Biochemical Pharmacology, Vol. 28, pp. 3285–3290.
© Pergamon Press Ltd. 1979. Printed in Great Britain.

0006-2952/79/1115–3285 $02.00/0

EVIDENCE FOR THE INVOLVEMENT OF *N*-ACETYL-*p*-QUINONEIMINE IN ACETAMINOPHEN METABOLISM

DAVID J. MINER and PETER T. KISSINGER
Department of Chemistry, Purdue University, West Lafayette, IN 47907, U.S.A.

(*Received* 2 *November* 1978; *accepted* 11 *May* 1979)

Abstract—Evidence for the presence of *N*-acetyl-*p*-quinoneimine (NAPQI), a postulated toxic intermediate of acetaminophen metabolism, in mouse liver microsomal incubations is reported. The intermediate was tentatively identified by comparison with synthetic NAPQI generated electrochemically from acetaminophen in a coulometric flow reactor. All but one of the reaction products of NAPQI with a number of nucleophiles were found *in vitro* as well, and in similar relative amounts. The NAPQI intermediate is moderately stable at physiological pH and temperature with a lifetime which is dependent on the components of the medium.

Acetaminophen is a widely used analgesic drug. It is believed to be quite safe at therapeutic doses, but it can cause severe liver damage in overdose. The probable mechanism of this toxicity has been worked out primarily by J. R. Mitchell and coworkers [see Ref. 1 and references therein]. Their scheme is illustrated in the top portion of Fig. 1. A small fraction of a therapeutic dose is believed to be *N*-hydroxylated, primarily in the liver. The synthesis of *N*-hydroxy acetaminophen has been reported recently [2, 3], and it was shown to be more toxic than acetaminophen itself. This metabolite is relatively unstable at physiological pH and temperature, presumably losing water to form *N*-acetyl-*p*-quinoneimine (NAPQI). The NAPQI is probably detoxified *in vivo* by reaction with glutathione, but when large doses are administered, hepatic glutathione levels are depleted and covalent binding to hepatic protein occurs. While there is significant indirect evidence to support this mechanism it has proven difficult to verify it by directly detecting either the *N*-hydroxy acetaminophen or *N*-acetyl-*p*-quinoneimine.

We report here the application of modern electrochemical techniques, together with high performance liquid chromotography, as a new approach to the study of the mechanism of acetaminophen (APAP) toxicity. Quinoneimines are readily generated by two electron oxidation of the corresponding aminophenols, either chemically [4] or electrochemically [5]. The use of electrochemical oxidation avoids the need for addition and subsequent removal of oxidizing agents. Quantities of electrogenerated products sufficient for study can be conveniently and rapidly generated through the use of a porous flow-through coulometric reactor. These reactors offer a number of advantages over traditional approaches to coulometry, but it is only recently that they have been used for practical laboratory syntheses [6, 7]. Liquid chromatography with electrochemical detection (l.c./e.c.) is a sensitive and selective technique well suited to the study of acetaminophen metabolism [8, 9]. With these tools, NAPQI has been generated, its relative stability determined, and evidence for the existence of NAPQI *in vitro* has been obtained.

MATERIALS AND METHODS

Coulometric flow cell. The cell constructed in our

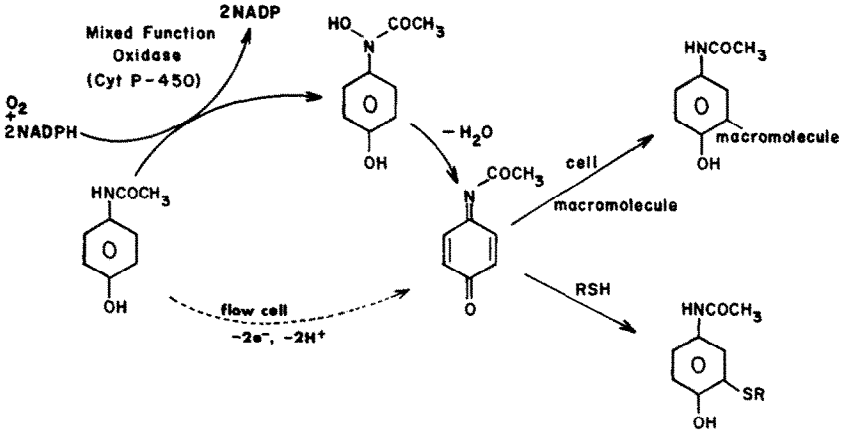

Fig. 1. Proposed mechanism of acetaminophen toxicity.

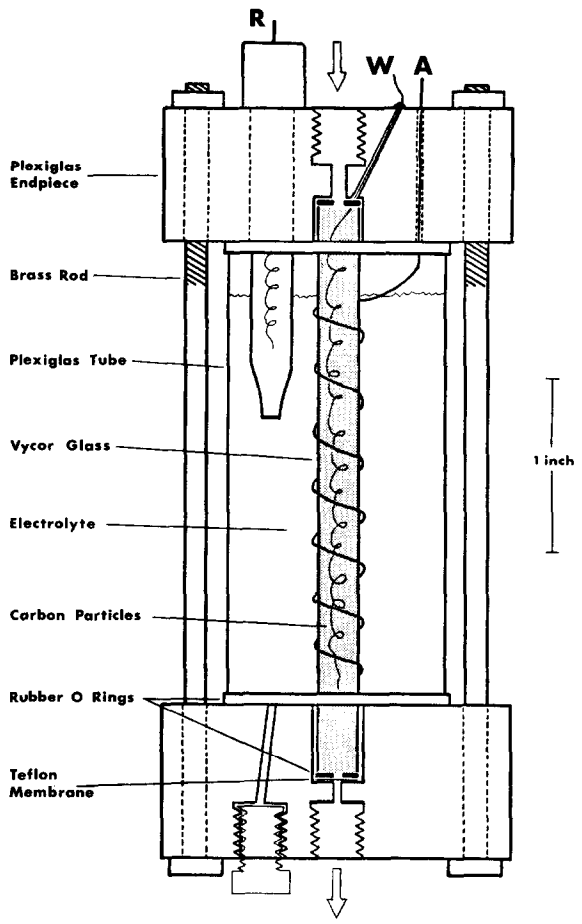

Fig. 2. Flow-through coulometric reactor. The direction of flow is indicated by arrows. Key: (R) Ag/AgCl reference electrode, (W) working electrode lead, and (A) platinum wire auxiliary electrode.

laboratory is depicted in Fig. 2. It consists of a porous glass tube (Vycor, Corning 7930 glass), packed with graphite particles (150–180 μm) and wrapped with a platinum wire auxiliary electrode. Contact with the working electrode is made with a length of platinum wire. The tube is placed in a plexiglas capped jacket containing electrolyte and a Ag/AgCl reference electrode. The assembly is held together by three threaded brass rods. Solutions were pumped through the cell at 1 ml/min, using a peristaltic pump (Gilson Medical Electronics, Middleton, WI, Minipuls II), and the potential was maintained at + 0.72 V. This cell was used for determination of the number of electrons involved in the oxidation of acetaminophen, and subsequently for routine synthesis of NAPQI. An n value for the oxidation of acetaminophen was obtained by measuring the current produced in the cell when 1.75 mM acetaminophen in 0.1 M citrate (pH 6.8) was passed through it at 1.0 ml/min.

Liquid chromatography with electrochemical detection. A commercial liquid chromatograph (LC-50, Bioanalytical Systems, Lafayette, IN) equipped either with a carbon paste or a glassy carbon electrode was employed. The injection volume was 20 μl. The column was slurry packed in this laboratory with 10 μm reverse-phase (C_{18}) packing material (μ-Bondapak, Waters Associates, Milford, MA). All sample solutions and mobile phase constituents were prepared using distilled, deionized water.

For determination of NAPQI, the mobile phase consisted of 100 ml of redistilled methanol plus 500 ml 0.1 M citrate (pH 6.8). The citrate buffer was prepared fresh each day from stock solutions of 0.1 M citric acid and 0.1 M trisodium citrate. The mobile phase was pumped at 1.0 ml/min and the detector electrode potential was set at −0.24 V vs Ag/AgCl.

Stability studies. The response of the liquid chromatograph to injected NAPQI was determined by using the coulometric flow cell to convert known concentrations of acetaminophen. The flow cell exit port was connected directly to the injection valve of the liquid chromatograph, minimizing transfer time. The decomposition occurring during the transfer time (20 sec) was calculated from rate constants determined subsequently and found to be insignificant. System response was found to be linear over the range 10^{-4} to 10^{-5} M. A slight decay of the response with time was noted at concentrations below 10^{-5} M.

Decomposition of NAPQI (starting concentration 1.1×10^{-4} M) under various solution conditions was

followed by repeated injections into the liquid chromatograph. When microsomes were added, the solutions were filtered prior to analysis. Peak heights were recorded manually and converted to concentrations. Half-order rate constants were determined from the slope of plots of $[NAPQI]^{1/2}$ vs time.

Reaction products with nucleophiles. *N*-acetyl cysteine, glutathione, cysteine, thioglycolic acid, methionine and diethyl dithiocarbamate were obtained from Sigma (St. Louis, MO), cysteamine from Aldrich (Milwaukee, WI). α-mercaptopropionylglycine from Cal-Biochem (LaJolla, CA), and glycine from Matheson, Coleman & Bell (East Rutherford, NJ). The compounds containing a sulfhydryl group were reacted with the electrogenerated NAPQI under the following conditions. Eight ml of 2 mM sulfhydryl were incubated at 37°. Eight ml of 1 mM acetaminophen (in 75 mM KCl, 15 mM $MgCl_2$ and 20 mM phosphate, pH 7.4) was oxidized in the flow cell and added with constant agitation. The mixture was then incubated an additional 8 minutes, at which time the reaction was quenched by addition of solid ascorbic acid. The samples were diluted 1:6 prior to analysis. The non-sulfhydryl nucleophiles were reacted with NAPQI in a similar fashion, except that acetaminophen was 0.4 mM in 150 mM KCl and 40 mM phosphate.

Microsomal incubations were carried out in the conventional manner. Two Swiss male mice (Laboratory Supply, Indianapolis, IN) were decapitated and their livers were removed and homogenized in cold 20 mM Tris buffer (pH 7.4) containing 1.15% KCl. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant fraction was centrifuged for 60 min at 100,000 *g*. The pellet was resuspended and recentrifuged at 100,000 *g* for 50 min. The microsomal fraction was reconstituted in 8.2 ml KCl/phosphate. Five hundred μl of this were added to tubes containing acetaminophen and a nucleophile. This mixture was equilibrated at 37° for 2 min, then NADPH (P-L Biochemicals, Milwaukee, WI) in $MgCl_2$ was added. Final concentrations were acetaminophen, 2×10^{-4} M; NADPH, 6×10^{-4} M; nucleophiles, 2 mM; $MgCl_2$, 15 mM; KCl, 75 mM; microsomal protein, 2 mg/ml; and pH 7.4 phosphate, 20 mM. The tubes were incubated and shaken for 20 min after which time 500 μl of ice-cold methanol were added. They were then centrifuged for 10 min and the supernatant fractions were filtered (0.22 μm pores, Millipore, Bedford, MA). Control incubations with acetaminophen, NADPH or nucleophiles omitted were also carried out. Samples were diluted 2:7 prior to analysis. In some cases the very large acetaminophen peak obscured some product peaks; thus, 300 μl of the incubation mixture were extracted with 2 ml of ethyl acetate. This served to remove much of the acetaminophen.

Formation of NAPQI in vitro. Microsomal incubations were carried out as described above, except that the following concentrations were increased: acetaminophen, 10^{-3} M; and NADPH, 2×10^{-3} M. Sulfhydryls were omitted to maximize the lifetime of NAPQI. After incubation the samples were rapidly filtered, and a portion was injected into the liquid chromatograph. Flow cell-generated standard solutions of NAPQI were chromatographed alternately with incubation solutions.

Thin-layer chromatography. The thin-layer chromatography (t.l.c.) behaviour of the major reaction product of *N*-acetyl cysteine was examined using the following systems: DEAE cellulose (Analtech, 250 μm), developed with *n*-propanol–0.4 M ammonium hydroxide (80:20) and silica (Kieselgel 60 F-254, Merck) developed first with ethyl acetate–methanol–water–acetic acid (60:30:9:1) and then in the other direction with *n*-butanol–water–acetic acid (4:1:1). The major product with cysteine and 3-[(5-acetamido-2-hydroxyphenyl)thio]alanine were compared using the silica system.

RESULTS AND DISCUSSION

The performance of the coulometric flow cell was evaluated by monitoring the current produced upon oxidation of hydroquinone. It was found to effect coulometric conversion of up to 10 mM solutions at a flow rate of 1 ml/min. Using the flow cell, an n value of 2.1 ± 0.1 electrons was found for the oxidation of acetaminophen, indicating that its redox behavior is analogous to that of the *p*-aminophenols [5].

An l.c./e.c. determination of NAPQI was developed with chromatographic conditions chosen such that the stability of NAPQI would be maximized. Thus, citrate buffer (pH 6.8) was used at 0.1 M to ensure buffering capacity while minimizing buffer-mediated decomposition. This system permitted linear determination of NAPQI over a useful range of concentrations (10^{-4}–10^{-5} M). NAPQI concentrations as low as 10^{-7} M could be determined with the use of standards. A typical chromatogram for the determination of NAPQI is shown in Fig. 3.

The hydrolysis of quinioneimines to form quinones is a well-documented reaction [5, 10, 11]. The hydrolysis of NAPQI appears to be no exception, it being hydrolyzed to yield *p*-benzoquinone (see Fig. 3). At neutral pH the hydrolysis appears to have a half-order dependence on the concentration of NAPQI, i.e. reasonably linear fits of $[NAPQI]^{1/2}$ vs time were obtained. We are currently investigating the mechanism and rate of hydrolysis further. Rate constants as a function of several factors are given in Table 1. These results show the stability of NAPQI to be very dependent on solution conditions. The intermediate is most stable around pH 7.2. At higher or lower pH than those listed, the rate of hydrolysis was too fast to be measured chromatographically. Most of these rate constants were obtained at room temperature; the one case examined at 37° showed 2-fold rate increase. Buffer strength has a pronounced effect on the rate, the rate increasing with increased phosphate concentration. This suggests that general acid/base catalysis may be involved. The influence of the presence of the microsomal fraction on the decomposition of NAPQI was explored, and it was found to accelerate greatly the disappearance of NAPQI. Under these conditions, and assuming half-order kinetics and a starting NAPQI concentration of 10^{-6} M, the half-life is expected to be < 7 sec. The mechanism of microsomal involvement may be either as an acid/base catalyst or providing nucleophilic sites for 1, 4-addition to NAPQI. In a series of experiments, the direct detection of NAPQI in microsomal incubations was attempted using the same l.c./e.c. system used for the hydrolysis studies. All of these experiments

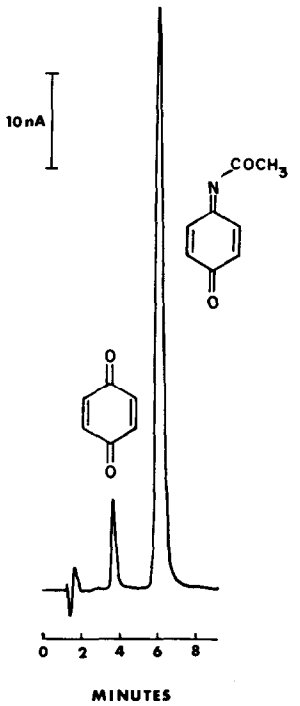

Fig. 3. Chromatogram illustrating determination of NAPQI in aqueous media by l.c/e.c. Conditions: 15 cm μ-C_{18} column, mobile phase: 500 ml of 0.1 M citrate (pH 6.8) and 100 ml of CH_3OH; flow rate 1.0 ml/min; detector potential = −0.24 V. Represents approximately 90 ng of NAPQI and 10 ng of *p*-benzoquinone injected.

failed to detect any NAPQI. Efforts to detect NAPQI are continuing, but it appears that levels of NAPQI are quite low. Concentrations greater than 10^{-7} M are known to be detectable in aqueous media and no interfering peaks were noted in the microsomal incubations. At this point one could conclude that NAPOI is not formed at all or that the level of NAPQI is less than 0.01 per cent of the 10^{-3} M acetaminophen concentration during the course of the incubation. The choice between these two possibilities was determined by trapping experiments.

Evidence for the involvement of NAPQI in acetaminophen metabolism was obtained by studying the reaction products of NAPQI with various sulfhydryl nucleophiles. Synthetic NAPQI was reacted with a number of nucleophiles under conditions approximating those of microsomal incubations. The reaction mixtures were analyzed by l.c./e.c. The product peaks which were found were then sought in microsomal incubations containing acetaminophen and the same nucleophile. An example of the chromatograms obtained is shown in Fig. 4. With *N*-acetylcysteine as the nucleophile, four products were found under these chromatographic conditions. Peaks with matching retention times are seen in similar relative amounts in the microsomal incubation as well. These peaks are not observed if NADPH, acetaminophen, or the nucleophile is omitted from the incubation. To verify further the identity of the matching peaks, the chromatographic conditions were changed and the samples reanalyzed. The results of these experiments with a number of nucleophiles are listed in Table 2. For all of these compounds, all of the synthetic products were present in the microsomal incubations. The only exception was one of the products of NAPQI with α-mercaptopropionylglycine, which could not be found in microsomal incubations. In all cases, the relative amounts of the synthetic products were similar to the relative amounts of the microsomal products. No products were detected under the conditions of these experiments when glycine, methionine or diethyldithiocarbamate was mixed with NAPQI.

Additional evidence for the identity of peaks with matching retention times was obtained in some cases by hydrodynamic voltammetry. A hydrodynamic voltammogram is generated by repeatedly injecting a sample into the liquid chromatograph with the detector potential set to a new value for each injection. This process is analogous to obtaining a point by point u.v. spectrum of a peak in conventional chromatography. The resultimg peak heights are normalized to unity using the peak height at the greatest detector potential (the ratio is being called ϕ) and plotted versus the detector potential. A typical curve is depicted in the inset in Fig. 5. The voltammogram is characteristic of a given compound and the medium (i.e. chromatographic mobile

Table 1. Rates of decomposition of NAPQI*

Buffers	pH	Temp (°C)	K ($M^{1/2}$ $l^{-1/2}$ sec^{-1})
Citrate (0.01 M)	6.0	25	1.4×10^{-6}
Citrate (0.01 M)	7.2	25	5.0×10^{-7}
Citrate (0.01 M)	7.2	37	1.2×10^{-6}
Citrate (0.01 M)	8.3	25	8.1×10^{-7}
Phosphate (1 M)	7.2	25	1×10^{-5}
Phosphate (0.1 M)			2.5×10^{-6}
Phosphate (0.01 M)			6.2×10^{-7}
Phosphate (0.001 M)			4.4×10^{-7}
KCl/phosphate (75 mM/20 mM)	7.4	25	1×10^{-6}
KCl/phosphate + microsomes (2 mg/ml)			$\sim 8 \times 10^{-5}$

*Decomposition of electrochemically produced NAPQI (starting concentration 1.1×10^{-4} M) was monitored by repeated analysis using reverse phase liquid chromatography with electrochemical detection. Half-order rate constants were determined from the slope of plots of $[NAPQI]^{1/2}$ vs time.

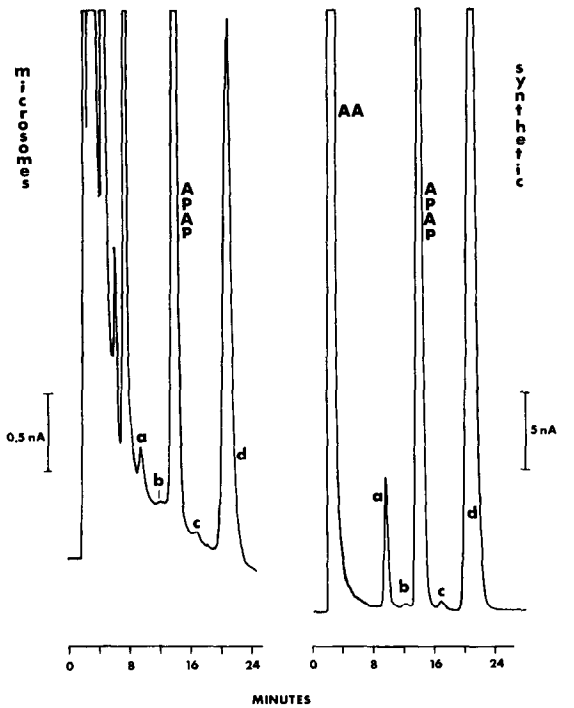

Fig. 4. Comparison chromatograms of a microsomal incubation containing acetaminophen and *N*-acetyl cysteine, and a reaction mixture of NAPQI and *N*-acetyl cysteine. Conditions: 15 cm μ-C_{18} column, 10/35/25/400, 1 M CH_3COOH/1 M CH_3COONH_4/CH_3OH/H_2O, $E = +0.75$ V. Key: (APAP) acetaminophen and (AA) ascorbic acid; a, b, c and d are reaction products.

phase). The excellent match of the graphs for the synthetic and microsomal peaks ($E_{1/2} = +0.45$ V) further confirms that the same products are formed.

The detection in microsomal incubations of almost all of the products of reaction between NAPQI and various sulfhydryls strongly supports the proposed involvement of *N*-acetyl-*p*-quinoneimine in acetaminophen metabolism.Taken together with our inability to detect NAPQI directly in these same incubations, this suggests that, as NAPQI is formed, it reacts quickly and thus could only be present at very low steady state levels.

Establishment of the structures of the various adducts should further confirm the production of NAPQI in biological systems. Efforts to isolate quantities of these adducts are currently underway. The major prod-

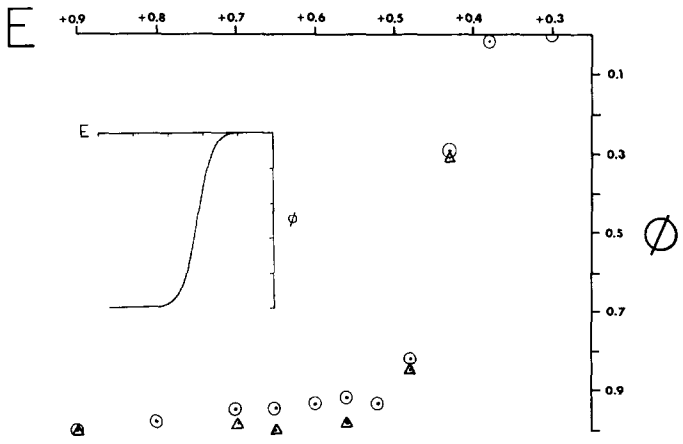

Fig. 5. Hydrodynamic voltammograms of major reaction products of cysteamine with NAPQI. Key: (⊙) synthetic product, and (△) corresponding peak from microsomal incubations. Inset is a theoretical hydrodynamic voltammogram. Conditions: 15 cm μ-C_{18} column, 10/35/20/400, 1 M CH_3COOH/1 M CH_3COONH_4/CH_3OH/H_2O, flow rate 1 ml/min.

Table 2. Number of reaction products of NAPQI found by l.c./e.c. in microsomal incubations*

Nucleophile	Chromatographic conditions	
	Acid mobile phase	Neutral mobile phase
N-Acetyl cysteine	3+	4+
Thioglycolic acid	2	1
Glutathione	2+	3
α-Mercaptopropionyl glycine	2‡	1‡
Cysteamine	4+	3+

* NAPQI was produced electrochemically and reacted with the nucleophiles under solution conditions approximating those of microsomal incubations. The reaction mixtures were analyzed by liquid chromatography with electrochemical detection. The product peaks were sought in mouse liver microsomal incubations of acetaminophen and the nucleophile. Chromatography: 15 cm μ-C_{18} column. Acid conditions: 40/5/28/400, 1 M CH_3COOH/1 M CH_3COONH_4/CH_3OH/H_2O. Neutral conditions; 10/35/25/400. $E = +0.75$ V.

+ In these cases the level of unmetabolized acetaminophen in the microsomal incubation was reduced by an ethyl acetate extraction prior to analysis.

‡ One reaction product of NAPQI was not found in these incubations.

ucts are almost certainly adducts like that shown in Fig. 1. For example, the major product peak in a reaction with cysteine has been identified to be 3-[5-acetamido-2-hydroxyphenyl)thiol]alanine by t.l.c. and h.p.l.c. comparisons with a synthetic sample. In addition, the major product was formed with *N*-acetyl cysteine chromatographed on cellulose and on silica, with an R_f value matching that for the previously identified mercapture metabolite [1, 12, 13]. The detection of several products being formed with these sulfhydryls contrasts with previous work wherein one product [14] or none at all was reported to be formed [15, 16]. This is probably due to a combination of factors: the simplicity of the microsomal incubation relative to urine, the superior resolution of l.c. as compared to t.l.c., and the selectivity and sensitivity of the electrochemical detector.

Acknowledgements—This work was supported by the National Institute of General Medical Sciences and the National Science Foundation. We wish to thank Dr. G. P. Carlson for his assistance with the microsomal studies, and McNeil Laboratories for their gift of the cysteine conjugate of acetaminophen.

REFERENCES

1. D. J. Jollow, S. S. Thorgeirsson, W. Z. Potter, M. Hashimoto and J. R. Mitchell, *Pharmacology* **12**, 251 (1974).
2. M. W. Gemborys, G. W. Gribble and G. H. Mudge, *J. med. Chem.* **21**, 649 (1978).
3. K. Healey, I. C. Calder, A. C. Yong, C. A. Crowe, C. C. Funder, K. N. Ham and J. D. Tange, *Xenobiotica* **8**, 403 (1978).
4. R. Adams and W. Reifschneider, *Bull. Soc. chim. Fr.* 23, (1958).
5. R. F. Nelson, in *Technique of Electroorganic Synthesis* (Ed. N. Weinberg), pp. 637–43. John Wiley, New York (1974)
6. B. R. Clark and D. H. Evans, *J. electroanal. Chem.* **69**, 181 (1976).
7. R. E. Sioda and W. Kemula, *Electrochim. Acta* **17**, 1171 (1972).
8. P. T. Kissinger, *Analyt. Chem.* **49**, 227A (1977).
9. D. J. Miner and P. T. Kissinger, *J. pharm. Sci.* **68**, 96 (1979).
10. D. Hawley and R. N. Adams, *J. electroanal. Chem.* **10**, 376 (1965).
11. K. T. Finley and L. K. J. Tong, in *The Chemistry of the Carbon-Nitrogen Double Bond* (Ed. S. Patai), pp. 680–687. John Wiley, New York (1970).
12. R. S. Andrews, C. C. Bond, J. Burnett, A. Saunders and K. Watson, *J. Int. med. Res.* **4**, (suppl. 4), 34 (1976).
13. M. Davis, C. J. Simmons, N. G. Harrison and R. Williams, *Q. Jl Med.* **45**, 181 (1976).
14. A. R. Buckpitt, D. E. Rollins, S. D. Nelson, R. B. Franklin and J. R. Mitchell, *Analyt. Biochem.* **83**, 168 (1977).
15. F. D. Harvey and T. E. Levitt, *J. Int med. Res.* **4**, (suppl. 4), 130 (1976).
16. D. Labadarios, M. Davis, B. Portmann and R. Williams, *Biochem. Pharmac.* **26**, 31 (1977).