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### An Automated Technique for the Clearing and Staining of Fetal Bone in the Autotechnicon. (32578)

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Since a large number of fetuses must be cleared and stained as part of the teratogenic studies required for complete safety evaluation of new drugs, it became necessary to find a method in which these processes could be conducted and controlled automatically. While several convenient clearing and staining techniques are presently available for the examination of fetuses(1,2,3) they are manual in their operation and are tedious and time consuming.

The Autotechnicon has been utilized for the automatic processing and staining of tissues for many years; therefore, it was decided to adapt the method of Cray(1) to this instrument. The results of this adaptation are reported herein.

*Methods and materials.* Rat and mouse fetuses, which are removed from the dam by Caesarian section, or newborn pups are

put into the autopsy trays of the Autotechnicon. These trays are fitted with removable dividers so that each fetus is maintained and processed separately. Each tray can hold at least 6 mice or 4 rats. A paper label containing the dam and fetus number is placed in each tray compartment with the fetus. This identifies the fetus so one may correlate skeletal anomalies with weight, length, or any other distinguishing characteristic of that particular offspring. 6 trays are placed in the Autotechnicon basket in such a fashion that one acts as a lid for another.

The Autotechnicon beakers are arranged so that the baskets containing the fetuses or pups are sequentially processed in the following solutions: a) Acetone; b) 1% Potassium Hydroxide; c) a solution of 6 mg alizarin red S stain per liter of distilled water; and d)

70% aqueous ethanol, glycerol, and benzyl alcohol in a ratio of 2:2:1.

Since the Duo-Autotechnicon contains 24 beakers and the complete clearing and staining cycle employs the use of only 4 beakers, a series of 6 baskets of fetuses can be run simultaneously resulting in 150 cleared and stained rats or 216 cleared and stained mice per cycle.

The basket containing the trays is submerged in 70% aqueous ethanol for fixation for a minimum of 24 hours. The schedule of the procedure which follows is shown in the accompanying table. The transfer of the

Reagent	Beaker No.	Time	Purpose
Acetone	1	4 hr	Defatting and dehydrating
1% Potassium hydroxide	2	24 hr	Maceration
Alizarin red S stain solution	3	24 hr	Staining
Benzyl alcohol, 70% aqueous alcohol & glycerol	4	6 days (maximum)	Destaining, dehydrating, and temporary storage
100% Glycerol		Indefinite	Final storage

baskets into beakers numbers 2, 3 and 4 are performed automatically by the Autotechnicon processing disc.

The fetuses are removed and transferred to the Autotechnicon beaker containing acetone\* where they remain for 4 hours, the time period being programmed on the time delay clock. During this time, the tissues are dehydrated and defatted. They are then automatically transferred into the 1% potassium hydroxide where they remain for 24 hours. At the completion of this interval, the tissues have been cleared and the bones are opaque. The baskets are then transferred to the alizarin red S stain where they remain for an additional 24 hours. It is here that the skeletal structures are stained. The fetuses are finally transferred to the solution of 70% aqueous ethanol,

glycerol, and benzyl alcohol where they are stored until they are to be examined. It is unnecessary to wash the fetuses after staining. The stained fetuses may be examined after they have been in this solution for 24 hours. Although Crary(1) states that the maximum time the fetuses can be kept in this solution prior to their examination is 24 hours, we have been able to keep them for up to 6 days prior to examination. After examination, the fetuses are stored in pure glycerol containing a few crystals of thymol to inhibit bacterial and fungal growth.

*Discussion.* An automated method for clearing and staining rat and mouse fetuses or neonates on the Autotechnicon has been described. The advantage of this technique is to enable one to obtain quick accurate clearing and staining of rat and mouse fetuses with a minimum of handling of the specimens. The use of the Autotechnicon shortens the processing time through a gentle mechanical churning and a slight elevation of the temperature of the reagents. It was found that better skeletal staining results from this combination of mechanical and thermal activity. During their immersion, the fetuses are constantly in motion in the fluids so that the body surfaces are continuously being presented to the reagents. This results in an equal distribution of stain throughout the skeletal system. In addition, the use of the Autotechnicon is time saving as the fetuses are shifted automatically from one solution to the next at a pre-set time interval. To date, this procedure has been used primarily for rats and mice, although other animals (hamsters, guinea pigs, and chickens) could be processed this way. So far, rabbits have been found to be too large for this procedure.

*Summary.* An automated technique for the clearing and staining of fetal bone of rat and mouse in the Autotechnicon is presented. By use of the Duo-Autotechnicon, 150 rats or 216 mice may be cleared and stained on each cycle. The entire cycle on the Autotechnicon requires 72 hours and the fetuses may be examined by 76 hours after the start of the procedure. This system could also be used for guinea pigs, hamsters and chickens; rabbits were found to be too large to be practical.

\* More recently, it was found that air-drying the fetuses for 24 hours in place of their immersion in acetone improves the clearing and staining of the fetal structures. In addition, if the air-drying method is utilized, 192 rats or 284 mice can be processed by the Duo-Autotechnicon per cycle. We are currently exploring this technique.

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## Metabolic Effects of Actinomycin in Rats.\* (32579)

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Largely because of its rather specific ability to block the synthesis of messenger RNA (1,2), the antibiotic, actinomycin D has been used widely by endocrinologists seeking to determine the modes of action of various hormones. Experiments in this laboratory revealed that the late, inhibitory effects of growth hormone on carbohydrate utilization in adipose tissue were blocked by pretreatment of the rats with actinomycin D(3). Further experiments initiated to determine what other effects of growth hormone might also be blocked by treatment with actinomycin D failed to yield useful information as far as growth hormone was concerned, but provided intriguing observations on the effects of actinomycin. For these studies, fasting rats were used, and it was found that actinomycin reversed many of the metabolic changes caused by fasting.

*Materials and methods.* Experiments were conducted using hypophysectomized rats (120 g) obtained from the Charles River Breeding Laboratories. They were fed a high carbohydrate, fat-free diet‡ until studied 2 weeks post-operatively. Fasting rats were deprived of food 18-24 hours before sacrifice. Actinomycin D§ (50 µg/rat) was injected intraperitoneally at the beginning of the fasting period. No attempt was made to study fed rats after actinomycin treatment because these animals spontaneously stopped eating several

hours after injection, at which time the gastrointestinal tract was quite hyperemic and distended with fluid.

The rats were anesthetized with pentobarbital and blood was drawn from the abdominal aorta into heparinized syringes. The hearts and samples of liver were rapidly excised, weighed and digested in hot 30% potassium hydroxide. The diaphragms were dissected free of the ribs, soaked for 3 minutes in cold Krebs-Ringer bicarbonate buffer containing 1 mg/ml of glucose and then incubated for 2 hours at 37° in Krebs-Ringer bicarbonate buffer containing 1 mg/ml of glucose. Epididymal adipose tissue was excised and a sample immediately placed in Dole's extraction mixture(4) for determination of initial content of free fatty acids (FFA). The remaining fat was dissected into several segments and incubated as previously described (5) with randomly labeled glucose for the determination of incorporation of carbon-14 into long chain fatty acids and CO<sub>2</sub>. To measure lipolysis, adipose tissue segments were incubated in bicarbonate buffer to which was added 1 mg/ml of glucose and 40 mg/ml of bovine serum albumin (Fraction V, Armour). Chemical analyses were made as described in previous publications(5-8).

*Results and discussion.* When the animals were deprived of dietary glucose, the concentration of glucose in the blood fell significantly below the fed level (Table I). The decline in blood glucose was less precipitous, but nevertheless present when actinomycin was given at the start of the fasting period. Even in the absence of the pituitary gland the concentration of FFA in the plasma increased more than twofold, in agreement with our earlier conclusions that the pituitary gland is not required for the mobilization of fatty acids

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