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Determination of Enzymic Demethylation of *p*-Chloro-*N*-methylaniline. Assay of Aniline and *p*-Chloroaniline

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The rate of demethylation of aromatic amines by liver microsomal systems has been often utilized to provide a measure of the activity of the NADPH oxygen-dependent "drug-metabolizing" enzymes. The demethylation reaction involves the cleavage of the N—C bond resulting in the formation of formaldehyde and a primary amine (1). Formaldehyde is usually determined with Nash reagent (2) and the primary aromatic amine is assayed by diazotization and coupling reactions (3, 4).

In the course of studies of the enzymic demethylation of *N*-methylaniline, we have observed that, when aniline was determined by the Bratton-Marshall procedure (4), there was about a threefold increase in intensity of the color formed due to the presence of residual substrate, *N*-methylaniline.[2] This interference was not correctable, since *N*-methylaniline alone did not yield a significant amount of color. This serious limitation warranted the development of another method for the determination of primary amines.

The present work describes a rapid and sensitive method for the measurement of primary aromatic amines [aniline and *p*-chloroaniline (PCA)]. Moreover, interference by the corresponding secondary amines [*N*-methylaniline and *p*-chloro-*N*-methylaniline (PCMA)] is negligible. The method involves a reaction of the amine with *p*-dimethylaminobenzaldehyde (PDAB) and is based on a previously described procedure for the determination of anthranilic acid in bacterial systems (5). This method was applied to the study of the rate of demethylation of

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[2] In 5 ml solutions the following absorbances were obtained: aniline (10 μmoles) = 0.750; *N*-methylaniline (10 μmoles) = 0.03; aniline (10 μmoles) + *N*-methylaniline (10 μmoles) = 2.40; aniline (1 μmole) = 0.146; aniline (1 μmole) + *N*-methylaniline (10 μmoles) = 0.292.

p-chloro-*N*-methylaniline by liver microsomal systems. The advantages of assaying the primary amine (PCA) rather than formaldehyde are discussed.

MATERIALS AND METHODS

NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Calbiochem; *p*-chloroaniline and *p*-dimethylaminobenzaldehyde were obtained from Eastman Organic Chemicals; *p*-chloro-*N*-methylaniline (sp.gr. 1.102; $n_D^{25°}$ — 1.5816, b.p. 91.0–5°C at 3.5 mm Hg) was obtained from Dr. R. E. Diehl of the Agricultural Division of American Cyanamid Company[3]; aniline was obtained from Merck and *N*-methylaniline·HCl from K & K Laboratories; nicotinamide was obtained from Sigma. All inorganic compounds were of reagent grade. Male albino Wistar strain rats (140–150 gm) were obtained from Royal Hart Farms.

Reagents. PDAB solution: 60 mg *p*-dimethylaminobenzaldehyde/ml 3 *N* H_2SO_4. Diluted PDAB solution: PDAB solution diluted 1:3 with H_2O.

Determination of an Aromatic Primary Amine (Aniline and PCA)

PDAB solution (1 ml) was added to a 4 ml (5 ml when aniline was used) aliquot of a solution containing the primary amine.[4] Absorbance was measured after 10 min at 430 mμ (aniline) with the Beckman model B spectrophotometer and at 445 mμ (PCA) with the Beckman DU monochrometer equipped with a Gilford model 2000 multiple-sample absorbance recorder.

Determination of Rate of Enzymic Demethylation of PCMA

(*a*) *Incubation procedure:* Rat liver supernatant (9000 *g*) was prepared as previously described (6). In a total volume of 2 ml in 25 ml Erlenmeyer flasks the following constituents were present:[5] 0.2 ml liver supernatant (0.0667 gm equiv of original liver); NADP (1.6 μmoles); magnesium chloride (30 μmoles); nicotinamide (20 μmoles); glucose 6-phosphate (16 μmoles)[6]; glucose-6-phosphate dehydrogenase (120 Buch-

[3] *p*-Chloro-*N*-methylaniline was prepared from *p*-chlorophenylisocyanate according to the method of Ried and Müller (10). Preparation of this compound by a different method has been described (11).

[4] Aniline stock solution was prepared in water containing 2% propylene glycol and PCA solution was prepared in water with equimolar concentrations of HCl.

[5] Larger volumes (containing same concentrations of constituents) were used when a time course of the reaction was studied. The assay was conducted on 2 ml aliquots which were taken at different intervals.

[6] The preparation was labeled by the manufacturer as 78% pure. Therefore, the quantity used contained only 12.5 μmoles of glucose 6-phosphate.

ner units); phosphate buffer (sodium), pH 7.4 (100 μmoles), and substrate PCMA (3.0 μmoles).[7] A parallel incubation was carried out in the absence of substrate. The contents of each flask were kept chilled on ice until incubation. Incubations were preformed at 37°C in a rotary shaker in an atmosphere of air for 10 min.

(*b*) *Assay procedure:* The reaction is terminated by addition of 3 ml of a diluted PDAB solution (20 mg/ml 1 *N* H_2SO_4). The resulting mixture is centrifuged at 8000 *g* for 30 min in a refrigerated (0–4°C) Servall centrifuge.[8] The supernatant solutions are brought to room temperature and the absorbance determined at 445 mμ. The quantity of PCA formed with a given liver preparation is determined from a standard curve[9] constructed from the absorbance of solutions containing the complete incubation mixture (less substrate) and with varied concentrations of PCA.[10]

RESULTS

Assay of Aniline

The reaction of PDAB with aniline in aqueous solutions yielded a yellow solution with a maximal absorbance at 430 mμ. There was no interference in color formation due to the presence of *N*-methylaniline (Table 1). Absorbance of the solution as a function of concentration of aniline is shown in Figure 1. The curve is linear to an absorbance of at least 1.40 in the Beckman model B spectrophotometer.

Assay of p-Chloroaniline (PCA)

The reaction of PCA with PDAB in aqueous solutions was found to yield a yellow color. The absorbance spectrum of the colored solution obtained with a Cary recording spectrophotometer demonstrates a max-

[7] PCMA solution was prepared by dissolving 30 μmoles of the compound per ml H_2O containing equimolar amounts of HCl; 0.1 ml aliquots of this solution were used in the described incubation studies.

[8] When this procedure yields opalescent solutions, the following is proposed: Reaction is terminated with 0.5 ml of 3 *N* H_2SO_4 followed by addition of 2.5 ml of a diluted PDAB solution (24 mg/ml 1.2 *N* H_2SO_4). However, under these conditions a marked decrease in extinction, presumably due to higher acidity, is observed (W. Pearl and D. Kupfer, unpublished results).

[9] PCA solution is prepared by dissolving the compound in water containing equimolar amounts of HCl.

[10] Absorbance of each solution is corrected for the color present in solutions in the absence of substrate or product. The principal portion of this color (pink) was formed by NADPH and faded almost entirely within 24 hr.

TABLE 1
Color Development[a] in Aqueous Solutions Containing Aniline and *N*-Methylaniline

Aniline, μmoles	*N*-Methylaniline, μmoles	Absorbance
0.2	—	0.302
0.2	1.0	0.308
0.2	3.0	0.322
0.6	—	0.900
0.6	1.0	0.906
0.6	3.0	0.911
1.0	—	1.46
1.0	1.0	1.47
1.0	3.0	1.48

[a] Color was developed by the addition of 1 ml PDAB solution to 5 ml aqueous solutions containing the respective amines. Tabulated values are absorbances at 430 mμ as measured with a Beckman model B spectrophotometer.

imum at 440–445 mμ (Fig. 2). The color formed was stable for several hours (Table 2). The interference in color formation due to the presence of *p*-chloro-*N*-methylaniline (PCMA) was negligible. Absorbance of an aqueous solution as a function of concentration of PCA is shown in Figure 3. The curve is linear to an absorbance of 2.2 in the Beckman DU monochrometer equipped with a Gilford model 2000 absorbance recorder.

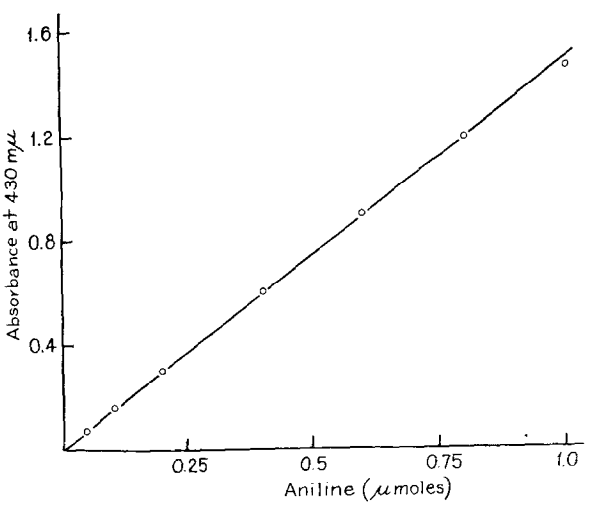

FIG. 1. Absorbance as a function of aniline concentration. Various aliquots of aniline solution (0.5 μmole/ml in water containing 2% propylene glycol) were adjusted to a volume of 5 ml with water. Color was developed by addition of 1 ml of PDAB solution; the absorbance measurements were carried out at 430 mμ against a reagent blank lacking aniline.

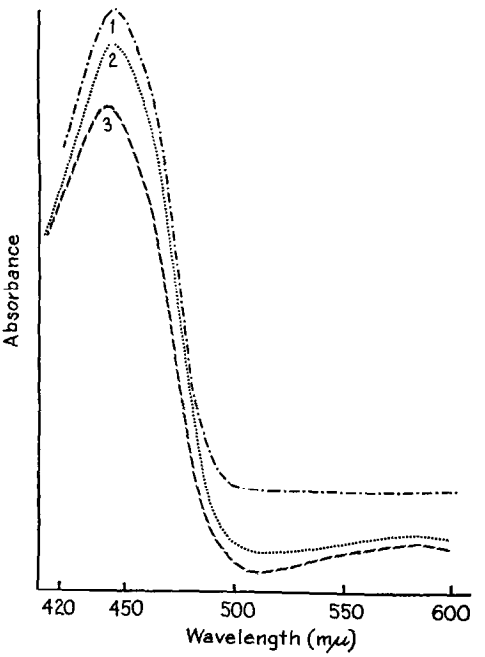

FIG. 2. Absorption spectra of colored product obtained from *p*-chloroaniline (PCA) and from incubation of *p*-chloro-*N*-methylaniline (PCMA): (1) 0.34 μmole PCA in 2 ml H_2O, OD at 443 mμ = 0.73; (2) 0.36 μmole PCA incubated 10 min in the presence of 9000 *g* rat liver supernatant and the complete incubation mixture in a volume of 2 ml, OD at 443 mμ = 0.76; (3) 3.0 μmoles PCMA incubated 10 min as described for (2), OD at 442 mμ = 0.70. Conditions of incubation and work up are as described under "Materials and Methods." Color was developed by addition of 3 ml of a diluted (20 mg/ml) PDAB solution. Spectra were obtained with a Cary recording spectrophotometer model 14 M. For the purpose of visual clarity, the curves were plotted on the same graph without regard for the magnitude of the absorbance peak.

TABLE 2
Stability of the Colored Product of *p*-Chloroaniline with Time[a]

Sample No.	Time of color development prior to measurement, hr	
	0.5	24
1	1.162	1.180
2	1.229	1.240

[a] Each solution contained 0.5 μmole *p*-chloroaniline in 4 ml water. Color was developed with 1 ml PDAB solution. Tabulated values are absorbances at 445 mμ in a Beckman DU monochrometer equipped with a Gilford model 2000 absorbance recorder measured against a water blank and corrected for absorbance of the reagent (absorbance of the reagent blank remained 0.100 after 0.5 or 24 hr of color development).

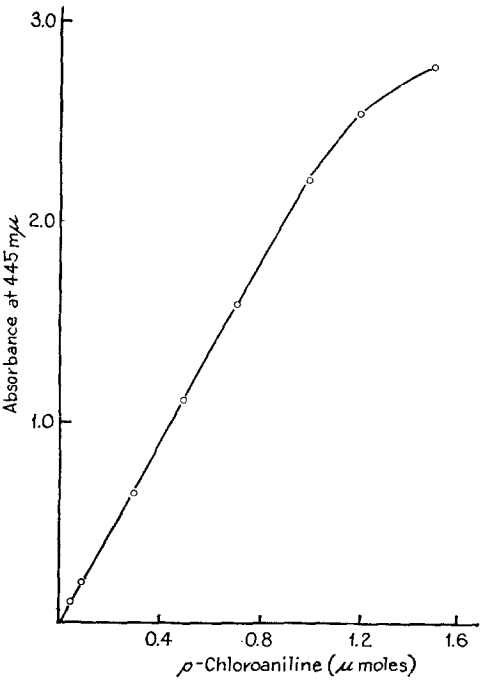

FIG. 3. Absorbance as a function of *p*-chloroaniline concentration in aqueous solutions. PDAB solution (1 ml) was added to a 4 ml sample containing the amine. Measurements were made at 445 mμ against a reagent blank.

Determination of Rate of Demethylation of Aromatic Secondary Amines by 9000 g Liver Supernatant

(*1*) Disadvantages in the use of *N*-methylaniline and advantages in the use of PCMA as a possible substrate: 30 min incubation of aniline in the presence of a liver microsomal system resulted in a 20% disappearance of the amine as observed by a decrease in the intensity of the color formed with PDAB. Moreover, a loss of aniline was observed when this compound was merely heated for 30 min at 37°C in distilled water, suggesting evaporation and/or destruction of aniline under these conditions. These results indicate that *N*-methylaniline is not a suitable substrate for enzymic demethylation under our incubation conditions, if the product aniline is to be measured. Furthermore, a hydroxylation of the aromatic ring of aniline in the para position by liver microsomes (7) would certainly further complicate the colorimetric determination of this compound. Thus, it was advantageous to use an aromatic secondary methylamine with a substituent in the para position which upon demethylation would yield a less volatile primary amine than aniline.

p-Chloro-*N*-methylaniline (PCMA) seems to satisfy these requirements:

(*a*) The para position is blocked by a halogen.

(*b*) The demethylated product (PCA) is a solid with melting point and boiling points being 70° and 230°C, respectively, as compared with aniline (liquid) with a boiling pont of 184°.

(*2*) Enzymic demethylation of PCMA: The above observations indicated that PCA could be rapidly and accurately assayed in aqueous solutions. However, the application of this assay to enzymic demethylation reactions was contingent on satisfying the following conditions: (*a*) PCMA can serve as a substrate for the enzymic demethylation. (*b*) PCA is a product of the reaction. (*c*) PCA is stable, fully recoverable under incubation conditions, and can be accurately assayed in the presence of the constituents of the incubation mixture.

Preliminary observations demonstrated that the incubation of PCMA with a 9000 *g* supernatant, yielded a substance which formed a yellow color with PDAB. The absorbance spectrum of the product was identical to that obtained with PCA in aqueous solution, and to that obtained from the incubation of PCA with a 9000 *g* supernatant (Fig. 2). The observation that *p*-fluoroaniline could be converted to *p*-aminophenol by liver microsomes (8) suggested that the incubation of PCMA with liver preparations might yield, in addition to PCA, products such as *p*-aminophenol or *p*-hydroxy-*N*-methylaniline. *p*-Aminophenol, but not *p*-hydroxy-*N*-methylaniline, was found to react with PDAB, forming a color with an absorption spectrum similar to that obtained with PCA, but with a lower extinction as compared with PCA. Thus, the formation of *p*-aminophenol instead of the expected PCA would yield lower, and therefore erroneous, values for the rate of demethylation of PCMA. The finding that the product formed by the incubation of PCMA was entirely extractable by ether from a basic solution (pH 11), while *p*-aminophenol when added to the incubation mixture was not extractable under similar conditions, indicated that the product of the reaction was an amine which was not contaminated by significant quantities of *p*-hydroxyaniline. Furthermore, the product from a 10 min incubation of PCMA with a 9000 *g* liver supernatant yielded a purple color on diazotization and coupling by the conventional Bratton-Marshall procedure (4). The resulting solution had an absorption curve identical to that observed with PCA ($\lambda_{max} = 560$ mμ). These results provided indirect evidence that the product of the incubation of PCMA was the expected PCA.

To examine whether the assay of PCA was a satisfactory measure of the enzymic demethylation of PCMA, the stability and recovery of PCA under incubation conditions were determined. Thus, a 10 min incubation of PCA with a 9000 *g* rat liver supernatant resulted in a total recovery

TABLE 3
Absorbance of *p*-Chloroaniline (PCA) in the Presence of Incubation Constituents[a]

Sample No.[b]	PCA, μmole/2 ml	Incubation time, min 0	10
1	0.2	0.446	0.440
2	0.4	0.892	0.897
3	0.6	1.306	1.351
4	0.8	1.717	1.710
5	0.2	0.438	
6	0.4	0.866	
7	0.6	1.306	

[a] The volume of each incubation mixture was 5 ml; 2 ml aliquots were taken at 0 time and after a 10 min incubation. Color development was achieved by addition of 3 ml of a 1:3 diluted PDAB solution. Conditions were as described under "Materials and Methods." Tabulated values are absorbances at 445 mμ corrected for nonspecific absorbance by solutions lacking PCA.

[b] Samples 1–4 contained a complete incubation mixture and 9000 *g* liver supernatant from different animals. Samples 5–7 contained the indicated amount of PCA in water.

of this compound (Table 3); there was no difference in absorbance of PCA solutions in the presence or absence of incubation constituents. A standard curve constructed from solutions containing the total incubation mixture with liver supernatant from different rats indicates a linear relationship to an absorbance value of at least 1.70 (Fig. 4). When PCA was added to the product of a 10 min incubation of PCMA, and the resulting solution was reacted with PDAB, a complete recovery (102%) of the added PCA was obtained. The colored product obtained from a 10 min incubation of PCMA with a 9000 *g* liver supernatant is stable

TABLE 4
Stability of the Colored Product from the Incubation of *p*-Chloro-*N*-methylaniline with Time[a]

Sample No.[b]	Time of color development prior to measurement, hr 0.5	24
1	0.732	0.733
2	0.536	0.582
3	0.430	0.484

[a] Tabulated values are absorbances at 445 mμ measured with a Beckman DU monochrometer equipped with a Gilford Model 2000 absorbance recorder. Measurements were made against a water blank and corrected for nonspecific color formed by the incubation mixture in the absence of substrate.

[b] Each incubation contained 3.5 μmoles of *p*-chloro-*N*-methylaniline and the respective 9000 *g* rat liver supernatant. Preparations, conditions of incubation and assays are as described under "Materials and Methods."

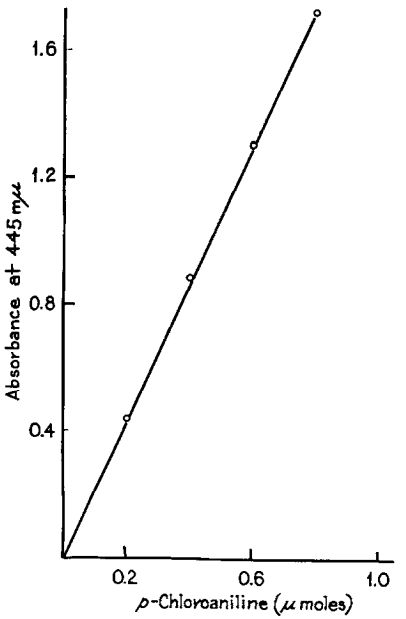

FIG. 4. Absorbance as a function of *p*-chloroaniline concentration in the presence of a complete incubation mixture. Color was developed with 3 ml diluted (20 mg/ml) PDAB solution which was added to 2 ml aliquots of the incubation mixture containing the amine. Measurements were made at 445 mμ against a reagent blank.

for at least 24 hr (Table 4), indicating that the method is suitable for the determination of a large number of samples.

For comparison purposes, the rate of demethylation of PCMA was determined by the conventional formaldehyde assay (2) and by the presently described assay of PCA. Results (Table 5) indicated that

TABLE 5

Rate of Demethylation of *p*-Chloro-*N*-methylaniline by a Male Rat Liver 9000 *g* Supernatant as Measured by Formation of Formaldehyde and *p*-Chloroaniline

Incubation time, min[a]	Formaldehyde, μmoles/gm liver	*p*-Chloroaniline, μmoles/gm liver
10	1.88	2.22
30	4.05	5.30

[a] Incubation mixture contained in a total volume of 15 ml: 3 ml 9000 *g* liver supernatant (1 gm liver equivalent); PCMA (22.5 μmoles); $MgCl_2$ (225 μmoles); NADP (6 μmoles); phosphate buffer, pH 7.4 (750 μmoles); nicotinamide (150 μmoles); glucose 6-phosphate (120 μmoles); glucose 6-phosphate dehydrogenase (900 Buchner units); semicarbazide (60 μmoles). Two parallel incubations were carried out in the absence of substrate (PCMA), one of which contained both HCHO (9 μmoles) and *p*-chloroaniline (2.70 μmole). Assays were performed on 2 ml aliquots, taken at 0 time, 10 min, and 30 min, as described under "Materials and Methods."

higher PCA values were obtained by the PCA assay as compared with those obtained by formaldehyde determination.

DISCUSSION

The studies described demonstrate a rapid and simple assay for the determination of two primary aromatic amines (aniline and *p*-chloroaniline) in aqueous solutions. Furthermore, previous observations on the color formation with anthranilic acid (5) and the finding that *p*-aminophenol forms color with PDAB would suggest that this method could be easily adapted to the quantitation of other aromatic amines as well.

The method for the quantitative determination of PCA was adapted to determine the rate of enzymic demethylation of the corresponding secondary amine (PCMA). The ease, rapidity, and sensitivity of the method combined with the stability of the color formed permitted the determination of a large number of samples and eliminated the problems arising from having to stagger the assay of numerous samples. The observation that there was no loss in PCA in the course of incubation (Table 3) permitted the construction of standard curves from solutions which were not incubated (Fig. 4).

Our studies demonstrated that hydroxylation of the aromatic ring of PCA did not occur. However, the possibility that incubations of PCMA under different conditions will yield phenolic products could not be ruled out. Such side reactions, if not recognized, could result in erroneous values of the rates of *N*-demethylation. These complications, however, could be easily remedied by the differential extraction of the products. In fact, when a given enzymic system is found to yield both *p*-aminophenol and PCA, the differential extraction of the latter compound from a basic solution could permit the adaptation of the method to the determination of the rate of formation of both compounds.

The observation that the incubation of PCMA with rat 9000 *g* liver supernatant yielded lower amounts of formaldehyde as compared with those of PCA (Table 4) suggested that the cleavage of N—C bond did not result in a quantitative transformation of the methyl group into measurable formaldehyde. However, the less likely possibility that a significant quantity of the formaldehyde was lost in the course of the incubation could not be ruled out. Furthermore, the determination of formaldehyde as an indication of the extent of demethylation of a given secondary amine could at times lead to erroneous conclusions—namely, the formaldehyde generated in the incubation might originate from sources other than the amine. In fact, a recent study of the enzymic demethylation of aminopyrine demonstrated the formation of larger amounts of formaldehyde than could be accounted for by the total de-

methylation of the substrate (9). Thus, in each new study it would be necessary to examine the source of the formaldehyde formed prior to its quantitation. These considerations indicate that in studies of enzymic demethylation of secondary amines it is advantageous to be able to measure the formation of the amine rather than formaldehyde.

SUMMARY

A rapid and sensitive colorimetric method has been developed for the quantitative determination of aromatic amines in aqueous solutions. The method is based on a reaction of the amine with *p*-dimethylaminobenzaldehyde and the intensity of the color formed is determined spectrophotometrically. This method has been adapted to assay the rates of demethylation of *p*-chloro-*N*-methylaniline by liver enzyme systems. The advantages of this method over the previously described procedures are discussed.

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REFERENCES

1. GAUDETTE, L. E., AND BRODIE, B. B., *Biochem. Pharmacol.* **2**, 89 (1959).
2. COCHIN, J., AND AXELROD, J., *J. Pharmacol. Exptl. Therap.* **125**, 105 (1959).
3. BRODIE, B. B., AND AXELROD, J., *J. Pharmacol. Exptl. Therap.* **99**, 171 (1950).
4. BRATTON, C., AND MARSHALL, E. K., JR., *J. Biol. Chem.* **128**, 537 (1939).
5. KUPFER, D., AND ATKINSON, D. E., *Anal. Biochem.* **8**, 82 (1964).
6. KUPFER, D., AND PEETS, L., *Biochem. Pharmacol.* **15**, 573 (1966).
7. KATO, R., AND GILLETTE, J. R., *J. Pharmacol. Exptl. Therap.* **150**, 285 (1965).
8. RENSON, J., *Federation Proc.* **23**, 325 (1964).
9. WILSON, J. T., *Federation Proc.* **25**, 417 (1966).
10. RIED, W., AND MÜLLER, F., *Chem. Ber.* **85**, 470 (1952).
11. ROBERTS, R. M., AND VOGT, P. J., *J. Am. Chem. Soc.* **78**, 4778 (1956).