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:

Supplementary Corporate Source (if applicable):

NASA Patent Case No. : A2C-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
Automated Analysis of Oxidative Metabolites

Fig. 2.

Fig. 3.
WITH OXYGEN

\[ \Delta \text{O.D.} \]

WITHOUT OXYGEN

TIME

FIG. 5.

FIG. 6.
SUPERNATANT was then mixed with a special color forming reagent. This reagent 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 metabolizing 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 pressure 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.
The present invention relates to a procedure for monitoring the hepatic metabolism 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 with phenol solution through line 12 until it reaches equilibrium, normally about 30 minutes. To calibrate the system, samples containing 10 \( \mu \)g/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 10, 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 18; a few minutes before sample pickup. The preparation and constitution of the incubation mixture for the development 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 x g. The supernatant 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.

The cofactor-substrate solution contained: 4.0 \( \mu \) moles NADP, 30.0 \( \mu \) moles glucose-6-phosphate, 20.0 \( \mu \) moles nicotinamide, 25.0 \( \mu \) moles MgCl\(_2\), 10.0 \( \mu \) moles amine-HCl or 5.0 \( \mu \) moles ethylamine-HCl. The above was made up to 4.0 ml. with 0.1 M phosphate buffer. If the substrate was p-nitroanilide, the volume was 3.9 ml. and 10.0 \( \mu \) moles p-nitroanisole in 0.1 ml. acetic acid was added.

The incubation mixture was made by mixing 4.0 ml. of the cofactor-substrate solution (3.9 ml. when p-nitroanilide was the substrate) with 1.0 ml. of the homogenate 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 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 calibrating 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 connection 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 plates 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 diluted 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 0.025% CuSO\(_4\), 0.04% potassium tartrate and 2% Na\(_2\)CO\(_3\) in a solution which is 0.1 N with NaOH from source 20 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 through coil 23 (24 turns) and the filter 660 \( \mu \)m 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 insure 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 dialyzing from the supernatant 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 m\(\mu\), while formaldehyde requires a reagent prepared as follows: 150 gm. ammonium acetate, 3 ml. glacial acetic acid, 2 ml. acetylaconte diluted to 400 ml. with distilled water. When combined with formaldehyde this reagent yields a bright yellow color with an absorption maximum near 412 m\(\mu\). In the analysis of formaldehyde the color forming reagent is prepared in 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.

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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 solution, 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 45 of the aluminum plate is deep enough to accept ice, forming reagent consists essentially of 150 g. ammonium acetate, 3 ml. glacial acetic acid, and 2 ml. acetylacetone in 400 ml. distilled water.

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 extinctions, 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-nitroaniline.

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 .02% CuSO₄, .04% sodium potassium tartrate, and 2% Na₂CO₃ and which is 0.1 N with NaOH mixed with a phenol-cloacite-water solution in the ratio of 1:6.

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