Nov. 5, 1974 JAMES C. FLETCHER 3,846,243
ADMINISTRATOR OF THE NATIONAL AERONAUTICS
AND SPACE ADMINISTRATION
AUTOMATED ANALYSIS OF OXIDATIVE METABOLITES
Filed Aug. 18, 1972 4 Sheets-Sheet 1

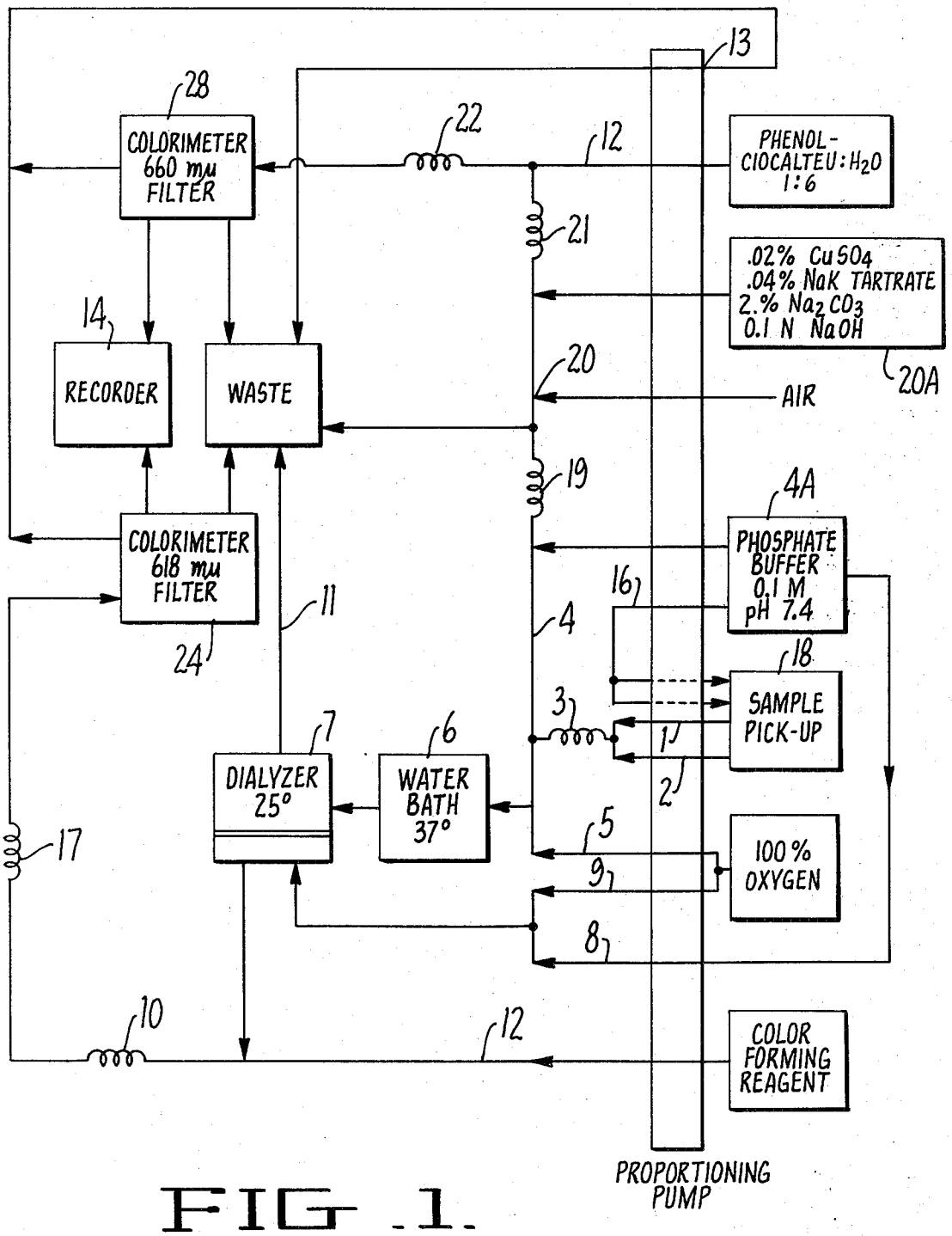


FIG. 1.

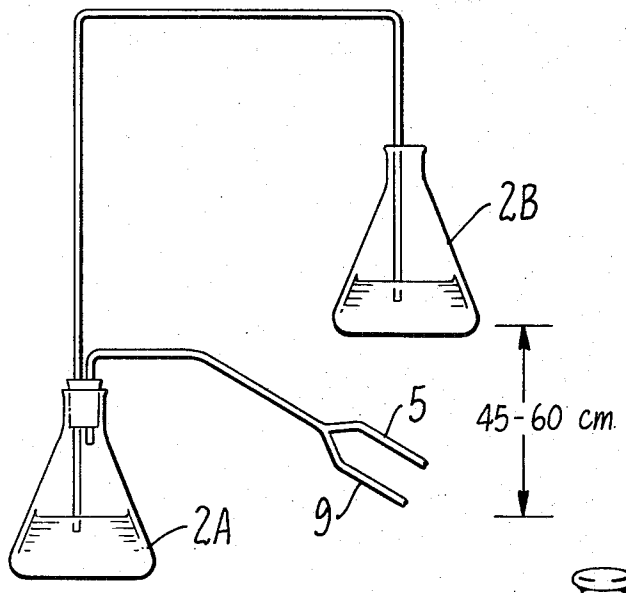


FIG. 2.

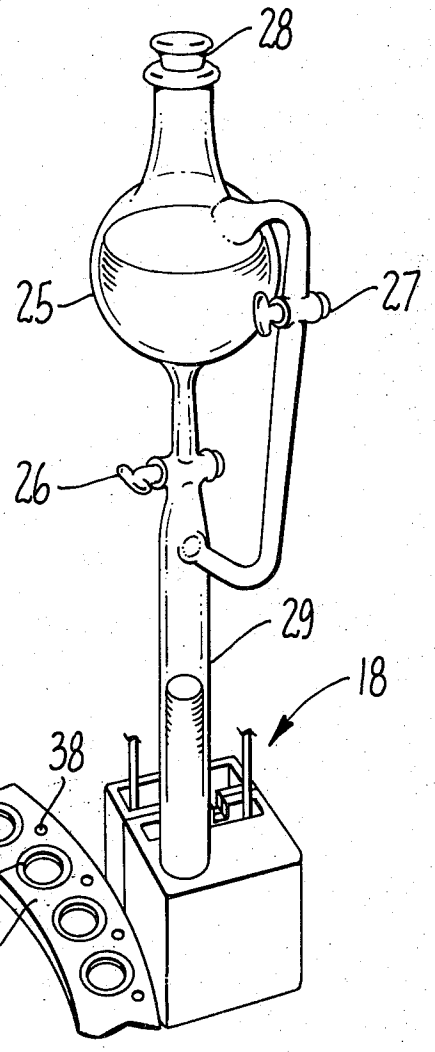


FIG. 3.

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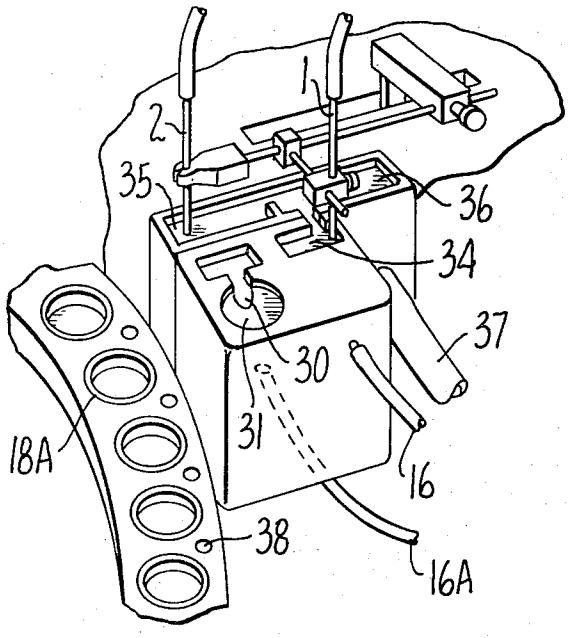


FIG. 4A.

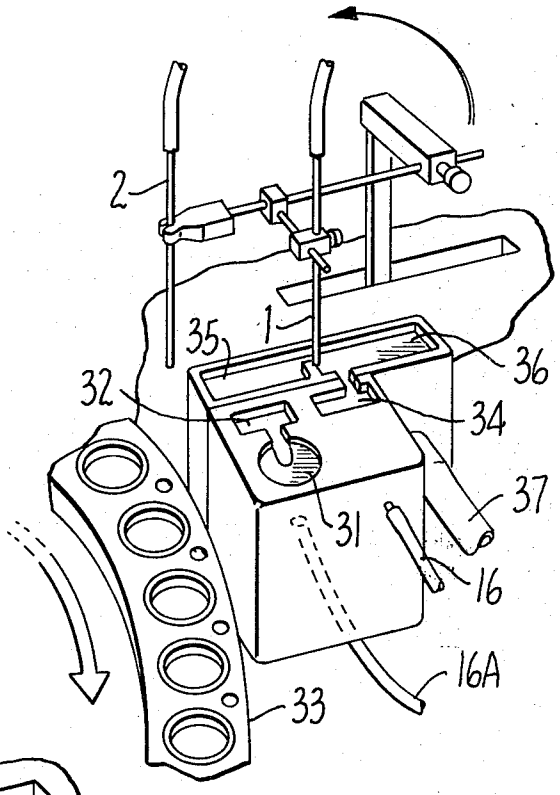


FIG. 4B.

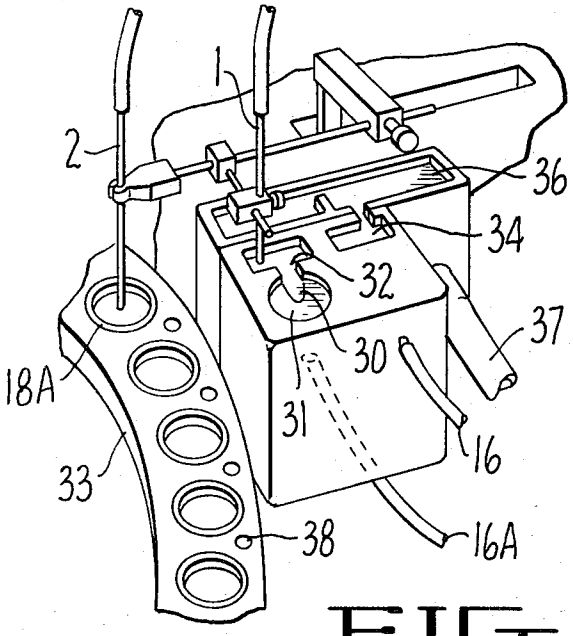


FIG. 4C.

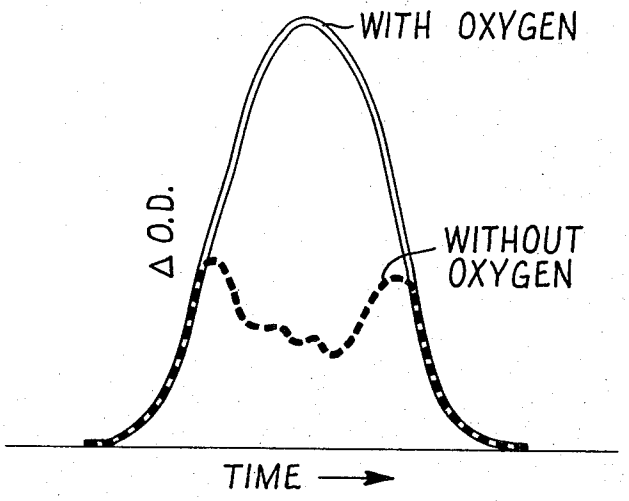


FIG. 5.

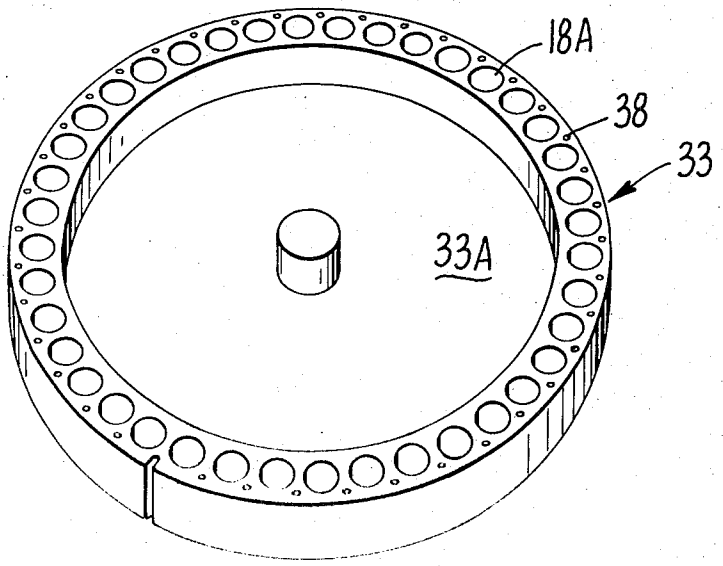


FIG. 6.

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3,846,243

AUTOMATED ANALYSIS OF OXIDATIVE METABOLITES

James C. Fletcher, Administrator of the National Aeronautics and Space Administration, with respect to an invention of Raymond L. Furner, Birmingham, Ala.

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Int. Cl. C12k 1/00
U.S. Cl. 195—103.5 R 6 Claims

ABSTRACT OF THE DISCLOSURE

An automated system for the study of drug metabolism is disclosed. The system monitors the oxidative metabolites of aromatic amines and of compounds which produce formaldehyde on oxidative dealkylation. It includes color developing compositions suitable for detecting hydroxylated aromatic amines and formaldehyde.

The invention described herein was made by a Research Associate of National Academy of Sciences while under contract with the United States Government and may be manufactured and used by or for the Government for governmental purposes without the payment of any royalties thereon or therefor.

BACKGROUND OF THE INVENTION

Field of the invention

An automated system for the study of drug metabolism.

Description of the prior art

The metabolism of aniline to p-aminophenol by oxidative microsomal enzymes has been studied in considerable detail by numerous investigators. Previous methods for studying oxidative metabolism required that the enzyme, substrate and cofactors be mixed in a flask and placed in a constant temperature water bath for incubation. Time of incubation was generally an arbitrary unit varying from 5 minutes to an hour or more depending on the substrate.

Termination of the oxidative metabolism was accomplished by extracting the metabolite with an organic solvent. The solvent also inactivated the oxidative enzymes.

An aliquot of the organic solvent was transferred to another vessel containing an alkaline mixture containing phenol. The metabolite was then re-extracted into the alkaline medium containing phenol to form a blue color with an absorption maximum near 620 mμ. Although this extraction procedure has been made more sensitive by increasing the phenol concentration in the alkaline extraction medium, it has certain inherent inconveniences and disadvantages; namely, it requires an ether extraction; it is time consuming (1½ hours for a single sample) and it requires at least four individual transfers or additions of reagent. These numerous steps increase the potential for error. Working with organic solvents is both hazardous and unpleasant.

With these points in mind an assay for the oxidative metabolism of aniline has been automated from the incubation stage through quantitative analysis by color formation of the metabolite, p-aminophenol.

To measure the formation of formaldehyde from substrates which are oxidatively N-demethylated or O-demethylated, the oxidative enzymes were first inactivated and precipitated by $ZnSO_4$; an equimolar amount of $Ba(OH)_2$ was used to precipitate the excess $ZnSO_4$, and the mixture was centrifuged to remove the precipitated protein and the zinc and barium salts. An aliquot of the supernatant was then mixed with a special color forming reagent, commonly called Nash Reagent, and incubated for 30 minutes at 60° C. in order to assure maximum color development and maximum color stability. The

colored mixture had an absorption maximum near 412 mμ.

The prior art method for measuring the formaldehyde had a number of inherently inconvenient and disadvantageous procedures; namely it required the use of heavy metals for the precipitation of proteins; it was time consuming, requiring about 1½ hours for a single sample; it required at least five individual transfers or additions of reagents; it required a water bath for incubation during the color forming phase, and close attention to the duration of the color forming phase. These numerous steps increase the potential for error. The use of heavy metals is messy at best since special care is required in the cleaning of the analytical glassware.

Following a similar procedure to that described herein for the measurement of p-aminophenol, an automated assay has been developed for the measurement of formaldehyde; the assay begins with sample constitution in the incubation stage and proceeds through the analysis by colorimetric techniques.

In these analyses dialysis has replaced ether extraction and protein precipitation; transfer steps have been completely eliminated; a method for using a minimal amount of valuable cofactor has been provided; an aluminum sample plate has been devised that keeps the enzyme suspension cold until analysis actually is begun; the time between the start of incubation and recording of results has been made uniform; time required for one analysis has been reduced to 20 minutes plus 2 minutes for each additional sample or duplicate. A multiplicity of operations can be performed inexpensively, accurately, with a high degree of reproducibility and with the results automatically recorded while the analyses are being performed.

Initially, the product of the oxidative metabolism of aniline appeared as small dual peaks (FIG. 6), even though successful recovery of p-aminophenol as a single peak had been achieved. The system did not respond to increased cofactor concentrations or increased protein 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 metabilizing aniline to p-aminophenol except at the ends of the sample.

The peaks coincided with the sample extremities, and so it was reasoned that lack of sufficient oxygen might be responsible for reduced metabolism in the middle portion of the sample. The replacement of air bubbles with 100% oxygen, using an oxygen to sample ratio of 0.31 by volume yielded symmetrical, reproducible peaks.

The problem of introducing pure oxygen into the system without distortion of the system was solved by using a water gravity pump. The flask containing oxygen is a closed system with a water head of 45–60 centimeters, so that a small but constant pressure can be maintained. Too much pressure could distort the system and disrupt the bubble pattern, overload the de-bubbler and ultimately cause pen noise.

The problem of determining the protein content in the incubation mixture was solved by splitting the sample after exit from the mixing coil and diverting a portion of the sample for automatic dilution and analysis by a modification of the Lowry method.

SUMMARY OF THE INVENTION

The present invention relates to a system for the automatic analysis of drug metabolites which consists of mixing the drug with an amount of enzyme and cofactor in the presence of 100% oxygen to form the metabolite, dialyzing the so-formed solution against phosphate buffer and developing color by adding a suitable reagent to the recipient stream of the dialysis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic flow diagram illustrating a novel system for automatically analyzing drug metabolites and protein content in incubation mixtures.

FIG. 2 is a side view of a water gravity pump for introducing 100% oxygen at a low, steady pressure into the automated system in FIG. 1.

FIG. 3 is a perspective view of a gravity fed cofactor dispensing system.

FIGS. 4A, 4B and 4C illustrate the dual pipette pickup assembly in action. FIG. 4A shows the resting buffer pickup position. FIG. 4B shows both pipettes raised out of the buffer. FIG. 4C illustrates the enzyme-cofactor pickup position.

FIG. 5 is a plot of peaks in systems with and without oxygen representing diffusion of oxygen from buffer into sample.

FIG. 6 is a perspective view of a solid aluminum sample plate.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to a procedure for monitoring the hepatic metabolites of aromatic amines such as aniline and compounds which form formaldehyde on oxidative dealkylation such as ethylmorphine and p-nitroanisole.

The system as shown schematically in FIG. 1 is first flushed with buffer which is introduced through pipettes **1** and **2** and with phenol solution through line **12** until it reaches equilibrium, normally about 30 minutes. To calibrate the system, samples containing 10 μg./ml. of freshly prepared p-aminophenol or formaldehyde in distilled water (with or without protein) are introduced into the system via pipette **2** from a conventional sampler system **18**, later described in detail. In the initial phase of development when metabolism was studied the complete incubation mixture was previously prepared in an ice bath near 0° C. and placed in sample cups on the sample plate **18A** a few minutes before sample pickup. The preparation and constitution of the incubation mixture for the developmental phase is described next.

The rat liver homogenate used in the incubation mixture was prepared by placing five grams of rat liver in a vessel containing 10 ml. 1.15% KCl and homogenized for 30 seconds with a conventional homogenizer. The homogenate was then centrifuged for 20 minutes at 9,000× gravity and the supernatant decanted. This supernatant was used as the source of enzyme, and after dilution, each ml. contained the soluble portion of 333 mg. whole liver or 333mg. whole liver equivalents (WLE). It was maintained on ice until used.

The cofactor-substrate solution contained: 4.0μ moles NADP, 50.0μ moles glucose-6-phospate, 20.0μ moles nicotinamide, 25.0μ moles $MgCl_2$, 10.0μ moles aniline·HCl or 5.0μ moles ethylmorphine·HCl. The above was made up to 4.0 ml. with 0.1 M phosphate buffer. If the substrate was p-nitroanisole, the volume was 3.9 ml. and 10.0μ moles p-nitroanisole in 0.1 ml. acetone was added.

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 supernatant. Tissue concentration was 67 mg./ml. in terms of while 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 a 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 less than 1% transmission difference between duplicates or triplicates of the same sample.

In the preferred procedure, after equilibrating the system in FIG. 1, as described above, the cofactor-substrate part of the sample is picked up in line **1** of the sample pickup system **18** from a specially designed trough supplied by a feed mechanism as is later described in detail in conjunction with FIG. 4. The enzyme is picked up by the second line **2** from the sampler system **18**. The two parts of the sample pass over the platen of the proportion pump in the ratio 4225:1225 cofactor-substrate to enzyme respectively and then pass through a mixing coil **3** to assure homogeneity.

After exiting from the mixing coil **3** the sample is split. One portion of the sample, equal to the original volume of the enzyme alone, is reacted with Lowry's reagents to colorimetrically determine the protein content. The remaining portion enters the water bath **6** where incubation occurs. The protein analysis is described first.

The protein analysis sample is diverted through line **4**, automatically diluted approximately 100 times with phosphate buffer from source **4A** and mixed in coil **19** (30 turns). A portion of this sample is aerated at **20** and reacted with Lowry's reagents in two steps, the remaining portion going to waste. The first step consists of reaction with a solution containing 0.02% $CuSO_4$, 0.04% sodium potassium tartrate and 2% Na_2CO_3 in a solution which is 0.1 N with NaOH from source **20A** followed by mixing in coil **21** (24 turns). In the second step the sample is reacted with phenol-ciocalteu-water 1:6 from line **12**, passed through the reaction coil **22** (84 turns) and the colored solution is moved on the colorimeter **28** with 660 mμ filters, where the air bubbles are removed by exhaust action of the proportioning pump **13** and the colored material is forced through a flow cell with a 1.5 cm. light path. Percent transmittance is measured by a photomultiplier tube and recorded on a strip chart **14**.

The remaining portion of the original sample, after the portion for protein analysis has been diverted, is oxygenated from line **5** by bubbling in 100% oxygen from the water gravity pump later described in detail in FIG. 2. The oxygen separates the sample into segments, facilitates mixing, helps to eliminate deposit formation in the lines, and insures uniform reaction of the sample with enzyme. Oxygen to sample ratio is about 0.31 by volume.

The oxygenated sample moves into an incubation coil in water bath **6** maintained at 37° C. Time in the coil is about 10.5 minutes (time depends upon coil length and flow rate). During the period the substrate is metabolized the oxygenated sample containing the metabolite is forced through the continuous flow dialyzer **7** maintained at 25° C. and equipped with a standard membrane.

Dialysis is against oxygenated buffer provided by lines **8** and **9** with a flow rate identical to the oxygenated sample. The metabolite dialyzes across the membrane is carried out of the dialyzer by the buffer. The dialyzed sample containing protein exits to waste via line **11**. The dialysate containing the metabolite is reacted with a suitable color forming reagent provided by line **12** and the mixture allowed to incubate for approximately 6 minutes in reaction coil **10** and 1 minute in coil **17**.

The color forming reagent is selected according to the metabolite being analyzed. p-Aminophenol requires IN-NaOH containing 5% phenol to form a blue color at room temperature with an absorption maximum near 620 mμ, while formaldehyde requires a reagent prepared as follows: 150 gm. ammonium acetate, 3 ml. glacial acetic acid, 2 ml. acetylacetone diluted to 400 ml. with distilled water. When combined with formaldehyde this reagent yields a bright yellow color with an absorption maximum near 412 mμ. In the analysis of formaldehyde the color forming coils **10** are placed in a water bath at 60° C. Time for color formation is about 6 minutes. After the heating bath the colored material is passed through the air-cooled coil **17** before proceeding to a de-bubbler and the flow cell in the colorimeter **24**. Cooling prevents overloading the de-bubbler by contracting the gas bubble. Percent transmittance is measured by a photomultiplier tube and recorded on a strip chart recorder **14**. Concentration of the metabolite is proportional to the log of the peak height.

In FIG. 2 there is shown a method for introducing oxygen into the system without distortion of bubble patterns. This consists of a water gravity pump having a flask 2A with the flask about half filled with water and with pure oxygen standing over the water. Additional water can now be introduced by siphon action from flask 2B so that oxygen from flask 2A is forced out through the lines 5 and 9. In this way a very gentle but controlled positive pressure can be maintained on the oxygen lines so that bubbles are introduced into the system in a regular and uniform fashion.

FIG. 3 illustrates a gravity fed dispenser for cofactor-substrate which feeds into the dual pipette pickup assembly shown in FIGS. 4A, B and C, both of which are at the sample pickup location 18 in FIG. 1. Cofactor-substrate solution is placed in the 1000 ml. container 25 equipped with a stop-cock 26 for passage of liquid, a stop-cock 27 in the internal pressure equilibration arm, an air tight stopper 28 and a 4-inch delivery barrel 29. After the delivery barrel 29 is placed in opening 30 (FIG. 4) and allowed to rest on the ledge 31, the stop-cocks 26 and 27 are opened allowing cofactor-substrate solution to spill into trough 32. As the level of liquid rises an equilibrium of hydrostatic pressure is reached which prevents further flow from 25 until the liquid level is decreased by withdrawal through pipette 1. Thus, the reservoir is continually refilled, but does not overflow; the cofactor-substrate is used only for each sample and is not continuously introduced into the system.

Pipette 1 is in a continual exhaust state and therefore it must be in liquid to avoid introduction of air bubbles into the system. When it is not in the cofactor-substrate trough 32, it is in the buffer trough 34.

Pipette 2 is in buffer trough 35 or in a sample cup 18A in the sample changer. Buffer is presented to trough 34 by inflow line 16. Excess buffer overflows into spillover chamber 36 and hence through the waste exit 37. The buffer supply 16A to trough 35 is standard on existing equipment and is partially obscured by the cofactor substrate-buffer reservoir.

The sequence of steps is clearly shown in FIGS. 4A, 4B and 4C which show the successive stages of the movements of the two pipettes.

The sample plate 33 (see also FIG. 6) is made of solid aluminum for high thermal conductivity. The center well 33A of the aluminum plate is deep enough to accept ice, frozen CO_2, or other material to maintain a desirable temperature in the sample cup 18A. When cooled with ice, the solutions in the sample cup are maintained between 0.5 and 1.5° C. A microswitch lever on the sample changer is bent so that the sample plate turns freely until a stop pin in a stop pin hole 38 engages the microswitch lever and interrupts the cycle.

FIG. 5 shows the results obtained with oxygen and without oxygen. The broken line shows the result without oxygen wherein the oxidative metabolism of aniline appeared as small twin peaks. The system did not respond to increased cofactor concentrations or increased tissue concentrations. The peaks coincide with sample extremities, 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. It was found that this difficulty could be solved by adding 100% oxygen to the system to replace the standard air bubbles and, as the solid line shows, a single sharp peak is achieved by the addition of oxygen. The method of adding the oxygen without distorting the bubble pattern of the system was previously described in conjunction with FIG. 2.

While I have illustrated a preferred mode of practicing the present invention, it will be understood that the invention may be practiced otherwise than illustrated here. Various changes may be made and will occur to those skilled in the art without departing from the underlying idea of this invention within the scope of the appended claims.

I claim:

1. A method for the continuous-flow analysis of oxidative metabolites of aromatic amines and of compounds which produce formaldehyde on oxidative dealkylation consisting essentially of the steps of:

(1) combining a fluid stream comprising a substrate-cofactor in a phosphate buffer with a rat liver homogenate comprising oxidative microsomal enzymes,

(2) dividing the resulting mixture into a first stream for determining the quantity of protein and into a second stream for metabolizing the substrate and for determining the quantity of metabolite generated during such metabolism,

(3) diluting said first stream with phosphate buffer,

(4) aerating said diluted first stream,

(5) reacting said aerated first stream with a color forming reagent for protein,

(6) colorimetrically determining the amount of protein,

(7) intermittently adding small aliquots of 100% oxygen to said second stream,

(8) incubating said oxygenated second stream at a controlled temperature to promote the formation of metabolite by enzyme action on said substrate,

(9) dialyzing said second stream against phosphate buffer, and

(10) colorimetrically determining the concentration of metabolite after reacting the dialysate with a color forming reagent for said metabolite.

2. The method of claim 1 wherein said substrate consists essentially of aniline.

3. The method of claim 2 wherein the metabolite color forming reagent is 1 normal NaOH containing 5% phenol.

4. The method of claim 1 wherein the substrate consists essentially of materials selected from ethylmorphine and p-nitroanisole.

5. The method of claim 4 wherein the metabolite color forming reagent consists essentialy of 150 g. ammonium acetate, 3 ml. glacial acetic acid, and 2 ml. acetylacetone in 400 ml. distilled water.

6. The method of claim 1 wherein the color forming reagent for protein consists essentially of a solution containing .02% $CuSO_4$, .04% sodium potassium tartrate, and 2% Na_2CO_3 and which is 0.1 N with NAOH mixed with a phenol-ciocalteu-water solution in the ratio of 1:6.

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