TO: KSI/Scientific & Technical Information Division  
Attn: Miss Winnie M. Morgan

FROM: GP/Office of Assistant General Counsel for Patent Matters

SUBJECT: Announcement of NASA-Owned U.S. Patents in STAR

In accordance with the procedures agreed upon by Code GP and Code KSI, the attached NASA-owned U.S. Patent is being forwarded for abstracting and announcement in NASA STAR.

The following information is provided:

U.S. Patent No.: 3,846,243

Government or Corporate Employee: Nat'L Academy of Sciences

Supplementary Corporate Source (if applicable): Nat'L Research Council, Wash., DC

NASA Patent Case No.: ARC-10,469-1

NOTE - If this patent covers an invention made by a corporate employee of a NASA Contractor, the following is applicable:

YES □  NO □

Pursuant to Section 305(a) of the National Aeronautics and Space Act, the name of the Administrator of NASA appears on the first page of the patent; however, the name of the actual inventor (author) appears at the heading of column No. 1 of the Specification, following the words "...with respect to an invention of ..."

Bonnie L. Woerner

Enclosure
FIG. 5.

FIG. 6.
AUTOMATED ANALYSIS OF OXIDATIVE METABOLITES

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

Filed Aug. 18, 1972, Ser. No. 281,908
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 m.$\mu$. 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; the solvent also inactivated the oxidative enzymes.

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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 metabolizing aniline to p-aminophenol except at the ends of the sample.

The peaks coincided with the sample extremes, 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 a 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 to 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.

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 1/4 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 metabolizing aniline to p-aminophenol except at the ends of the sample.

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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 system as shown schematically in FIG. 1.

FIG. 3 is a perspective view of a head holding, mixing, and measuring system for automatically analyzing drug metabolites and protein content in incubation mixtures. The system is calibrated with 10 ml of a solution containing 0.02% CuSO_4, 0.04% potassium tartrate and 2% Na_2CO_3 in a solution which is aerated at 20 and re-acted with Lowry's reagents in two steps, the remaining portion entering the water bath where incubation occurs. The protein analysis is described first.

The protein analysis sample is introduced 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 re-acted 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% potassium tartrate and 2% Na_2CO_3 in a solution which is aerated at 20 and passed through the reaction coil 22 (84 turns) and the colored solution is moved through a column of 660 µm 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 recorder 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. Dialysis is against oxygenated buffer provided by lines 8 and 9 with a flow rate identical to the oxygenated sample. The mixture is passed 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 is 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 nm. p-Aminophenol 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 nm. The analysis of formaldehyde is described next.
In FIG. 2 there is shown a method for introducing oxygen into the system without distorting the bubble pattern. 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 sub-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 45 is made of aluminum for high thermal conductivity. The center well 45 of the aluminum plate is deep enough to accept ice, which is 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 substitute consists essentially of materials selected from ethylmorphine and p-nitroanisole.

5. The method of claim 4 wherein the metabolite color forming reagent consists essentially 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 0.02% CuSO4, 0.04% sodium potassium tartrate, and 2% Na2CO3 and which is 0.1 N with NaOH mixed with a phenol-cloacal-water solution in the ratio of 1:6.

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