a concentration of 16.67% (v/v) was chosen for further studies to facilitate adding 1.0 ml into the samples. Very little sensitivity is sacrificed to obtain this experimental simplification.

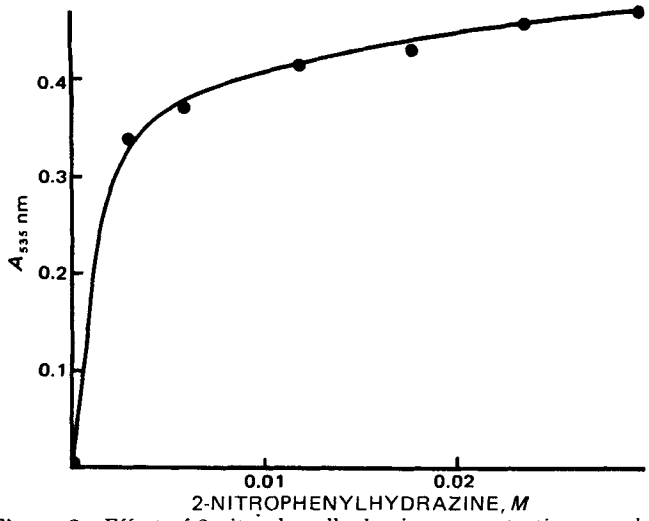

Figure 2—*Effect of 2-nitrophenylhydrazine concentration on color formation resulting from the reaction of acetic acid.*

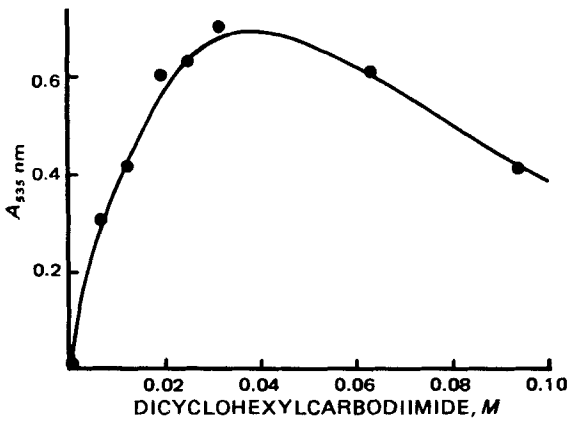

Figure 3—*Effect of dicyclohexylcarbodiimide concentration on color formation resulting from the reaction of acetic acid.*

error of the estimate (S_{xy}) of 0.049. Six separate determinations of 4.2×10^{-4} *M* acetic acid gave a standard deviation of 0.0092 (mean absorbance of 0.620). The concentration range for the linear regression analysis was $1\text{–}10 \times 10^{-4}$ *M*.

Several other carboxylic acids were studied under the optimum conditions as determined for acetic acid. At a reaction time of 45 min, butyric, succinic, and indoleacetic acids were detectable at concentrations of 10^{-4} *M* (absorbance greater than 0.1). Under the same conditions, phenylacetic, 2-methyl-2-hydroxypropanoic, and cinnamic acids were detectable at 10^{-3} *M*. Aromatic acids did not react under these conditions. Ethyl acetate, γ-butyrolactone, and acetamide at concentrations of 10^{-3} and 10^{-2} *M* gave no measurable absorbance.

The analysis of butyric acid extracted from aqueous solutions (five samples) produced a linear calibration curve described by:

$$\text{absorbance} = 333\ [\text{butyric acid } (M)] - 0.037 \qquad \text{(Eq. 2)}$$

The correlation coefficient for this equation was 0.991 with a standard error of the estimate (S_{xy}) of 0.054. The sensitivity was less than that expected from studies of butyric acid in nonaqueous solvents. This result was probably due to an incomplete recovery of butyric acid from the aqueous sample. The concentration range for this study was $1\text{–}5 \times 10^{-3}$ *M*. Nevertheless, these results demonstrate the feasibility of analyzing aliphatic acids in aqueous solution by this method.

Condition optimization is required for acids other than the simple aliphatic acids discussed here. This method does not appear to be suitable for the analysis of aromatic acids.

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