DEVELOPMENTAL CHANGES OF FREE AMINO ACIDS IN BOVINE FETAL FLUIDS WITH GESTATIONAL AGE AND THE INTERRELATIONSHIPS BETWEEN THE AMINO ACID CONCENTRATIONS IN THE FLUID COMPARTMENTS

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(Received 5th November 1974)

Summary. Thirty ninhydrin-positive compounds were determined in the sera, amniotic fluid, stomach content and allantoic fluid from 29 bovine fetuses of various gestational ages. Fetal serum was found to contain about 3-fold higher concentrations of free amino acids (FAA) than maternal serum, and allantoic fluid contained about 3-fold higher concentration of FAA than fetal serum. Decreasing concentrations of FAA were found in serum as a function of the crown-rump length for the amino acids taurine, aspartic acid, threonine, serine, ornithine and lysine. Decreasing concentrations of FAA in allantoic fluid were found for threonine, alanine and ornithine, whereas increasing concentrations were found for phosphoserine and methionine as a function of the crown-rump length. Correlations were found between the concentrations of most amino acids in amniotic fluid and stomach content, but fewer correlations were found between the other fluid compartments. The transport of amino acids between compartments is discussed.

INTRODUCTION

Only one report has been found in the literature about the free amino acid (FAA) concentrations in the bovine fetus. In that paper, Martincic *et al.* (1970) reported the determination of FAA by paper chromatography and compared the FAA concentrations at various gestational ages for serum, amniotic fluid and allantoic fluid. Because of the limitations of paper chromatography, they were able to quantitate only the FAA that were present in high concentrations. The use of ion-exchange chromatography has allowed other workers to make much more comprehensive studies of fetal fluids in sheep and women. For example, Alexander *et al.* (1970) and Hopkins *et al.* (1971) reported that fetal sheep had plasma FAA levels that were more than twice the maternal FAA levels and that the fetal levels did not vary significantly even though the maternal FAA levels altered over a wide range by perfusion or starvation. In studies with human fetuses, Scott *et al.* (1972) examined the changes in indivi-

dual FAA concentrations in amniotic fluid and proposed that the FAA concentrations could be used as an indicator of fetal age. The determination of FAA in amniotic fluid was used by Thomas *et al.* (1971) to detect phenylketonuria in the fetus and by Emery *et al.* (1970) to detect central nervous system disorders of the fetus. On the basis of FAA concentrations found in various fetal fluid compartments, A'Zary *et al.* (1973) proposed a model for the transfer of FAA between maternal plasma, fetal plasma, and amniotic fluid and fetal urine.

Tsoulos *et al.* (1971) reported that only half of the metabolic requirements of the fetal sheep was being met by the transfer of glucose from the mother and proposed that the FAA might be an energy source. This supposition was supported by the calculations of Gresham *et al.* (1972) on urea clearance studies which revealed that up to 25% of the O₂ consumption of the fetal lamb could be accounted for by the catabolism of amino acids.

The present report extends the work of Martincic *et al.* (1970) by reporting the concentrations of FAA and other ninhydrin-positive compounds that can be determined by ion-exchange chromatography and that were found in bovine fetal serum, amniotic fluid, allantoic fluid and stomach contents. Tests were made for the interrelationships between FAA concentration and gestational age and between individual FAA concentrations in each fluid compartment.

MATERIALS AND METHODS

Samples

Fetuses were collected at a local abattoir from 29 pregnant cows. Fetal allantoic and amniotic fluid and stomach contents was collected and subsequently cultured for bacterial pathogens; none was found. The fluids were centrifuged at 5000 *g* for 10 min to remove cells and frozen at -70°C. Fetuses were bled, and the serum was harvested and stored at -70°C. Fetuses were weighed and measured for estimation of gestational age by the criteria developed by Hubbert (1972).

Analysis

The fluids were deproteinized by adding 50 mg sulphosalicylic acid/ml fluid, centrifuging and filtering the supernatant fluid through a 0.3- μ m filter. The fluid was then analysed directly on a Model 121 amino acid analyser (Beckman Instruments, Inc., Palo Alto) according to the instructions in the Technical Manual 121-1M-1A (1970). The two-column system for physiological fluids was used with sodium acetate buffers. The acidic column was 0.9 $\times$ 56 cm of Beckman Type PA-38 resin and the basic column was 0.9 $\times$ 20 cm of Beckman Type PA-35 resin. Norleucine was used as an internal standard. The ninhydrin compounds were identified by comparing the elution time to known standards. Allantoic fluid had to be diluted 1:4 in order to get the acidic and neutral free amino acids in the analytical range of the instrument; otherwise, 0.25 ml of supernatant fluid was analysed.

Statistics

A graph was constructed for each amino acid in each fetal fluid as a function of gestational age. If a trend was observed, a best line was fitted by computer to the points, and an equation of the line was derived. A correlation coefficient, r , was calculated for the line and tested for significance by the t test. (A significant correlation coefficient implied a significant slope of the regression line.) If the data were too variable to discern a trend, a mean value $\pm$ S.E.M. was calculated to represent the amino acid concentration over the gestational period studied. Graphs were also constructed by plotting the concentration of a single amino acid in each of two fluids against each other to see whether there was a relationship between the concentrations in the fluid compartment. A line was fitted by computer to these points, and a correlation coefficient was calculated and tested for significance.

RESULTS

Of the 120 possible graphs of fetal fluid FAA as a function of crown-rump length, only eleven combinations were statistically better represented by a

Table 1. Concentrations ($\mu\text{mol}/100\text{ ml}$) of amino acids in various fluids of the bovine fetus

Amino acid	Fetal serum	Amniotic fluid	Stomach content	Allantoic fluid
Phosphoserine	1.37 ± 0.14	1.51 ± 0.35	1.22 ± 0.33	58.09 (Incr.)*
Phosphoethanolamine	0.76 ± 0.12	0.59 ± 0.11	1.29 ± 0.25	33.65 ± 4.07
Taurine	11.51 (Decr.)*	3.17 ± 0.49	5.79 ± 0.87	58.23 ± 11.18
Hydroxyproline	7.18 ± 0.95	2.29 ± 0.53	0.20 ± 0.20	33.37 ± 5.67
Aspartic acid	6.95 (Decr.)*	0.35 ± 0.13	1.21 ± 0.38	7.77 ± 1.45
Threonine	15.37 (Decr.)*	3.04 ± 0.38	3.40 ± 0.38	59.27 (Decr.)*
Serine	29.36 (Decr.)*	3.88 ± 0.39	5.02 ± 0.73	96.80 ± 12.40
Asparagine + glutamine	49.38 ± 5.38	16.27 ± 2.53	16.02 ± 2.39	128.00 ± 15.80
Proline	1.80 ± 0.97	2.79 ± 0.60	2.05 ± 0.54	19.10 ± 3.52
Glutamic acid	67.58 ± 4.15	7.33 ± 1.10	16.75 ± 2.38	88.67 ± 20.80
Citrulline	10.31 ± 0.49	3.11 ± 0.44	1.97 ± 0.23	44.10 ± 6.40
Glycine	64.17 ± 3.56	21.99 ± 2.83	17.83 ± 2.31	327.70 ± 41.80
Alanine	92.46 ± 3.62	22.24 ± 2.31	23.26 ± 2.73	225.80 (Decr.)*
α -Amino adipic acid	1.54 ± 0.57	0.30 ± 0.13	0.16 ± 0.08	22.70 ± 4.97
α -Amino-n-butyric acid	1.99 ± 0.58	0.69 ± 0.26	0.54 ± 0.21	3.65 ± 0.71
Valine	32.69 ± 1.16	6.96 ± 1.32	5.32 ± 1.12	47.25 ± 9.66
Cystathionine	3.34 ± 0.17	1.43 ± 0.18	1.69 ± 0.32	41.74 ± 3.12
Methionine	5.76 ± 0.21	1.24 ± 0.26	1.58 ± 0.37	22.55 (Incr.)*
Isoleucine	16.41 ± 0.71	2.18 ± 0.49	2.89 ± 0.61	27.66 ± 2.69
Leucine	27.14 ± 0.60	3.40 ± 0.55	4.78 ± 1.09	36.16 ± 4.56
Tyrosine	8.07 ± 0.47	1.15 ± 0.15	1.72 ± 0.33	24.89 ± 5.10
Phenylalanine	12.10 ± 0.45	1.62 ± 0.23	2.21 ± 0.49	13.92 ± 2.22
Hydroxylysine	N.D.	N.D.	N.D.	13.62 ± 2.23
Lysine	20.10 (Decr.)*	3.93 ± 0.33	5.10 ± 1.00	58.94 ± 6.52
Histidine	6.43 ± 0.53	1.61 ± 0.25	1.07 ± 0.34	36.58 ± 3.73
3-Methyl-histidine	N.D.	N.D.	N.D.	18.01 ± 4.71
Anserine	1.93 ± 0.22	1.11 ± 0.22	0.32 ± 0.09	23.84 ± 3.40
Carnosine	3.61 ± 0.45	N.D.	N.D.	12.49 ± 1.64
Arginine	8.48 ± 1.27	3.03 ± 0.35	1.88 ± 0.42	39.38 ± 3.90
Total amino acids	523.67	120.35	129.40	1659.34

Values are Means $\pm$ S.E.M. N.D. = not detected.

* Amino acids that varied with the gestational age; the concentration at a crown-rump length of 56 cm (190 days of gestation) is given and the trend to parturition is indicated: Incr. = increasing; Decr. = decreasing.

TEXT-FIG. 1. Free amino acid concentrations in bovine fetal fluids. (a) to (k) Concentrations ($\mu\text{mol}/100\text{ ml}$) in serum and allantoic fluid as a function of crown-rump length (cm) and the best fit line; (l) an example of the comparison between fluid compartments for taurine in amniotic fluid and serum. (a) Taurine in serum ($y = 22.04 - 0.188(x)$ (estimated equation), $r = 0.647$ (correlation coefficient)); (b) aspartic acid in serum = $13.78 - 0.122x$, $r = 0.697$; (c) threonine in serum = $3.17 + 0.61 - 0.007^2(x^2)$, $r = 0.748$; (d) serine in serum = $47.84 - 0.33x$, $r = 0.662$; (e) ornithine in serum = $48.05 - 0.51x$, $r = 0.867$; (f) lysine in serum = $33.82 - 0.245x$, $r = 0.591$; (g) phosphoserine in allantoic fluid = $-13.03 + 1.27x$, $r = 0.86$; (h) threonine in allantoic fluid = $142.15 - 1.48x$, $r = 0.63$; (i) alanine in allantoic fluid = $468.30 - 4.33x$, $r = 0.622$; (j) methionine in allantoic fluid = $53.69 - 1.62 + 0.019x^2$, $r = 0.786$; (k) ornithine in allantoic fluid = $56.28 - 0.59x$, $r = 0.672$; (l) $r = 0.822$.

Table 2. Comparison of essential (E) and nonessential (NE), and glucogenic (G) and nonglucogenic (NG) amino acids in bovine fetal fluids at 190 days of age

	<i>Serum</i>	<i>Amniotic fluid</i>	<i>Stomach content</i>	<i>Allantoic fluid</i>
Essential amino acids* ($\mu\text{mol}/100\text{ ml}$)	142.55	28.16	28.37	359.27
Nonessential amino acids ($\mu\text{mol}/100\text{ ml}$)	371.12	92.19	101.03	1300.10
Ratio E:NE	0.411	0.305	0.280	0.276
Glucogenic amino acids† ($\mu\text{mol}/100\text{ ml}$)	381.85	91.78	88.69	1074.60
Nonglucogenic amino acids ($\mu\text{mol}/100\text{ ml}$)	141.82	28.57	40.71	584.74
Ratio G:NG	2.70	3.22	2.18	1.84

* Considered to be lysine, phenylalanine, threonine, valine methionine, leucine, isoleucine, histidine, arginine and tyrosine.

† Considered to be glycine, alanine, serine, threonine, valine, glutamic acid, asparagine, histidine, arginine, proline, and hydroxyproline.

Table 3. Correlation coefficients between fetal fluid compartments for individual amino acids

<i>Amino acid</i>	<i>Amniotic fluid versus serum</i>	<i>Stomach content versus amniotic fluid</i>	<i>Allantoic fluid versus serum</i>	<i>Allantoic fluid versus amniotic fluid</i>
Phosphoserine	0.42*	0.86**	0.49*	0.57**
Phosphoethanolamine	0.31	0.43*	0.28	0.19
Taurine	0.82**	0.61**	0.52*	0.58*
Hydroxyproline	0.34	0.18	0.13	0.11
Aspartic acid	0.23	0.23	0.24	0.02
Threonine	0.47*	0.27	0.48*	0.14
Serine	0.07	0.68**	0.68**	0.11
Asparagine+glutamine	0.21	0.53**	0.17	0.33
Proline	0.27	0.32	0.01	0.37
Glutamic acid	0.13	0.61**	0.29	0.13
Citrulline	0.11	0.47*	0.17	0.37
Glycine	0.53**	0.71**	0.30	0.20
Alanine	0.14	0.42*	0.11	0.11
α -Aminoadipic acid	0.30	0.77**	0.41*	0.69**
α -Amino-n-butyric acid	0.45*	0.84**	0.18	0.10
Valine	0.10	0.18	0.21	0.11
Cystathionine	0.06	0.18	0.06	0.02
Methionine	0.12	0.05	0.18	0.18
Isoleucine	0.38†	0.32	0.03	0.27
Leucine	0.40	0.61**	0.23	0.03
Tyrosine	0.00	0.35†	0.24	0.22
Phenylalanine	0.24	0.66**	0.05	0.17
Ornithine	0.62**	0.45*	0.61*	0.37*
Lysine	0.05	0.68**	0.22	0.06
Histidine	0.03	0.37†	0.25	0.28
Anserine	0.37†	0.59**	0.23	0.00
Carnosine			0.11	
Arginine	0.01	0.40†	0.26	0.29

* $P < 0.01$; ** $P < 0.001$; † $P < 0.05$.

sloping line than by a mean (Text-fig. 1a to k). Only two of the amino acids in allantoic fluid (phosphoserine and methionine) increased with increasing gestational age (Text-figs 1g and 1j). Because of the relatively low amounts of free amino acids in amniotic fluid and stomach content and the variability from animal to animal, these data are summarized in Table 1.

Allantoic fluid contained about 3-fold more total FAA than did fetal serum. Amniotic fluid and stomach content contained about one-fourth the amount of total FAA that serum contained. The order of abundance of amino acids in each fluid compartment varied slightly between compartments; alanine, glycine, glutamic acid and asparagine plus glutamine were the most abundant.

The data from Table 1 were used to group the amino acids into essential and nonessential amino acids and to calculate a ratio of essential to nonessential for each fluid compartment (Table 2). A similar grouping was made to compare glucogenic and nonglucogenic amino acids (Table 2).

The data were examined for a correlation between the concentration of the individual amino acids and the various fluid compartments. A graph of the concentration of taurine in amniotic fluid versus the concentration of taurine in fetal serum is shown as an example in Text-fig. 1(l). Four fluid compartment interactions were tested, and the correlation coefficient and its significance are presented in Table 3. The concentrations of phosphoserine, taurine and ornithine had significant correlation coefficients for all of the fluid compartments tested.

DISCUSSION

The FAA concentrations in fetal serum or allantoic fluid can be used as an index of fetal age from 125 to 255 days. Serum concentrations of ornithine would be the best indicator because the correlation coefficient is high ($r = 0.87$) and the ornithine concentration decreases over a wide range as the fetus matures. Allantoic fluid concentrations of phosphoserine could also be used ($r = 0.86$) except that the phosphoserine concentrations increase as the fetus matures. The other amino acids (Text-figs 1a to 1k) can be used as an internal check on the estimate of the fetal age.

The total FAA in bovine fetal serum reported here is greater than the FAA reported for the adult cow by Verbeke *et al.* (1972) and by Love & Wiygul (1969), who used the same methodology. Threonine is $\times 3$ and alanine is $\times 6$ more concentrated in the fetus than in the adult. In order for this concentration difference to be maintained, there must be an active placental transport mechanism. Such a mechanism is also suggested by the results from studies in sheep, in which the FAA concentration differences between the mother and fetus are similar to those in cattle, since the fetal concentrations were very little changed by starvation or perfusion of the mother (Hopkins *et al.*, 1971; Slater & Mellor, 1972).

It can reasonably be assumed that FAA are transported from maternal to fetal plasma and from these to amniotic fluid, urine, and allantoic fluid. Protein formation and metabolism by fetal tissue would thus affect the amino acid concentrations in serum most extensively, and transfer from fetal serum to amniotic fluid would be in terms of excess amino acids. The ratio of essential

to nonessential amino acids found in the present study for serum and amniotic fluid indicates that nonessential amino acids are preferentially transferred, and the ratio of glucogenic to nonglucogenic amino acids between these fluids suggests that glucogenic amino acids are preferentially transported. The concentration of the individual amino acids in the two fluid compartments correlate with each other for only 7 of the 27 amino acids tested (Table 3) and thus diffusion does not seem to be an important transport mechanism for most of the amino acids.

The correlation coefficients for amino acids between amniotic fluid and stomach content are highly significant for most of the amino acids. This finding was to be expected because stomach content is mainly swallowed amniotic fluid, but hydroxyproline was found in only one sample of stomach contents and in most of the amniotic fluids.

Correlation coefficients for amino acids in comparing allantoic fluid with either serum or amniotic fluid were similar. Significant coefficients were found for phosphoserine, taurine, α -amino adipic acid and ornithine in both comparisons. These amino acids are considered neither essential nor glucogenic and are considered metabolites of other processes. Their concentration may not be controlled by an active transport mechanism, so a correlation between compartments on the basis of concentration gradients may exist.

Allantoic fluid has been considered to be a fluid into which the fetus shunts waste materials that are not readily transferred back to the mother. The concentration of total amino acids in allantoic fluid was 3-fold greater than that in serum and more than 10-fold greater than that of amniotic fluid. Comparisons of the ratios of glucogenic to non-glucogenic amino acids and essential to non-essential amino acids indicate that nonglucogenic and nonessential amino acid concentrations were increased, in allantoic fluid, as compared with the ratios for serum. That the allantoic sac is a waste container is also suggested by the experiments in the pig of Stanier (1965), who injected [^3H]water, [^{14}C]urea, and [^{14}C]glycine into the allantoic sac. Only tritium was found in maternal blood or in fetal tissues. No transfer of urea or glycine was detected. This finding indicates that the constituents in allantoic fluid are normally rather static but does not preclude the possibility that the fetus might re-utilize some of those amino acids during times of stress.

The origin of amniotic and allantoic fluids and the secretions that contribute to them seem to be very complex and fetal urine presumably contributes to these fluids. Reeves *et al.* (1972) have tried to trace the changes in urinary flow in the bovine fetus; the concentrations in urine were much higher than in serum but lower than in allantoic fluid although they varied greatly from sample to sample. Thus, the fetal kidney also contributes to the distribution of amino acids in the various fluid compartments. Much more extensive studies of turnover rates and transport mechanisms are needed to understand the distribution of amino acids in these fluids.

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