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Received January 3, 1961. P.S.E.B.M., 1961, v107.

Carbonic Anhydrase Activity in Fetal and Young Rhesus Monkeys.*† (26625)

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The distribution and physiologic significance of the enzyme carbonic anhydrase has been extensively investigated since the initial report of Meldrum and Roughton(1,2). In the mammalian species high activities have been measured in erythrocytes, renal cortex, gastric mucosa and pancreas(2,3); the enzyme appears to play an essential physiologic role in these tissues(2-5). Significant activities of unknown significance have been reported in brain, spinal cord, testis, salivary glands and the lens and retina of the eye (2,3).

Several reports have dealt with activity of carbonic anhydrase in human fetal tissues obtained by induced abortion and in premature and newborn infants(6-11). Enzyme activity in erythrocytes and/or kidney has been compared in fetal and premature subjects, full term infants, children and adults in several of these reports(7,9-11). The present investigation reports maturation of carbonic anhydrase activity in several tissues of the rhesus monkey from 75 days of gestation to one year of age.

Experimental. Thirteen fetuses of rhesus monkeys were obtained by cesarean section at known gestational ages of 75, 90, 120 or 150 days calculated from the known date of conception (conceptional age). (Average duration of gestation in the rhesus monkey is 165 days). Portions of pancreas, kidney cortex, lung, gastric mucosa and erythrocytes were weighed and immediately frozen. Lung and kidney tissues were perfused with 0.9% NaCl; sections of fetal gastric fundus were

* Supported by grants from Oregon Heart Assn., U.S.P.H.S. and Ross Laboratories.

† Presented in part before Western Society for Pediatric Research meeting, Santa Monica, Calif., Nov. 2 and 3, 1959.

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weighed intact because separation of mucosa was impracticable. Heparinized cord blood was centrifuged in capillary tubes; erythrocytes were transferred to tared parafilm cups, weighed and immediately frozen.

Similar tissues were obtained at autopsy from 10 healthy 350-day-old rhesus monkeys. In these animals lung and kidney tissues were perfused with 0.9% NaCl as in the fetuses. Gastric mucosa and submucosa were dissected from the muscular components. Brain tissue was also obtained from these animals (predominantly gray matter) from the tip of the right temporal lobe. Erythrocytes were prepared as described for fetuses. Blood from 10 adult monkeys was obtained by venipuncture in heparinized syringes. Subsequently erythrocytes were prepared by centrifugation in capillary tubes and were weighed and frozen as described.

Carbonic anhydrase activity in frozen tissues was found to be stable over at least a 4-month period.

Eighty to 300 mg of tissue was homogenized using a pyrex tube with motor driven Teflon pestle with appropriate amounts of distilled water to produce 5 to 25 mg of tissue in 0.5 cc of homogenate. Fifteen to 25 mg of tissue was assayed in fetuses. In older animals 10 to 15 mg of non-erythrocyte tissue and 5 to 15 mg of erythrocytes was assayed.

Homogenates were centrifuged 10 minutes at 1500 RPM in a refrigerated centrifuge and placed in a refrigerator for immediate assay. Specimens were placed in the Warburg flasks immediately before each determination.

To evaluate erythrocyte contamination of tissues, hemoglobin content of the earliest assayed fetal and postnatal homogenates was determined as follows: Homogenates were diluted 1 to 10 with distilled water and centrifuged. The hemoglobin content of the supernatant was determined at 415 mμ on a Beckman DU spectrophotometer by comparison with a linear standard prepared with known hemoglobin solutions. Approximately one-third of tissue homogenates were directly assayed for hemoglobin content. Contamination did not exceed 2.5% by weight in any case. Hemoglobin contamination in the amount of 0.30 mg per ml of homogenate was easily detected with the naked eye, and tissue homogenates were prepared to contain at least 20 mg non-erythrocyte tissue per ml so that 2% contamination was easily detectable. Thus in the remainder of specimens only homogenates with visible hemoglobin contamination were assayed directly. In no case did hemoglobin contamination exceed 2.5%.

Assays were carried out by a modification of the Krebs and Roughton Warburg technic (12,13). Twenty ml Warburg flasks with magnet attached 0.3 ml sidearms were used. The apparatus was equipped with a motor and eccentric wheel for shaking. One and eight-tenths (1.8) ml of phosphate buffer (3 parts of 0.1 M Na_2HPO_4 and 2 parts of 0.1 M KH_2PO_4) with 0.5 ml of plain distilled water (blanks) or distilled water homogenate was placed in the Warburg flask; 0.3 ml of 0.33 M $NaHCO_3$ was placed in the sidearm. Each specimen was run with a blank as control. pH of the phosphate buffer was 6.8 at 20°C.

CO_2 evolution was measured after 10 minute equilibration at 0.2 to 0.4°C (ice bath) and CO_2 evolution read at 30 second intervals for 5 minutes. The average per minute CO_2 evolution in microliters was determined as the average per minute rate of CO_2 evolution during the first 3 minutes multiplied by the flask constants in the usual manner. There was a linear relationship between the activity (rate of CO_2 evolution per minute) and the logarithm of enzyme concentration over the range of homogenate concentrations utilized. This linear relationship is demonstrated for a partially purified carbonic anhydrase preparation and for erythrocyte and gastric tissue homogenates in Fig. 1.‖ Results were expressed in Meldrum-Roughton units (MRU). One MRU is the amount of enzyme to double the rate of the uncatalyzed reaction. The uncatalyzed reaction achieved a mean rate of 20 μl CO_2 evolution per minute by 3 to 4 minutes of reaction time. Thus

‖ The partially purified enzyme was obtained from Mann Laboratories (Lot #A-6836) prepared by the method of Keilin and Mann(14).

TABLE I. Carbonic Anhydrase Activity in Monkey Tissues.*

	Age, days	No. animals	Tissue activities RBC	Kidney	Stomach	Lung	Pancreas	Brain
Fetuses†	75	2	.03 .02	.03 .03	.07 .04	.03 .01	.02 .00	
	90	2	.08 .07	.04 .06	.08 .06	.02 .02	.02 .00	
	105	4	.11 ±.03	.10 ±.05	.11 ±.02	.03 ±.01	.01‡	
	120	2	.06‡	.07 .15	.07 .06	.04 .02		
	150	3	.19 ±.09	.09 ±.03	.07 ±.03	.02‡		
Postnatal animals	350	10	.74 ±.34	.21 ±.02	.17 ±.04	.06 ±.02	.07 ±.03	.04 ±.02
	Adult	10	.72 ±.32					

* Enzyme activity recorded in Meldrum-Roughton units/mg wet tissue. Mean and stand. dev. recorded for 3 or more specimens.

† Age determined from known date of conception.

‡ Single specimens.

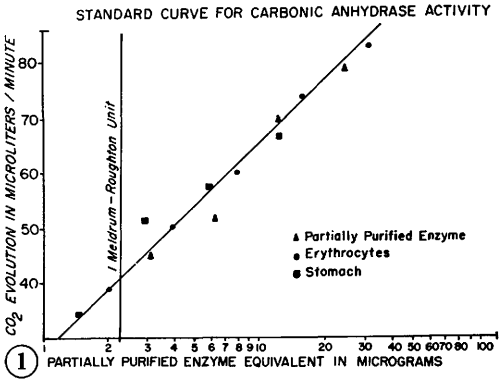
one MRU was equivalent to 2.3 μg of the partially purified enzyme preparation.

The MRU in the present system differs from the original descriptions which compared the catalyzed and uncatalyzed reaction rates during the second quarter of the reaction (E units). The MRU here employed compares the average per minute CO_2 evolution rate in the catalyzed reaction during the first 3 minutes with the stabilized uncatalyzed rate achieved by 3 to 4 minutes.

Reproducibility of the assay method as described is within ± 15%, which agrees with previously reported experience with the manometric technic(3,7).

Results. Table I presents the data derived from this investigation. The seeming rise in carbonic anhydrase activity of kidney and gastric tissues in the 105 day fetuses is of unknown significance and may relate to the small number of samples involved. There is a relatively greater carbonic anhydrase activity of erythrocytes, kidney and stomach, which progressively increased to age one year. This maturation of activity is shown graphically in Fig. 2.

As noted in Fig. 2 there is a 4-fold increase in erythrocyte (RBC) activity from 105 days of gestation to roughly one year of age in these animals and an increase in gastric and renal tissue activity of approximately 1½ times over the same period. Lung, pancreas and brain tissue activities remained low throughout these investigations. At 350 days



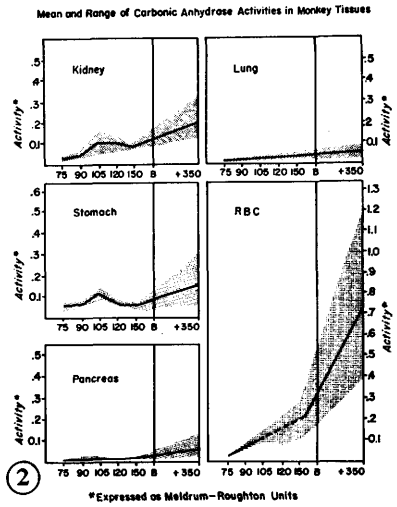


FIG. 1 and 2.

The erythrocyte and gastric curves in Fig. 1 are arbitrarily placed on the abscissa to demonstrate the similar linearity of the curves. Points on these curves represent serial dilutions of homogenate expressed as logarithm of tissue weight per 0.5 cc homogenate.

of age, activity of these tissues is equal to 1/10 to 1/20 of that found in erythrocytes. Adult human erythrocyte activity by the present method averages 15% more than activity in adult rhesus monkey erythrocytes.

Discussion. Erythrocyte carbonic anhydrase activities have been reported to be low in the human fetus and in premature infants. The activity increases 4- to 10-fold by adulthood(6,7,9,10). Berfenstam reported human erythrocyte activity to be about one-half of adult values by one year of age(9). Moreover, it has been observed that fetal tissue activities (kidney, gastric mucosa, pancreas, lung, liver and spleen) at various gestational ages were generally comparable to fetal erythrocyte activities and in many instances exceeded erythrocyte values(7,8).

The present results are in general agreement with these reported relative values. Erythrocyte carbonic anhydrase activity increased roughly 4-fold from 150 days of gestation to 350 days of age (equivalent to adult levels) and increased roughly 10-fold from 90 days of gestation to 350 days of age. Increases also occurred in renal and gastric tissue in contradistinction to the report of Day and Franklin in humans (also expressed as activity per mg wet weight of tissue)(7). Yaffee noted a 50% increase in carbonic anhydrase activity from newborn to adult human kidneys. These results were expressed on the basis of dry weight(11). Mean renal and gastric activity in the present study increased approximately 1½ times between 150 days of gestation and 1 year of age. Expressed on a dry weight basis values would be expected to increase only about 40% during this time in terms of the decrease in body water.¶ Thus the present results are in agreement with those of Yaffee in humans (11).

Direct comparison of reported values is difficult, however, because of dissimilar activity units, variation in reaction temperature and substrate concentration and variations in tissue preparation and homogenate concentrations. Adult human erythrocytes were observed in the present system to average 15% higher activity than adult monkey erythrocytes. Thus, human and rhesus monkey RBC activities appear similar, and the MRU in the present report is relatively greater than that in previous reports as summarized by Berfenstam(9). The present unit most closely approximates that of Meldrum and Roughton(1).

Summary. Maturation of carbonic anhydrase activity has been investigated in erythrocytes, gastric mucosa, kidney cortex, pancreas and lung tissues of the rhesus monkeys from 70 days of gestation to one year of age. Activity of brain tissue (cortical gray matter) was also assayed in the older animals. Significant maturation of activity in erythrocytes, gastric mucosa and renal cortex was noted during the investigation. Definite activity seemed present in fetal lung and pancreas, but values in the one year animals were minimal as were cerebral gray matter activities at one year. These results are in general agreement with reported studies in the human fetus and infant.

¶ Results were expressed on the basis of mg wet weight of tissue. Values for relative water content of monkey fetal tissues are not available but considerable information is available regarding water content of human fetuses, newborn infants and older children(15). Body water expressed as per cent body weight averages 89% at 4 months of gestation (roughly equivalent to 75 days of gestation in the monkey), 75% at birth and 65% at 2 to 3 years of age (roughly equivalent to 1 year in the monkey). Presuming similar relative content of body water in monkey tissues, the observed enzyme activities in tissue homogenates would have been expected to increase 2- to 2½-fold at birth and roughly 3-fold at 1 year of age over the values at 75 days of gestation if expressed on a mg dry weight basis. Water content of erythrocytes will vary minimally.

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Received January 9, 1961. P.S.E.B.M., 1961, v107.

In vitro Metabolism of C-14 Labeled β-Carotene.* (26626)

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Previous studies in this laboratory(1) and others(2,3) on the *in vivo* metabolism of C-14 labeled β-carotene have indicated that radioactivity in the rat is found in the non-saponifiable fraction, fatty acid and water soluble fractions isolated from liver and extra-hepatic tissue. The expired CO_2 is also found to be radioactive.

It has been well demonstrated that β-carotene is converted to Vit. A by the intact animal but it is questionable if such a conversion can be demonstrated by isolated tissue preparation. In view of this possible difference between *in vivo* and *in vitro* metabolism of β-carotene it seemed worthwhile to investigate the *in vitro* metabolism of C-14 labeled β-carotene.

Materials and methods. The β-carotene† used in these experiments was produced by growing *Phycomyces blakesleeanus* in a suitable C-14 acetate medium(4). Carotene samples used were recrystallized from methanol and petroleum ether to a constant specific activity and they had an $E^{1\%}_{1\,cm}$ of 2535 at 450 mμ in light petroleum ether. Booth(5) suggests that 2550 is the representative value for pure β-carotene.

Carotene suspensions were prepared by using 40 mg of Tween 80 and an appropriate volume of buffer solution. The amount of carotene used in each incubation is given in the tables.

All animals were fasted 24 hours before use. Wistar strain, white female rats, weighing between 110-125 g, two rabbits weighing approximately 2 lb each, and four 2-week old chicks were used in these experiments. Animals were decapitated and allowed to bleed freely. The organs were quickly removed, blotted, and various types of homogenates prepared.

Whole homogenates were prepared by taking one-gram samples of organ and homogenizing it for 90 seconds in 2.5 ml of cold 0.25 M sucrose with a loose fitting glass Potter-Elvehjem homogenizer. Cell-free homogenates were prepared by centrifuging whole homogenates, as prepared above, at 800 × g at 0°C for 10 minutes and the supernatant fraction was used. Protein content of the latter fractions was determined by the method of Siekevitz(6).

Mitochondria samples equivalent to one gram of tissue were prepared according to the method of Schneider and Hogeboom(7) and suspended in 2.5 ml of 0.25 M sucrose.

Each standard incubation mixture contained 2 ml of a carotene suspension (see tables for amount of carotene used) in Tris buffer (veronal, Table I), 2 ml of tissue homogenate (representing 1 g of tissue or its equivalent) suspended in 0.25 M sucrose and 6 ml of 0.2 M Tris hydrochloride, pH 7.5 (veronal buffer pH 8.5 used for experiments in Table I). Two mg of penicillin G and

* Supported by a grant from Nat. Vitamin Foundation, New York.

† The labeled carotene was made available by a grant from Rockefeller Foundation to Dept. of Biochemistry and Plant Pathology, West Virginia Univ.