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AUTOMATED METHOD FOR STUDYING THE HEPATIC METABOLISM OF ANILINE, ETHYLMORPHINE OR *p*-NITROANISOLE

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Abstract—A method for the study of drug metabolism in a continuous flow-through system is described. The system used for measuring the metabolism of aniline is presented in detail. Modifications of the system will allow the production of formaldehyde (HCHO) or *p*-nitrophenol to be studied.

The system requires 100% oxygen, a water bath at 37° for incubation of the sample, a dialysis system for resolution of the protein phase, and the appropriate color-forming system maintained at the proper temperature. Reproducibility is within ± 1 per cent transmission for a given sample.

Manual and automated methods for studying the hepatic metabolism of aniline are compared.

AN ASSAY for the oxidative metabolism of aniline has been automated from the incubation stage through quantitative analysis of the metabolite, *p*-aminophenol. Dialysis has replaced ether extraction; time for measurement of enzyme activity has been reduced to 20 min for one sample plus about 2 min for each additional sample or duplicate; and transfer steps have been completely eliminated, resulting in better reproducibility.

EXPERIMENTAL

Materials

1. 100% oxygen
2. 1 N NaOH containing 5% phenol; make fresh for each experiment
3. 0·1 M phosphate buffer, pH 7·4.
4. Co-factor substrate solution:
   4·0 μmoles NADP
   50·0 μmoles glucose 6-phosphate
   20·0 μmoles nicotinamide
   25·0 μmoles $MgCl_2$
   Substrate:
   10·0 μmoles aniline-HCl or
   5·0 μmoles ethylmorphine-HCl
   The above are made up to 4·0 ml with 0·1 M phosphate buffer. If the substrate is *p*-nitroanisole, the volume is 3·9 ml and 10·0 μmoles *p*-nitroanisole in 0·1 ml acetone is added to the incubation mixture as described in 6 below.

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5. Rat liver homogenate:
   Five grams of rat liver were placed in a vessel containing 10 ml of 1·15% KCl and homogenized for 30 sec with the Ultra-Turrax homogenizer. The homogenate was then centrifuged for 20 min at 9000 *g* and the supernatant fraction decanted. This supernatant fraction was used as the source of enzyme and after dilution each ml contained the soluble portion of 333 mg whole liver or 333 mg whole liver equivalents (WLE). It was maintained on ice until used.
6. Incubation mixture:
   The incubation mixture was made by mixing 4·0 ml of the cofactor substrate solution (3·9 ml when *p*-nitroanisole was the substrate) with 1·0 ml of the homogenate

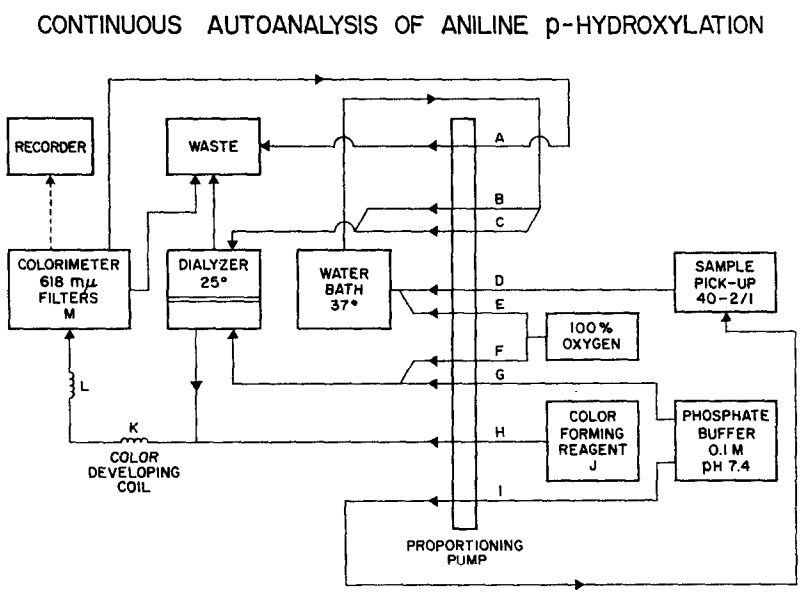

FIG. 1. Continuous autoanalysis of aniline *p*-hydroxylation. Colors are identification codes for Auto-Analyzer tubing, corresponding to the i.d. of the manifold tubing. (A) Blue–blue, i.d. 0·065. From debubbler to waste via proportioning pump. (B) Yellow–yellow, i.d. 0·056. Debubbler, rebubbler between water bath and dialysis. Used for pulse suppression. (C) Blue–blue, i.d. 0·065. Used for pulse suppression between water bath and dialysis. (D) Blue–blue, i.d. 0·065. Sample pickup. (E) Yellow–yellow, i.d. 0·056. Oxygen bubbler. Oxygen: sample ratio 0·31 on volume: volume basis. (F) Yellow–yellow, i.d. 0·056. Oxygen bubbler for buffer dialysis. (G) Blue–blue, i.d. 0·065. Buffer pickup. Continuous flow to dialyzer. (H) Orange–orange, i.d. 0·035. Color-forming reagent pickup. Color-forming reagent is diluted approximately 1:4·2 by buffer flow at color-forming coils. (I) Purple–purple, i.d. 0·081. From buffer reservoir to sample pickup reservoir. Maintains continuity of buffer between samples and during wash cycle. (J) Color-forming reagent. May be, 1 N NaOH containing 5% phenol for the analysis of aniline metabolism. May be NASH reagent as described elsewhere for the measurement of HCHO. May be 1 N NaOH for the measurement of *p*-nitrophenol formation. (K) Color developing coil. Contains 72 turns and is maintained in air for the measurement of aniline metabolism and the metabolism of *p*-nitroanisole. Coils are placed in water bath at 60° for color formation when HCHO is measured. (L) Air cooling coils. These are optional for measuring aniline and *p*-nitrophenol. They are essential for the cooling of the 60° material in the HCHO determination. The air coils have 12 turns, and serve to contract the oxygen bubbles after they emerge from the water bath. If the oxygen bubbles are not contracted, the debubbler may overload. (M) Colorimeter. 618 mμ filters are used for determining *p*-aminophenol production from aniline metabolism. 412 mμ filters are used for determining both HCHO and *p*-nitrophenol. All components with the exception of two water baths and the oxygen feed system were purchased from Technicon Corp. Water baths were from Cenco.

supernatant. Tissue concentration was 67 mg/ml in terms of whole liver. Approximately 1·5 ml of the incubation mixture was placed in a 2·0-ml sample cup, and the cup placed on the turntable of the sample changer. The remaining portion of the mixture was kept on ice until the next sample was to be inserted. Following this procedure, there was little or no difference in metabolism between the first and second sample.

7. Standard solutions:
   p-Aminophenol, formaldehyde (HCHO) or *p*-nitrophenol standards were prepared at 10 μg/ml in distilled water.
   p-Aminophenol and HCHO were prepared fresh immediately before each experiment.
8. The Auto-Analyzer system employed is described in Fig. 1 and in the accompanying legend.

Methods

Samples to be assayed, or standard solutions, were placed on the Auto-Analyzer sample turntable. The incubation mixture was picked up at the rate of 40 samples per hour with a 2:1 sample wash ratio. A buffer cup was interspersed between samples to allow better clearing of the preceding sample. Rate of pickup was 1·5 ml/55 sec. Air-bubbles were replaced by 100% O_2 with an oxygen to sample ratio of approximately 0·31 on a unit volume basis. The sample was incubated in a time delay coil at 37° for 10·5 min. To eliminate pulses, material was run again through the proportioning pump before going to the dialyzer. Dialysis was through a standard membrane at 25° against 0·1 M phosphate buffer, pH 7·4, maintained at an identical flow rate. The addition of 1 N NaOH containing 5% phenol to the diffusate–buffer mixture initiated color formation. Color formation was complete for *p*-aminophenol in less than 5 min, but time allowed was 7 min in order to accommodate other systems. Absorption was measured at 618 mμ.

Operating procedure

The system was run with buffer and phenol reagents until it reached equilibrium, normally about 30 min. Samples containing known amounts of *p*-aminophenol with or without tissue were introduced to the system via the sample changer. When metabolism was studied, the complete incubation mixture was prepared at 0° and placed in the sample changer only a few moments before sample pick-up. This procedure yielded less than 1 per cent transmission difference between duplicates or triplicates of the same sample.

It was necessary to deviate from the automatic pickup system when the influence of different alkaline mixtures and different phenol mixtures was examined. In this case a continuous stream of *p*-aminophenol solution (10 μg/ml) was pumped through the system and the solutions of alkaline-phenol were changed as indicated in the following section. No peak was formed, but rather a straight line at some elevated absorbance.

RESULTS

The standard curve for *p*-aminophenol is essentially linear. Color formation is completed in less than 5 min with the concentrations of NaOH and phenol used in

the automated system (Fig. 2) compared with 30 min with the ether extraction procedure. Since the color formation of *p*-aminophenol is not linear with time, there is need for either completeness of color formation or exact timing of color measurement. The automated system provides both.

In developing a useful method for continuous flow analysis of discrete samples of *p*-aminophenol, the first experiments were devoted to finding those components that would allow maximum sensitivity within the system. Table 1 shows the results

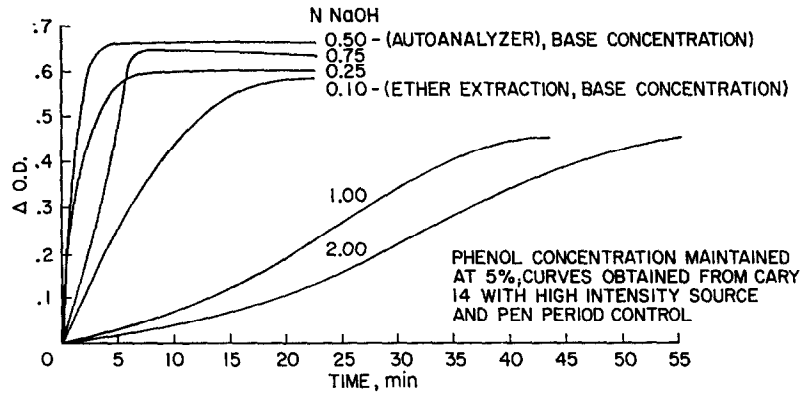

FIG. 2. Time course of color formation for *p*-aminophenol in an alkaline medium containing 5% phenol as the color-forming catalyst. Measurements were made with a Cary 14 dual beam recording spectrophotometer equipped with high intensity source, and pen period control. The proper mixture of NaOH containing 5% phenol was placed in both reference and sample cuvettes. Zero absorbance baseline was established and the machine switched to a nonscanning condition at 618 mμ. One ml *p*-aminophenol (10 μg/ml) was introduced into the sample cuvette resulting in a final concentration of 2·25 μg/ml. The recorder was quickly switched on and the color formation followed as described above. The addition of the *p*-aminophenol and the switching on of the recorder required normally 3–5 sec. In only one case of extremely rapid color formation did the pen leave the baseline immediately upon turning on of the recorder. All additions of *p*-aminophenol were from the same stock solution.

TABLE 1. INFLUENCE OF BASE AND PHENOL CONCENTRATIONS ON COLOR FORMATION*

Base concentration	Per cent phenol concentration	Δ O.D.
5 N NaOH	5	0·055
2 N NaOH	5	0·450
1 N NaOH	5	0·460
0·5 N NaOH	5	0·460
0·5 N NaOH	1	0·415
0·1 N NaOH	5	0·014
0·5 M Na_3PO_4	5	0·460
0·5 M Na_3PO_4	1	0·421

* All samples contained 10 μg/ml *p*-aminophenol in H_2O.

of those experiments. Maximum color developed in the system when the concentration of NaOH was greater than 0·5 N and less than 2·0 N before mixing with the diffusate. A dilution of the NaOH–phenol 1:4·2 with the phosphate buffer of the diffusate gives an NaOH concentration between 0·119 and 0·476 N. The effectiveness of this range is

supported by Fig. 2 which indicates that a concentration of 0·5 N NaOH yields optimal color formation.

Only two phenol concentrations were reported since no further color development occurred with phenol concentrations in excess of 5 per cent. Using 1% phenol as in the ether extraction procedure[1] does not produce optimum results in the automated system described here.

The color forming reagent selected for experimental use was 1 N NaOH containing 5% phenol.

Recovery of *p*-aminophenol from tissue ranged from 80 per cent to greater than 100 per cent depending upon the tissue concentration, the age of the animals and the species being studied. Recovery studies should be a routine part of each experiment to eliminate recovery as a potential variable.

Initially, the product of the oxidative metabolism of aniline appeared as small twin peaks as shown in Fig. 3, even though successful recovery of *p*-aminophenol had been achieved. The system did not respond to increased cofactor concentrations or

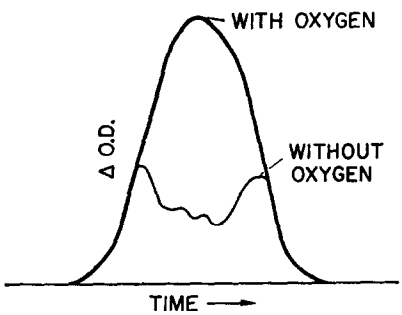

FIG. 3. System with or without oxygen supplement. Single peak is with oxygen in the system. Twin peaks are formed in the system without oxygen; they represent diffusion of oxygen from buffer into sample. Twin peaks also occur with very high tissue concentrations.

increased tissue concentrations. The possibility that one of the peaks might represent color formed from *o*- or *m*-aminophenol was investigated and found negative. The enzymes were simply not metabolizing aniline except at the ends of the sample.

The peaks coincided with the sample extremities, and so it was reasoned that the buffer might have contributed to metabolism by permitting some factor to diffuse out or permitting another factor to diffuse in. Since Fouts[2] had been able to increase drug metabolism by streaming oxygen over a system *in vitro*, oxygen was added to the automated system.

The problem of introducing pure oxygen into the system without distortion was solved by using a water gravity pump as illustrated in Fig. 4. The flask containing oxygen is a closed system, and so a small but consistent pressure can be maintained. Too much pressure could distort the system and disrupt the bubble pattern, overload the debubbler and ultimately cause pen noise. The introduction of 100% oxygen to the system, replacing air bubbles, produced symmetrical peaks which were quite reproducible. The oxygen to sample ratio was about 0·31 (v/v).

Having ascertained that the assay of aniline oxidizing enzymes could be carried out with a continuous flow system, a dual experiment was prepared for comparing

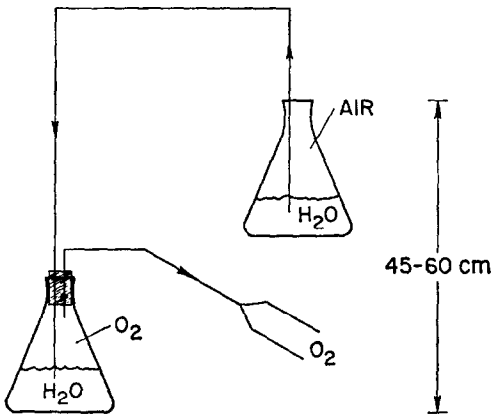

FIG. 4. Method for introducing oxygen into system without distortion of bubble pattern.

TABLE 2. EXPERIMENTAL COMPARISON OF ETHER EXTRACTION WITH AUTO-ANALYSIS*†

Pretreatment	Rat	Ether extraction	Group average	Auto-analysis	Group average	A.A.‡ / E.E.
Control	1	0·0325	0·0339	0·0260	0·0273	0.800
	2	0·0348		0·0290		0·830
	3	0·0343		0·0270		0·780
48 hr fast	4	0·0479	0·0495	0·0410	0·0399	0·856
	5	0·0551		0·0423		0·768
	6	0·0456		0·0363		0·796
Phenobarbital 100 mg/kg × 2	7	0·0818	0·0636	0·0642	0·0497	0·784
	8	0·0588		0·0446		0·758
	9	0·0504		0·0404		0·801

* Values are expressed as millimicromoles of aniline metabolized per milligram of whole liver equivalents per minute.

† Animals were male rats weighing about 300 g. The same cofactor and substrate solutions (as described in Methods) were used for both procedures; protein concentrations were also identical. The ether extraction method was essentially as described by Guarino *et al.*[1] except that the alkaline phase contained 5% phenol (w/v). Values for the ether extraction method are averages of duplicates; Auto-Analyzer values are averages of triplicates.

‡ A.A.—Auto-analysis : ether extraction ratio. $\frac{\text{A.A.}}{\text{E.E.}}$

the automated system with ether extraction. Table 2 indicates that on the basis of mμmoles/mg whole liver equivalents/min, the metabolism measured on the flow-through system was approximately 80 per cent of the ether extraction value. The difference is due to recovery and can be corrected for the automated system. A strict comparison of the values is difficult since the two systems are very different. An important factor is the relationship between control and experimental animals. Regardless of the method used, the ratio should not change, and in Table 2 it does not.

Although metabolism continued to increase with increasing tissue concentration

up to 134 mg whole liver equivalents (WLE)/ml, the most effective tissue concentration was between 40 and 60 mg WLE/ml. The latter range of tissue concentration preserves a margin for measuring increases in metabolism without overriding the cofactor oxygen availability. Beyond 134 mg WLE/ml, the formation of *p*-aminophenol decreased. Cofactors could not be maintained at optimal levels, and a typical oxygen deficiency curve with twin peaks was recorded.

DISCUSSION

Applications

A brief literature survey[1,3–6] indicates that only infrequently is a single drug metabolism pathway studied. More often two or more systems are studied. The system just described for measuring the metabolism of aniline is very flexible in that respect; with certain minor modifications it is possible to study the production of formaldehyde from a number of different substrates or the production of *p*-nitrophenol from *p*-nitroanisole.

Determination of formaldehyde

Components described in Fig. 1 are used. Color formation is achieved by a modification of the Nash procedure[7] with dialysis replacing protein precipitation. The color forming Nash Reagent is modified as follows: 150 g ammonium acetate, 3 ml glacial acetic acid, 2 ml acetylacetone, and q.s. to 400 ml with distilled H_2O. This concentration is approximately 20% stronger than that described by Stitzel *et al.*[7]

Color-forming coils were placed in a water bath at 60°. Time for color formation was limited to 6 min. After the heating bath, the colored material was passed through a 1-min air cooling coil before proceeding to the debubbler and the flow-cell. Cooling prevented overloading the debubbler by contracting the gas bubbles.

The colorimeter contained 412 mμ filters, and 2 μg/ml HCHO in the sample changed the optical density 0·123 units. Recovery of HCHO varied depending upon the specific animal investigated, but was normally about 80 per cent. As in the case of *p*-aminophenol, recovery studies should be a routine part of each experiment.

Both ethylmorphine and *p*-nitroanisole were employed as substrates to measure the production of HCHO from *N*- and *O*-dealkylation, respectively.

p-*Nitrophenol determination*

Major components are described in Fig. 1. The color-forming reagent is 1 N NaOH, and 412 mμ filters were used. In alkaline solutions *p*-nitrophenol forms a yellow color with a relatively wide band of absorption. In this assay dialysis replaces extraction with ether or another organic solvent. *p*-Nitrophenol (5 μg/ml) changes the optical density 0·075 units. Recovery is between 80 and 90 per cent depending upon the animals studied. With high concentrations of *p*-nitrophenol, the sample diffuses into the separating buffer wash, causing the baseline to shift. Lengthening the buffer wash or perhaps separating the samples by more than one buffer cup might stabilize the baseline, but each additional cup increases time by 2 min. The best approach is to eliminate the shift mathematically.

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