Radioscan of Electrophoretically Separated Thymine-H^3 Compounds From Bone Marrow Cultures.* (31551)

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The need for a rapid method for separation and determination of either nonradioactive or tritiated thymine compounds became apparent while studying the utilization of thymidine-H^3 in bone marrow cultures in DNA synthesis studies. The technique for determination of radioactive thymine compounds often involves lengthy column chromatography procedures. Rapid separation of mixtures of adenine nucleotides by electrophoresis in agarose gel with subsequent quantitation by ultraviolet spectrophotometry has been described(1). An attempt to use this procedure for the direct scanning of tritium-labeled thymine, thymidine and thymidine-5'-monophosphate was unsuccessful because of poor separation in the electrophoretic phase. A modification was developed which allowed these compounds to be separated easily and rapidly. The modification involved a difference in buffer content and pH for dilute agar gel contained on microscope coverglass. This technique was suitable for subsequent scanning of tritium yielding reproducible curves. The process was useful for the separation of a mixture of thymine-H^3 compounds from materials such as tissue culture fluids, and it seemed especially beneficial with the low concentrations of isotope often encountered in cell or bacterial cultures.

Materials and methods. Reagents. 1. Electrode vessel buffer: 0.1 M sodium borate at pH 10.5. Boric acid, 6.18 g was added to approximately 500 ml of distilled water, titrated to a pH of 10.5 with 1.0 N sodium hydroxide and then diluted to 1 liter. 2. Gel buffer: Dilute the electrode buffer 1:4 with distilled water. 3. Thymine, thymidine and thymidine-5'-monophosphate controls: Add 10 mg of each compound to 1.0 ml of distilled water. Prior to use, thymine tubes must be warmed to 90°C to insure solution. 4. Thymine-H^3, thymidine-H^3, and thymidine-H^3-5'-monophosphate controls: Specific activities were 8.03, 6.7 and 1.8 curies per millimole, respectively. 100 μc/ml stock solutions of each were mixed 1:1:1 and 20 microliters of the mixture were applied to the gel. 5. Dowex 1X8 (chloride), 200-400 mesh was converted to the hydroxyl form by cycling the resin with 1.0 M ammonium bicarbonate until the washings were free of chloride when tested with silver nitrate. The resin was washed with large volumes of distilled water and transferred to 0.02 M ammonium hydroxide.

Procedures. Electrophoresis. The gel was prepared as previously described(2) by heating 500 mg of Ionagar No. 2 in 100 ml of buffer. Four No. 1 microscope cover slips (24 mm × 60 mm) were placed on a 3¼ × 4 inch lantern slide and 13 ml of the hot agar-buffer solution were pipetted onto the plate. Schleicher and Schuell No. 900 wicks (3¼ × 4 inch) were connected between the 4 inch side of the lantern slide and the buffer vessels. The level of the liquid in the buffer vessels was the same as that of the surface of the gel-plate. The nonradioactive samples were applied by absorbing them into a strip of Whatman #3 MM filter paper (1 mm × 15 mm) and putting the strips onto the gel 5 mm from the end of the cover glass nearest the cathode. Electrophoresis was carried out at 200 volts across the total system for approximately 20 minutes until the thymidylic acid migrated 5 cm when the electropherogram was viewed under ultraviolet light. The plates were dried in a blower oven at 100°C. Final migrations of nonradioactive samples were marked by circling the separated and dried spots with ink.

Bone marrow cultures were prepared as previously described(3). In brief, bone mar-

* Supported in part by PHS grant CA 08217 from Nat. Cancer Inst., Michigan Heart Assn. and the Detroit General Hospital Research Corp.

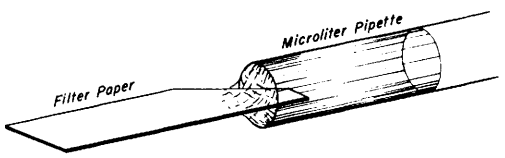

FIG. 1. Concentration system used for radioactive samples. Whatman 3MM filter paper sample applicator is shown inserted in the end of a capillary pipet. As evaporation takes place from the filter paper the sample is drawn into the paper.

row from either humans or animals was cultured in medium consisting of balanced salt solution and plasma. Isotopic tracer in the form of thymidine-H^3 was added and the culture was incubated at 37°C for periods up to 24 hours. The supernatant fluid of the culture was then separated by centrifugation.

Electrophoresis of such supernatant fluids applied as described above resulted in rather poor separation of thymine from thymidine. In addition, the high salt concentration present in these fluids caused noticeable depression of the gel near the point of application. Therefore, it was deemed necessary to remove salts before carrying out the electrophoretic separation. For desalting radioactive samples, a glass column (4 mm, I.D.) was packed with anion exchange resin to a height of 1.0 cm. One part of supernatant culture fluid was diluted with 2 parts of 0.02 M ammonium hydroxide and applied to the resin. The resin was then washed with 2 ml of 0.02 M ammonium hydroxide to insure removal of all unwanted salts and the sample was eluted with 1.0 ml of 1.0 M ammonium bicarbonate. The eluant was evaporated to dryness in an oil bath at 110-115°C, redissolved in 0.1 ml of gel buffer and 50 microliters (1.8 μc) was applied to the gel.

Radioactive control and desalted radioactive samples could be further concentrated as shown in Fig. 1. A tapered end of a filter paper strip was inserted into the end of a disposable microliter capillary pipet. The opposite end was then filled with sample by capillary action and pipet slightly tilted from the horizontal to increase the flow of the material into the filter paper. Evaporation left the concentrated radioactivity on the paper ready for application to the gel. The first of the two methods described for concentrating radioactivity has the advantage of desalting the sample.

A sample of nonradioactive mixture was included as a control with each run because the concentrations of the radioactive thymine compounds were too low to be seen under ultraviolet light. When the control achieved the desired separation, as visualized by U.V. light, the electrophoresis was stopped and the plates dried as described. The radioactive samples on the coverslips were removed from the supporting glass lantern slides, taped by their ends to a piece of carrier paper ($1\frac{1}{2} \times 11$ inches) and scanned in a windowless gas flow counter (Vanguard, model 880) at a rate of 1.9 cm per minute using a slit width of 0.33 cm. The optimum concentration of total applied sample necessary for counting was in a range of 0.5-2.0 μc.

Results and discussion. The electrophoretic separation of thymine compounds is shown schematically in Fig. 2A. The deoxyriboside, thymidine, moved 1 cm from the starting point; the base, thymine, moved 2 cm while the deoxyribotide, thymidylic acid, moved 5 cm during the formation of the 20-minute electropherogram. The mobility of each compound examined individually was identical to that of the same compound in a mixture when subjected to electrophoresis under the same conditions. The separation and radioscan of a radioactive control mixture is shown in Fig. 2B. Each electrophoretic peak represents 0.67 μc/cm^2. Aliquots of supernatant fluid from bone marrow cultures containing thymidine-H^3 were desalted and concentrated as previously described. The same samples were then subjected to both electrophoresis and column chromatography. Column chromatography for the comparative study was similar to the method of Hurlburt *et al*(4).

The results of 2 experiments using supernatant fluids taken from centrifuged tissue cultures at 0 and 24 hours of incubation are graphically illustrated in Fig. 3. At 0 hours, both the columns and the electropherogram showed thymidine-H^3 as expected. After 24 hours incubation, tissue culture supernatants showed thymine-H^3 and an unidentified radioactive compound designated as peak A on agar gel. An unidentified peak, B, was also

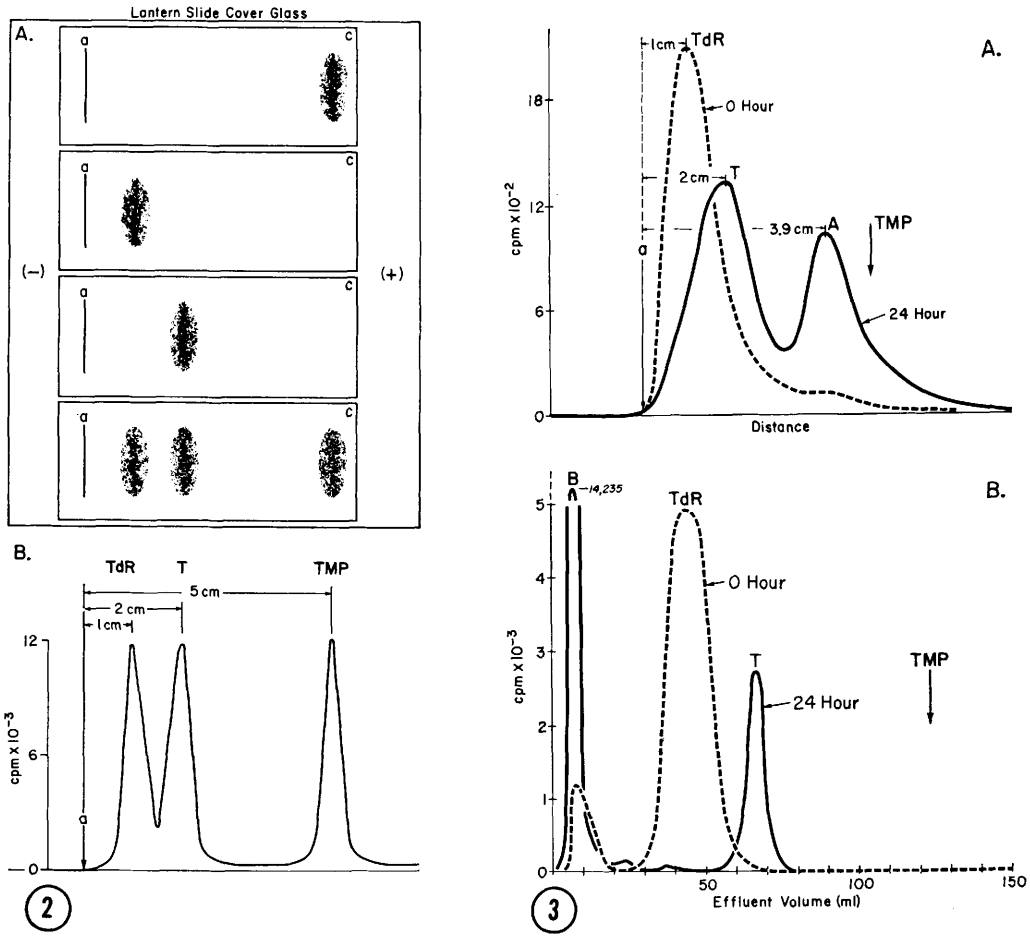

FIG. 2A. Separation of individual thymine compounds and a mixture of thymine (T), thymidine (TdR) and thymidylic acid (TMP). (a) application point of sample. (c) coverglass placed on lantern slide.

FIG. 2B. Radioactive scan obtained with electropherogram from a control mixture of the thymine-H³ compounds. (a) application point of the sample.

FIG. 3A. Agar gel separation showing the radioactive scan of the thymine-H³ compounds from the desalted, concentrated supernatant fluid of bone marrow tissue cultures. Peak A is an unknown compound from two cultures taken at 0 and 24 hr of incubation. (a) application point of samples, (T) thymine, (TdR) thymidine, (TMP) thymidylic acid.

FIG. 3B. Column chromatography of the same samples as in Fig. 3A showing radioactivity of the resolved thymine-H³ compounds and unknown radioactive component designated as peak B.

shown in the column chromatographic separation of the 24 hours supernatant fluid. Peaks A and B may very well be dihydrothymine-H³, a known degradation product of thymine-H³.†

The thymine-H³ compound identified indicates close correlation between the agar gel and chromatography separation scheme. While column chromatography required 1½ days for a single determination, 3 samples were completed by the electrophoretic method in a few hours. Since the study of the degradation of thymidine-H³ to thymine-H³ entails the examination of multiple cultures of bone marrow at intervals, the advantages of the described electrophoretic technique over column chromatography become obvious.

† Control experiments have shown that peaks A and B are not radioactive contaminants secondary to decay during storage.

Summary. An agar gel electrophoretic method has been described for separation of both nonradioactive and tritiated thymine, thymidine and thymidine-5′-monophosphate. The results obtained by this technique were faster and simpler, but comparable to those of column chromatography of bone marrow fluids. The radioactive samples separated on a microscope coverglass were subsequently scanned using an automatic gas flow counter. This quantitative procedure was useful for the study of nucleosides and nucleotides separated by agar gel electrophoresis.

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Received July 14, 1966. P.S.E.B.M., 1966, v123.

Liver and Plasma Malic Dehydrogenase Activities in the Exercised Rat.* (31552)

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Nikkila *et al*(1) have reported increased activity of serum malic dehydrogenase (MDH), and of other enzymes, as a consequence of exercise in untrained subjects and, to a lesser extent, in trained athletes Gardner *et al* (2) have reported similar observations with human subjects, noting a relationship of degree and duration of exercise (treadmill) with serum enzyme levels; training *per se* did not alter basal enzyme activities. More recently, we have reported (3) that liver and plasma MDH activities were increased in rats made to swim for 2 hours. Repeated daily exercise (training) caused an increased basal MDH activity in liver but not plasma; in the trained rat, a 2-hour swimming exercise did not elicit a further elevation of MDH activity in liver nor did it elicit an elevation in plasma. Neither acute nor repeated exercise caused any alteration in liver or plasma glutamic-pyruvate transaminase activities.

The lack of unanimity in observations from several laboratories on effects of exercise on enzyme activities indicates that several factors are involved in enzyme response. Critz(4) and Gardner *et al*(2) have provided evidence that the degree and duration of exercise are important in determining enzyme response. Another possible factor is the nature of the exercise; whereas some workers have used swimming, others have used treadmill running. A difficulty with swimming exercise is that it is not possible to quantitate. Further, at least on removal from the water, swimming could induce some degree of cold exposure which might alter enzyme activities. The present experiments were undertaken to investigate liver and plasma MDH in rats exercised on a treadmill to compare observations with those made using the swimming exercise(3).

Materials and methods. In these experiments, young male rats of the Wistar strain weighing 240-270 g were used. Prior to experimentation, the rats were housed in individual wire cages at an environmental temperature of 24 ± 1°C with laboratory chow and drinking water provided *ad libitum*. Exercise consisted of making the rats run on a circular treadmill at a speed of 1044 meters per hour; the exercise period consisted of 2 hours' continuous running and a total running

* This investigation was supported in part by a grant from Defence Research Board of Canada.

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