PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 864–869 (1975)

Placental Transport of Creatine in the Rat[1] (38649)

THOMAS R. KOSZALKA, RONALD P. JENSH, AND ROBERT L. BRENT

Harry Bock and Eleanor Roosevelt Research Laboratories Departments of Radiology, Biochemistry, Anatomy and Pediatrics, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Although the placental transport of amino acids in mammals has been studied in considerable detail, little work has been published on the maternal-fetal exchange of physiologically important derivatives of amino acids such as creatine. In this regard, we recently observed that ^{14}C-labeled creatine injected iv into pregnant rats crossed the placentae and entered the blood of the fetus late in gestation (1). Furthermore, the concentration of creatine in fetal blood on the 20th, 21st, and 22nd days of gestation was several-fold higher than that in the maternal blood. These findings indicate that creatine can be transferred from the maternal circulation to the fetus against a concentration gradient, suggesting that creatine transport across the placentae may be an active process, at least in the rat. In this connection, Fitch and Shields (2) have shown that creatine enters the isolated extensor digitorum longus muscle of the young rat by a saturable, energy dependent process rather than by a process of simple diffusion. In a later paper, Fitch *et al.* (3) also showed that the mediated entry process for creatine in muscle was specific for compounds containing an amidino group and could be inhibited both *in vivo* and *in vitro* by guanidinopropionic acid as well as by other derivatives of guanidine.

In the present study, we have defined in more detail the kinetics of creatine transfer across the entire rat placental unit *in vivo*.

The term *placental unit* is used here rather than placenta to emphasize the fact that in the rat this organ consists anatomically of the yolk sac placenta and the chorioallantoic placenta (labyrinth plus junctional zone) and represents the entire exchange surface separating the mother and fetus. We also have compared the results with a similar study using an end-product of creatine metabolism, namely, creatinine. Since creatinine is a metabolic "dead-end", and as such is excreted in the urine, it appeared worthwhile to determine whether the transfer of creatinine from mother to fetus exhibits the same or different kinetics than that of creatine, a metabolite which is known to be important in muscle metabolism and whose content increases rapidly in the rat fetus during development (1). Finally, since it is thought that the visceral yolk sac is an important organ for the transport of nutrients during the early stages of gestation in the rat (4), we attempted to determine if the visceral yolk sac plays a role in creatine transport from mother to fetus late in gestation.

Materials and Methods. Pregnant rats of the Wistar strain were used in all experiments. Females were placed in cages with males overnight. The beginning of the first day of gestation was considered to be at 9:00 AM the following morning, if sperm were found in the vagina. Pregnant dams were anesthetized on the 21st or the 22nd day of gestation with a subcutaneous (sc) injection of sodium pentobarbital (5 mg/100g body wt) which was administered in one or two doses. A tracheostomy was performed on those animals which were anesthetized for periods longer than 2 hr. Either the left or right femoral vein was exposed and injected with 0.2 ml of Ringer's solution containing 2.5 μCi of creatine-1-^{14}C hydrate (sp act, 0.22 mCi/mg) or creatinine-1-^{14}C hydrochloride (sp act, 0.096 mCi/mg). At specified times after the injection of isotope, the tip of the tail of each rat was transected and 0.1 ml of blood was collected in a calibrated capillary tube containing heparin and transferred into a conical centrifuge tube. Immediately thereafter a laparotomy was performed, the uterine

[1] This work was supported by NIH Research Grant No. HD-06360.

veins were exposed and incised, and 0.1 ml of blood was collected in capillary tubes. The thorax then was opened quickly and 0.1 ml of mixed venous and arterial blood was obtained from the heart after incising the ventricles. The fetuses and placentae were removed from the uteri, blotted on filter paper to remove residual amniotic fluid, and the fetuses were decapitated. Fetal blood was collected from the severed neck vessels in the same manner as described for tail blood. The chorioallantoic placenta was isolated and the labyrinth and junctional zone portions of the placenta were separated from each other by blunt dissection and stored at −20°. In most instances, the visceral yolk sac was separated from the fetus and was also stored at −20°. Samples of maternal or fetal blood (0.1 ml) were deproteinized with $Ba(OH)_2$ and $ZnSO_4$ according to the method of Van Pilsum *et al.* (5) and centrifuged. Aliquots of the protein-free supernatant solutions were added to PCS solubilizer (Amersham/Searle Company) or, in earlier experiments, to a toluene ethanol "cocktail" containing a mixture of 95% PPO and 5% POPOP. Radioactivity was measured using a Beckman Model 250 liquid scintillation spectrometer. The appropriate quench corrections were made using the external standard method.

The junctional zone and labyrinth portions of the chorioallantoic placenta and the entire visceral yolk sac were homogenized in distilled water to a final concentration of 10% (W/V) at 4°. Aliquots of the homogenates were deproteinized with $Ba(OH)_2$ and $ZnSO_4$ according to the method of Van Pilsum *et al.* (5). Radioactivity in the protein-free supernatant solution was determined as described. Creatine content of blood, placental tissues and visceral yolk sac was determined in the protein-free supernatant solution by the α-naphthol-diacetyl method as described by Gerber *et al.* (6). In one experiment, each of two pregnant rats on the 22nd day of gestation were injected with 2.5 μCi creatine-1-^{14}C and sacrificed after 30 min. The labyrinth portions of 15–20 chorioallantoic placentae as well as samples of fetal blood were pooled and treated with $Ba(OH)_2$ and $ZnSO_4$ to obtain protein-free supernatant solutions which then were lyophilized. The dried samples were dissolved in a minimal volume of water (0.5 ml) and analyzed by descending paper chromatography (Whatman Company, No. 3 MM) in three different solvent systems: (A) *n*-propanol-water (3:1), (B) ethyl acetate–acetic acid–water (3:1:1), and (C) methanol–isopropanol–ammonium hydroxide–water (35:35:5:15) according to Kammermeier (7). Guide strips were cut from both edges of a chromatogram and were developed with the α-naphthol-diacetyl reagent (6) or the alkaline picrate reagent (7) to detect creatine, creatinine and related substances. The remainder of the chromatograms was cut into small pieces and placed in scintillation vials containing PCS. Radioactivity was determined by scintillation spectrometry as described earlier. The R_f values observed for the derivatives of guanidine in each of the three chromatographic systems studied are listed in Table I.

TABLE I. CHROMATOGRAPHY OF DERIVATIVES OF GUANIDINE.[a]

	System A	System B	System C
		R_f	
Creatine	0.27	0.39	0.44
Creatinine	0.47	0.46	0.57
Phosphocreatine	0.05	0.28	0.10

[a] Paper chromatography was carried out in a descending system on Whatman No. 3 MM paper at room temperature for 7–16 hr. System A: *n*-propanol-water (3:1); System B: ethyl acetate-acetic acid-water (3:1:1), and System C: methanol–isopropanol–ammonium hydroxide–water (35:35:5:15). R_f values are averages of six or more determinations.

Results. Figure 1 shows the appearance of radioactivity in the labyrinth and junctional zone portions of the chorioallantoic placenta, the visceral yolk sac, and in blood obtained from the heart of pregnant rats and the neck vessels of fetuses at various times after the iv injection of creatine-1-^{14}C into the dam. Although the data are not shown here, the radioactivity observed in blood obtained from the heart was similar to that obtained from the uterine and tail veins of the mother at each time point

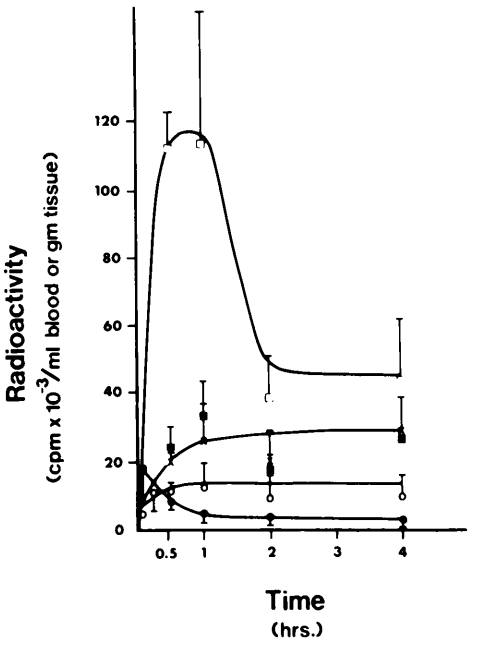

FIG. 1. Radioactivity found in fetal and maternal blood and extraembryonic membranes after iv injection of 2.5 μCi creatine-1-^{14}C into pregnant rats on the 21st or 22nd day of gestation. Three to five fetuses and associated chorioallantoic placentae and visceral yolk sacs were sampled from each litter. Each group consists of four to six pregnant rats and the fetuses obtained therefrom; standard deviation is indicated by vertical line at each point. Radioactivity was determined in (●) maternal blood, (○) fetal blood, (△) visceral yolk sac, (□) labyrinth portion of the chorioallantoic placenta, and (■) junctional zone of the chorioallantoic placenta.

studied. An exception was the 5 min time point when the radioactivity was determined in blood obtained from the uterine veins and heart. The radioactivity in the blood (heart) of the mother reached a maximum during the first 5 min and then decreased rapidly and leveled off after 1 hr. Considerable radioactivity was already detectable in fetal blood after 5 min. The level of radioactivity in fetal blood approximated that in maternal blood 15 min after injection of creatine-1-^{14}C into the dam, and at 30 min was somewhat higher than that in the maternal blood. After 30 min, radioactivity in fetal blood reached maximal levels and remained essentially constant for the duration of the experiment (4 hr). The radioactivity observed in both portions of the chorioallantoic placenta and yolk sac was maximal after 30–60 min and was always considerably higher than that in the fetal and maternal blood at each time point studied. Furthermore, the level of radioactivity in the labyrinth was several times higher than that in the junctional zone portion of the placenta in which the radioactivity remained essentially unchanged after 1 hr.

The radioactivity in the labyrinth began to decline rapidly after the first hour and reached levels which were about one-third of the maximal value after 4 hr. The level of radioactivity in the visceral yolk sac was much less than that in the labyrinth and was comparable to that in the junctional zone of the chorioallantoic placenta at each time point studied. When the protein-free supernatant solution obtained from the labyrinth and the fetal blood was examined by paper chromatography after 30 min, the results indicated that >99% of the total radioactivity recovered from the chromatograms developed in each of the three systems (A, B, and C) was associated with creatine. None of the radioactivity was found in creatinine or phosphorylcreatine, the only known products of creatine metabolism in mammals. The results of this experiment support the view that ^{14}C-creatine injected iv into pregnant rats was not converted in the chorioallantoic placenta to any low molecular weight metabolic products, but was transferred intact across the placenta to reach the fetal circulation as free creatine.

Table II shows the concentration of

TABLE II. CREATINE CONTENT OF MATERNAL AND FETAL BLOOD AND EXTRAEMBRYONIC MEMBRANES.

Creatine in whole blood (μg/ml)	
Maternal	Fetal
16.6 ± 6.6 (6)[a]	61.2 ± 7.4 (33)

Creatine in placental unit (μg/g)		
Chorioallantoic placenta		Visceral yolk sac
Junctional zone	Labyrinth	
145 ± 14 (22)	127 ± 12 (22)	171 ± 31 (22)

[a] Values are means ± SD. Numbers in parenthesis represent number of observations.

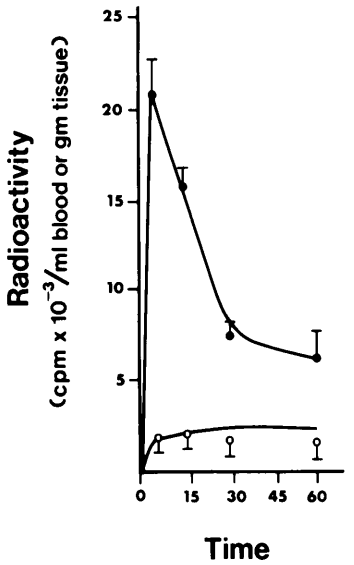

FIG. 2. Radioactivity found in fetal and maternal blood after iv injection of 2.5 μCi creatinine-1-^{14}C into pregnant rats on the 21st or 22nd days of gestation. Three to five fetuses and associated chorioallantoic placentae and visceral yolk sacs were sampled from each litter. Each group consists of four to five pregnant rats and the fetuses obtained therefrom; standard deviation is indicated by vertical line at each point. Radioactivity was determined in (●) maternal blood and (○) fetal blood.

creatine in fetal and maternal blood as well as in the labyrinth and junctional zone portions of the chorioallantoic placenta and visceral yolk sac. As shown previously (1), the creatine concentration in fetal blood was several times higher than that in the maternal blood at 21–22 days of gestation. At the same time, the concentration of creatine in the labyrinth was not significantly different from that observed in the junctional zone portion of the placenta. Creatine concentration in both portions of the chorioallantoic placenta as well as the visceral yolk sac was higher than that in the fetal blood, indicating that a downhill concentration gradient existed between creatine in placental tissues and that in the fetal circulation.

Figure 2 illustrates the appearance of radioactivity in blood obtained from the heart of pregnant rats and the neck vessels of fetuses at various times after the iv injection of creatinine-1-^{14}C into the dam. As in the case of experiments with creatine-1-^{14}C, the level of radioactivity in the blood of the mother reached a maximum after 5 min and then decreased progressively thereafter. On the other hand, the level of radioactivity in fetal blood rose to a maximum 5–15 min after injection of ^{14}C-labeled creatinine and remained approximately constant for at least 1 hr. In contrast to the results obtained with ^{14}C-labeled creatine, the level of radioactivity in the maternal blood after the iv injection of creatinine-1-^{14}C was always considerably higher than that in the fetal blood at each time point studied. After 1 hr, the level of radioactivity in the maternal blood was higher than that in either the chorioallantoic placenta or in the visceral yolk sac. At the same time, the radioactivity in the junctional zone and the labyrinth portions of the chorioallantoic placenta and the visceral yolk sac was comparable to that in the fetal blood.

Discussion. These results, taken together with our earlier observation (1) that the concentration of creatine in fetal blood is 2–3 times higher than that in maternal blood on the 20th, 21st and 22nd day of gestation in the rat, support the view that the transfer of creatine across the placental unit is mediated by an active process rather than by a process of simple diffusion. Our results with creatine are similar to those that have been observed when free amino acids were injected into pregnant animals. For example, when ^{14}C-labeled α-aminoisobutyric acid (AIB) was injected into pregnant guinea pigs late in gestation, the level of AIB in the fetal blood was found to be higher than that in the maternal blood after 30 min (8). At the same time, the level of AIB in the chorioallantoic placenta was about 20 times higher than that in the maternal blood, suggesting that the mammalian chorioplacenta actively transports AIB. Other investigators (9–11) have shown that the blood levels of naturally occurring α-amino acids are higher in the fetus than in the mother shortly after injection into pregnant animals; these observations also have been considered as evidence that the mammalian placenta actively transports α-amino acids.

Although our present evidence is in favor of an active mechanism for creatine trans-

port across the placental unit, our working hypothesis clearly requires further testing. In a preliminary report, Davis *et al.* (12), using the technique of continuous maternal infusion of labeled creatine under steady-state conditions, showed that fetal to maternal ratios of plasma ^{14}C-creatine greater than one can be maintained in rats for several hours, a finding which supports the view that creatine transport across the placenta is an active process. Furthermore, *in vitro* studies using the tissue slice technique have been carried out in our laboratory with placental slices to determine the characteristics of creatine uptake by embryonic membranes from both man and the rat. These studies (13) are in agreement with the hypothesis that the placental transport of creatine is an energy dependent process and appears to have a high degree of specificity. Thus, the present results taken together with our earlier findings (1) are consistent with the view that an active transport process for creatine exists in the rat placental unit, and that this process has similar characteristics to the transport system for creatine observed in rat muscle by Fitch et al. (2, 3). Although the data indicate that at least part of the ^{14}C-creatine found in the fetal blood could have come from the vitelline vessels by way of the visceral yolk sac, our experimental approach did not permit us to quantify more precisely the relationship between the yolk sac and the chorioallantoic placental transport systems for creatine. However, since the chorioallantoic placenta is in direct contact with the circulating maternal blood while the visceral yolk sac has several cellular and membrane layers between blood and tissue, it is likely that more material is presented to the chorioallantoic placenta per unit time than to the visceral yolk sac late in gestation. Thus, our results are consistent with the hypothesis that a major fraction of the injected ^{14}C-creatine was concentrated in the chorioallantoic placenta, and then was released into the fetal circulation. The kinetics of release of radioactivity from the chorioallantoic placenta (Fig. 1) very likely accounts for the observation that the level of radioactivity in the fetal blood remained essentially constant for at least 4 hr after reaching its maximal value 30 min after ^{14}C-creatine administration. Unfortunately, it cannot be ascertained from these "pulse" type experiments whether the placental transfer of creatine from mother to fetus is a physiologically important pathway for creatine accumulation in the fetus during intrauterine growth. The liver of the fetal rat is known to contain the enzyme (guanidinoacetate *N*-methyltransferase) which is involved in creatine biosynthesis, but there is disagreement with respect to the level of activity of the enzyme in the liver during development (1, 14); consequently, it is difficult to state at this time if the fetus can supply exclusively its own needs for growth by the hepatic synthesis of creatine late in gestation.

The results of the experiments described here indicate that the kinetics of creatinine transport across the rat placental unit is different from that of creatine transport. Creatinine-1-^{14}C is transferred less rapidly across the placentae and did not accumulate either in the placental unit or in the fetal blood to the same extent that labeled creatine did after injection into pregnant rats (Fig. 2). These studies are in agreement with the results observed by other investigators who have shown that creatinine is transported passively across the chorioallantoic placenta in several different animals (15–17).

In conclusion, our studies indicate that creatine is actively accumulated and released by the placental unit to the growing fetus, at least in the later stages of gestation. On the other hand, creatinine is only passively transported from mother to fetus across the placenta in keeping with its function as a nonutilizable end product of creatine metabolism.

Summary. Pregnant rats near term were injected iv with creatine-1-^{14}C or creatinine-1-^{14}C and the distribution of radioactivity was studied in maternal and fetal blood as well as in the visceral yolk sacs and chorioallantoic placentae. After injection of creatine-1-^{14}C into the dam, the level of radioactivity in fetal blood was somewhat higher than that in maternal blood at 30 min,

reached maximal levels after 30 min, and remained essentially constant for at least 4 hr. The radioactivity in the labyrinth and junctional zone portions of the chorioallantoic placenta and the yolk sac was maximal after 30–60 min and was always considerably higher than that in the fetal and maternal blood. Furthermore, the level of radioactivity in the labyrinth was several times higher than that in the junctional zone portion of the chorioallantoic placenta and the yolk sac in which the radioactivity remained essentially constant after 1 hr. On the other hand, the radioactivity in the labyrinth declined rapidly after the first hour and reached levels which were about one-third of the maximal value after 4 hr. When labeled creatine was injected into the dam, 99% of the radioactivity present in the fetal blood and chorioallantoic placenta was found in free creatine as shown by paper chromatography in three different systems. Ancillary studies showed that the creatine concentration in fetal blood was several times higher than that in the maternal blood on the 21st–22nd day of gestation. Creatine concentration in both portions of the chorioallantoic placenta as well as the visceral yolk sac was higher than that in the fetal blood, indicating that a downhill concentration gradient existed between creatine in placental tissues and that in the fetal circulation. When creatinine-1-^{14}C was injected iv into pregnant rats, the level of radioactivity in fetal blood rose to a maximum after 5–15 min, but the radioactivity in the maternal blood was considerably higher than that in either the fetal blood or the chorioallantoic placenta and yolk sac. These studies indicate that creatine is actively accumulated and released by the placental unit to the growing rat fetus, while creatinine is passively transported from mother to conceptus across the placentae, at least during the later stages of rat gestation.

We are indebted to Drs. Richard K. Miller and Barbara Davis for their constructive criticism of the manuscript and to Carole L. Andrew for her excellent technical assistance.

1. Koszalka, T. R., Jensh, R., and Brent, R. L., Comp. Biochem. Physiol. **41B**, 217 (1972).
2. Fitch, C. D., and Shields, R. P., J. Biol. Chem. **241**, 3611 (1966).
3. Fitch, C. D., Shields, R. P., Payne, W. F., and Dacus, J. M., J. Biol. Chem. **243**, 2024 (1968).
4. Padykula, H. A., Deren, J. J. and Wilson, T. H., Develop. Biol. **15**, 311 (1966).
5. Van Pilsum, J. F., Martin, R. P., Kito, E., and Hess, J., J. Biol. Chem. **222**, 225 (1956).
6. Gerber, G. B., Gerber, G., Koszalka, T. R., and Emmel, V., Amer. J. Physiol. **202**, 453 (1962).
7. Kammermeier, H., Anal. Biochem. **56**, 341 (1973).
8. Hyter, C. J., Hutchinson, E. A., Karvonen, M. J., and Young, M., J. Physiol. **175**, 11 (1964).
9. Christensen, H. N., and Streicher, J. A., J. Biol. Chem. **75**, 95 (1948).
10. Dierks-Vetling, C., Cone, A. L., and Wapnir, R. A., Biol. Neonate. **17**, 361 (1971).
11. Young, M., *in* "Fetus and Placenta" (A. Klopper and E. Diczfalusy, ed.) p. 139, Blackwell, Oxford (1969).
12. Davis, B. M., Koszalka, T. R., and Miller, R. K., Pharmacologist **15**, 199 (1973).
13. Miller, R. K., Koszalka, T. R., Davis, B. M., Andrew, C. L., and Brent, R. L., Teratology. **9**, A28 (1974).
14. Hommes, F. A., Oudman-Richters, A. R., Van Der Zwaag, P., and Luit-DeHaan, G., Develop. Biol. **29**, 250 (1972).
15. McGoughy, Jr., H. S., Corey, E. L., Scoggin, W. A., Ficklen, C. H., and Thornton, Jr., W. N., Amer. J. Obstet. Gynecol. **80**, 108 (1960).
16. Moore, W. M. O., and Ward, B. S., Amer. J. Obstet. Gynecol. **108**, 635 (1970).
17. Hodari, A. A., Mariona, F. G., Houlihan, R. T., and Peralta, J. Obstet. Gynecol. **41**, 47 (1973).

Received July 26, 1974. P.S.E.B.M. 1975, Vol. 148.