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IMPROVED AUTOMATED METHOD FOR THE STUDY OF DRUG METABOLISM

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Abstract—The system for assaying drug metabolism by a continuous flow-through technique has been modified with the following: (1) an aluminum sample plate containing a trough for cooling, (2) a dual pickup mechanism that allows the enzyme to be picked up separately from the cofactors and substrate, (3) a dispensing apparatus that allows the release of discrete aliquots of the cofactor-substrate solution upon demand, (4) a mixing coil where the enzyme solution and cofactor-substrate solutions are mixed in precisely determined amounts for a finite period of time, and (5) a sequential dilution system that allows for analysis of total protein. The advantages of these modifications include (1) preservation of enzyme activity, (2) elimination of manual preparation of incubation mixtures, (3) uniformity of incubation time, (4) increased reliability of analyses by separation of pickup procedures, and (5) greater general usefulness of the system.

AN AUTOMATED flow-through system for studying the oxidative metabolism of aniline, ethylmorphine and *p*-nitroanisole in hepatic tissue using a Technicon Auto-Analyzer has been previously described.[1] In that system, the fraction of liver homogenate (hereafter called the enzyme solution) used was mixed manually with the appropriate cofactors and substrate, and then placed in the sample cup for automatic pickup. The incubation mixture was then mixed with 100% oxygen and incubated for several minutes at 37°. After incubation, the metabolites were separated from the incubation mixture by continuous flow dialysis, and mixed with a reagent for color formation. Color intensity was measured in a flow cell using standard interference filters, and the results were recorded on a strip chart. Determinations with this system were more rapid and more accurate than with comparable manual extraction techniques, but considerable attention was still required to mix the samples and introduce them to the system at proper intervals.

To maintain a constant time of incubation, the sample was constituted by mixing the enzyme, substrate and cofactor solutions, placing the sample in a sample cup on the sample plate, and then having the sample picked up almost immediately. If the enzyme, cofactor and substrate could be picked up from different sources, mixed, and then analyzed, the system would be almost completely automatic. Two difficulties had to be overcome. First, the enzyme solutions, the unknowns to be analysed, were placed in the sample cups on the sample plate. This plate remained at

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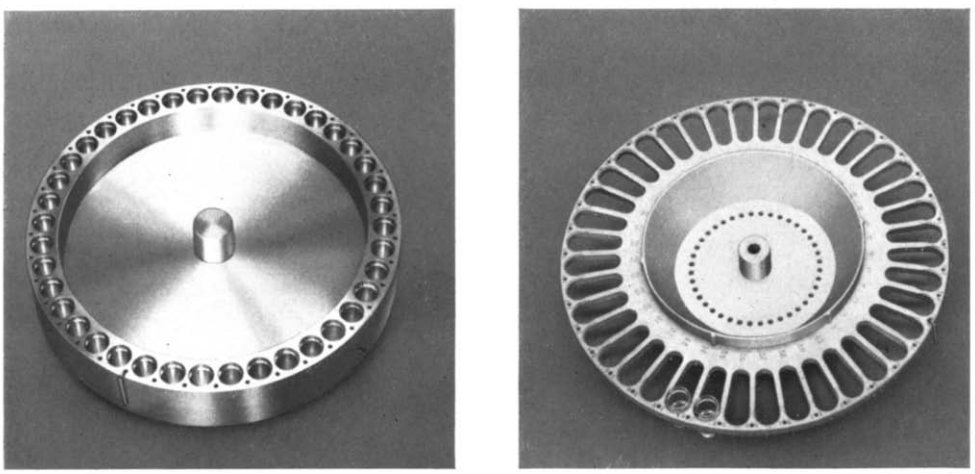
room temperature so the enzymes in the solutions lost activity in proportion to the time they were on the sample plate. Second, the cofactor solution and the substrate solutions were not varied with each sample and therefore would normally be introduced by continuous flow. However, continuous flow requires the preparation of large amounts of relatively costly compounds such as NADP, glucose 6-phosphate and glucose 6-phosphatase dehydrogenase.

Solutions to these problems were approached in two steps. First, a sample plate (Fig. 1A) was designed to keep the enzyme solutions cool. The plate was made of aluminum for high thermal conductivity. The dimensions are nearly identical to those of the original sample plate to be compatible with the sample changer. The major difference is the solid design of the aluminum plate versus the more open construction of the original plate (Fig. 1B). The center well 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. When cooled with ice, the solutions in the sample cups are maintained between 0·5 and 1·5°. Water that accumulates from melted ice may be removed by suction. The microswitch lever on the sample changer was bent so that the sample plate would turn freely until the stop pin engaged the microswitch lever and interrupted the cycle. Second, a dual mechanism was designed for the separate pickup of the cofactor and the enzyme solutions (Fig. 2). A block of Teflon was drilled to accommodate buffer and cofactor. A second pickup needle was attached to the original needle holder such that the second needle moved from buffer to cofactor as the first moved from the buffer to the enzyme sample. A similar solution was reached by Fasche and Rej.[2]

Figure 3 shows an apparatus for dispensing cofactor-substrate solutions in discrete aliquots. The delivery tube of the cofactor-dispensing apparatus is inserted into the dispensing trough (Fig. 2). The stopcocks are closed and the stopper is removed from the top. Cofactor solution is introduced and the stopper replaced. Stopcock A is opened to allow the cofactor solution to flow down the delivery tube and into the trough. As soon as the bottom of the delivery tube is covered by the cofactor solution, the flow decreases. The flow stops when the height of the column of liquid balances the external air pressure. When pickup needle 2 moves forward and removes material from the cofactor trough, the level of the cofactor solution recedes below the lip of the delivery tube, allowing an air bubble to enter and more cofactor solution to be released; again the cofactor solution flows into the trough and halts as the column height is balanced by external air pressure. Occasionally, surface tension in stopcock A prevents the release of cofactor solution from the reservoir in spite of the effects of gravity. At such a time, stopcock B may be opened to allow internal equilibration of pressures to overcome surface tension without loss of the cofactor solution from the dispensing trough.

For studying oxidative *N*-demethylation, a cofactor-substrate solution would be prepared in the following manner: 19·22 mg NADP; 107·4 mg glucose 6-phosphate; 43 units glucose 6-phosphatase dehydrogenase; 29·3 mg nicotinamide; 60·9 mg $MgCl_2$; 23·1 mg ethylmorphine; diluted to 46·8 ml with 0·1 M phosphate buffer adjusted to pH 7·4. Thus, the cofactor-substrate solution should be constituted approximately 27·9 per cent stronger in the stock solution than what is expected in the incubation mixture. The enzyme solution should be 444·9 per cent more concentrated in the sample cups than what will obtain after dilution. Any desired incubation



A. B.

FIG. 1. Comparison of the original sample plate (right) and one made of aluminum (left). The plate on the left was made of aluminum for high thermal conductivity. Dimensions are 3·81 cm thick by 24·77 cm diameter, compatible with the sample changer. A concentric disc 2·54 cm thick by 20·32 cm diameter and having a 2·54 cm i.d. center hole was removed from the plate to create a trough that could be filled with ice or other material for temperature regulation. Holes were drilled in the outside rim 1·43 cm i.d. by 2·54 cm deep to accommodate the sample cups. Holes for the stop pins were 0·238 cm i.d. drilled completely through the outside rim 0·318 cm on center from the edge. The center of the sample plate was counter drilled from the bottom 0·793 cm i.d. by 3·333 cm deep to accommodate the center post of the sample changer turntable. Smaller holes, 0·318 cm i.d. by 0·79 cm deep, were drilled in the bottom of the plate at a center radius of 4·445 cm to accept the stabilizers of the turntable. The microswitch lever on the sample changer was bent to accept the slightly different shape of the aluminum sample plate.

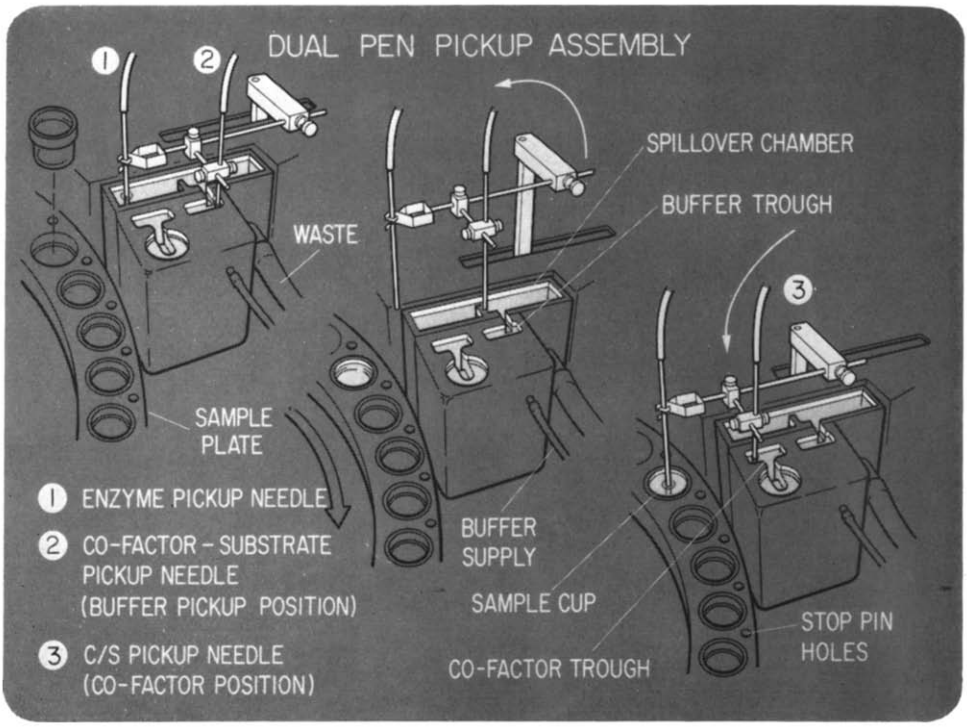


FIG. 2. Dual pickup mechanism is shown in action, moving from the resting buffer pickup position to the enzyme, cofactor pickup position. Not shown in the drawing is the buffer input to buffer trough 1; it enters under the cofactor trough in the Teflon block. The Teflon block, 5·0 cm long, 4·0 cm wide and 4·0 cm high, is designed as follows: the buffer trough is 2·0 cm long, 0·5 cm wide and 1·7 cm deep; it is 0·2 cm from the rear of the Teflon block and 0·25 cm from and parallel to the inside edge (for reference, the inside edge is mounted against the standard Technicon buffer chamber). A hole, 0·25 cm i.d., drilled from the outside of the block to the buffer trough admits the buffer supply. A notch, 0·6 cm wide × 0·3 cm deep, permits spillover into the waste chamber of the Technicon buffer trough. The cofactor trough begins 0·6 cm forward from the buffer trough, and is 1·7 cm long, 0·5 cm wide, and 1·7 cm deep. The barrel of the cofactor-dispensing apparatus fits loosely into a hole 2·3 cm i.d., 0·6 cm deep, and 2·4 cm from the center of the cofactor trough. The cofactor trough extends into the hole to the center or slightly beyond at a depth of 1·7 cm and a width of 0·5 cm. The cofactor-dispensing apparatus is supported within the hole on the lips of the trough, and the cofactor solution exits via the cofactor trough. The block is secured to the Technicon buffer trough with tape and rubber cement.

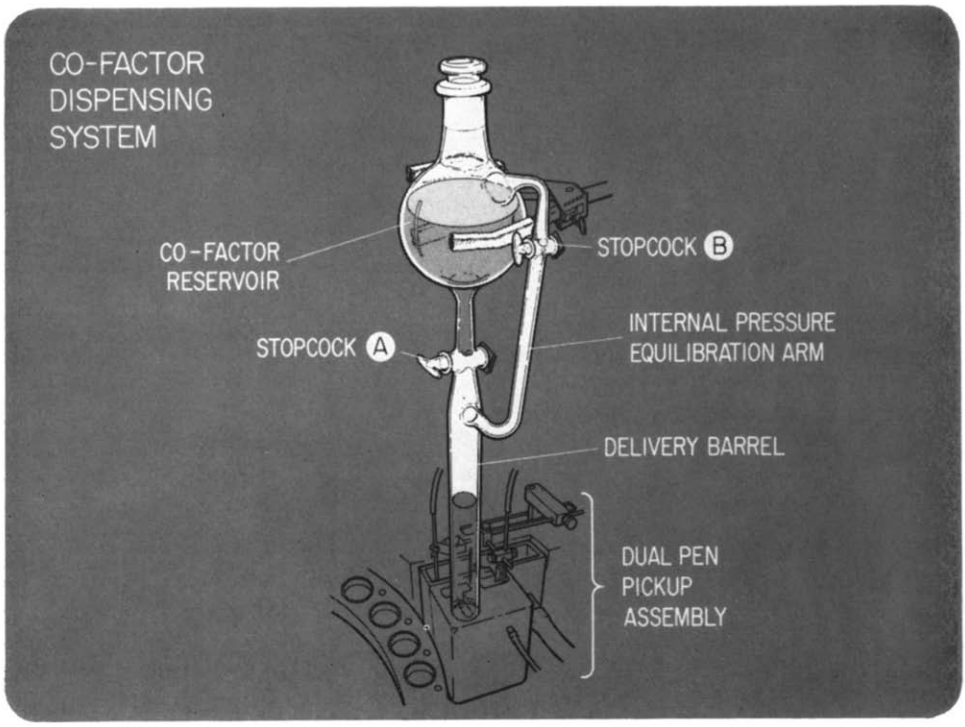


FIG. 3. Cofactor-substrate-dispensing apparatus shown here has a delivery barrel 10·16 cm long from the lip to the side arm bypass, and an internal diameter of 1·11 cm. Stopcocks are standard, and the side arm is 0·635 cm i.d. Maximum volume is about 75 ml. Mechanism of action is described in the text.

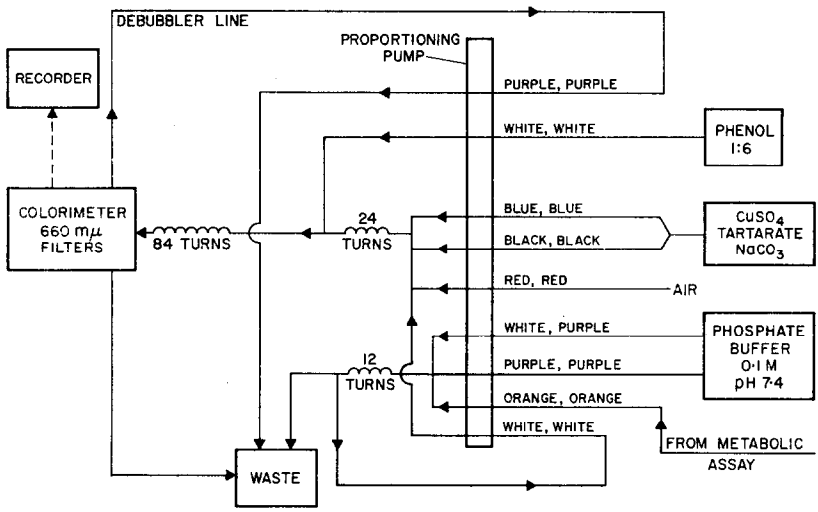


FIG. 4. Process by which an aliquot of the incubation mixture is drawn off at a continuous rate for protein analysis by the method of Lowry *et al.*[3] Lines passing over the plate of the proportioning pump are color-coded for internal diameter in inches: purple-purple, 0·081; white-white, 0·040; blue-blue, 0·065; black-black, 0·030; red-red, 0·045; white-purple, 0·110; and orange-orange, 0·035. Since the speed of the proportioning pump is a constant, the volumes transported are proportional to the cross-sectional area of individual tubes.

mixture can be obtained by adjustment of the stock cofactor solution and the enzyme samples.

The length of tubing from each pickup needle was adjusted so that the sample and cofactor streams reached the mixing coil at the same time. In the mixing coil, the cofactor-substrate solution is diluted 1:1·279 and the enzyme solution, 1:4·449 as the streams merge. After mixing, the incubation mixture is split into two streams; one is mixed with 100% oxygen and then directed to the time delay coil for incubation at 37° for 10·5 min,[1] while the other is diverted to protein analysis.[3]

The aliquot drawn off for protein analysis is mixed with phosphate buffer at a ratio of about 1:100 (Fig. 4). A portion of the diluted sample is then drawn off and mixed with 0·02% $CuSO_4$ + 0·04% NaK-tartrate in a solution that is 0·1 N with NaOH and also contains 2% $NaCO_3$. (An increase in the NaK-tartrate content helps prevent the formation of a blue precipitate, presumably $CuCO_3$.) After thorough mixing, Folin–Ciocalteu phenol, diluted 1:6 with distilled water, is added. Mixing continues for 5·5 min as color formation occurs, and then the color intensity is measured at 660 nm using standard interference filters.

The protein assay is effective when the enzyme solution in the sample cups contains no more than 10–20 mg protein/ml. Additional factors of dilution are needed for the system to work with higher protein concentrations.

DISCUSSION

The additions to the system described above present several advantages over the original automated analysis of drug metabolism.[1] The enzyme samples can be stacked on the cooled sample plate without damage. Manual preparation of the incubation

mixtures is eliminated, thus reducing the amount of time the operator must spend with the apparatus to insure proper functioning, and also reducing the number of potential errors that might result from inaccurate pipetting. Since the cofactor-substrate solutions and the enzyme-containing solutions are picked up separately and mixed automatically, all enzyme substrate reactions are continued for precisely the same length of time under identical conditions.

The system works well with the microsomal fraction, the 9000 *g* supernatant fraction and even with whole liver homogenate, provided the homogenate has been filtered to remove fibrous particles that might obstruct free flow in the system. Although the system was designed specifically for the study of oxidative drug metabolism, its versatility is such that it might well be used in the study of other enzyme systems, such as glucose 6-phosphatase, since there is a requirement for maintaining the enzyme at a low temperature until it is picked up and for limiting the flow of a relatively expensive substrate, glucose 6-phosphate.

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