Transport of Amino Acids into the Oestrogen-Primed Uterus ENHANCEMENT OF THE UPTAKE BY A PRELIMINARY INCUBATION

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(Received 14 February 1972)

1. Preincubation of the immature rat uterus under physiological conditions was found to increase its subsequent ability to transport α -aminoisobutyric acid, L-proline, L-alanine and 1-aminocyclopentanecarboxylic acid. Uptakes of L-valine, L-phenylalanine and L-leucine were not affected. With α -aminoisobutyric acid, a doubling of the uptake could be obtained after a 3–5 h preincubation period. Uteri from oestradiol-primed rats gave increases similar to those found in tissues from untreated animals. In both cases the preincubation increased the $V_{\max}$ of α -aminoisobutyric acid uptake but did not affect the K_m . 2. The conditions during the preincubation period determined the increase in subsequent uptake of α -aminoisobutyric acid. No increase in uptake was found if the preincubation was carried out at 1°C, in the presence of cyanide or dinitrophenol, under anaerobiosis or with a concentration of puromycin that depressed incorporation of L-leucine into protein by 95%. The puromycin was also shown to prevent the increase in $V_{\max}$ normally found after the preincubation period. In addition, no increase was found if Na^+ was omitted from the preincubation medium. Other inorganic ions had smaller effects. 3. The uptake of α -aminoisobutyric acid by uteri before and after a preincubation period showed the same general patterns of sensitivity to competitive inhibitors, K^+ , pH, temperature and 2,4-dinitrophenol. 4. The results suggest that the preincubation leads to an increase in a protein component of the 'A system' for amino acid transport in the uterus, and that metabolic energy is required for the reactions involved.

Our earlier studies on uptake of α -aminoisobutyric acid by the immature rat uterus *in vitro* revealed two unusual, related features: the presence of a lag in the uptake over the first 2 h of incubation and an increase in uptake of the amino acid after a preliminary incubation of the uterus in buffer at 37°C for several hours (Riggs *et al.*, 1968). Both effects can be attributed to a single cause, namely, that the tissue increases its ability to transport α -aminoisobutyric acid when it is incubated under these conditions. A further study of the phenomenon suggests that, during the incubation period, a metabolically dependent change occurs in the uterus that leads to an increase in the maximum capacity for transport of α -aminoisobutyric acid. The details of these and related experiments are reported here.

Materials and Methods

The techniques used were similar to those described in the first report of this series (Riggs *et al.*, 1968). Immature female rats (Sprague–Dawley–Spartan strain, Spartan Research Animals, Haslett, Mich., U.S.A.) were injected intraperitoneally with 1 μg of oestradiol-17 β in 0.1 ml of 25% ethanol in 0.9%

sodium chloride, or with the solvent alone. At 1 or 3 h later, the animals were decapitated and the uteri removed for incubation in buffered media of varying composition in 50 ml Erlenmeyer flasks. After a preliminary incubation of 1–5 h without added amino acid, each uterus was removed from its flask, blotted lightly on filter paper and transferred to a second flask containing fresh buffer plus ^{14}C -labelled amino acid. Transport was then studied by incubation of this flask under the desired conditions. The preincubations and incubations were carried out in Krebs–Ringer medium, or modifications of it, buffered with either bicarbonate, phosphate or tris. The gas phase was generally $\text{O}_2 + \text{CO}_2$ (95:5, v/v) when bicarbonate was used; 100% O_2 was used with the other buffers. Other details of experimental procedures and calculation of results were the same as reported previously (Riggs *et al.*, 1968).

Results

Variation in rate of uptake of α -aminoisobutyric acid with the time of incubation

The lower line of Fig. 1 shows that α -aminoisobutyric acid enters the uterus much more slowly over

Fig. 1. Effect of preincubation of the uterus on the subsequent uptake of α -aminoisobutyric acid

Uptake without preincubation (○) was obtained by incubating uteri with 5 mM- α -aminoisobutyric acid in oxygenated bicarbonate medium for the time shown on the abscissa. The preincubated uteri (●) were all incubated for a total of 2 h under the same general conditions, the time on the abscissa being the period during which uptake was measured after transfer to medium containing 5 mM- α -aminoisobutyric acid. The 2 h point is therefore common to the two curves. Two to six values were averaged for each point.

the first 30 min of incubation than it does over the second period of 30 min. In the experiment shown, the distribution ratio after 1 h was sevenfold that given at 0.5 h. On the other hand, if tissues were preincubated at 37°C in normal Krebs-Ringer bicarbonate medium before they were incubated in fresh, α -aminoisobutyric acid-containing medium, the uptake was markedly increased. The upper line of Fig. 1 was obtained by incubating all the uteri for a total of 2 h, divided into a period of preincubation without amino acid and one of incubation with amino acid. A 90 min preincubation, followed by a 30 min incubation with α -aminoisobutyric acid, led to a distribution ratio fivefold that given if the uptake was measured for 30 min with uteri that had not been preincubated. When the total incubation period was constant, the shape of the uptake curve over 2 h (upper curve of Fig. 1) was more typical of that found in other cells and tissues.

Effect of preincubation of the uterus on subsequent uptake of different amino acids

Preincubation of uteri in normal Krebs-Ringer bicarbonate buffer for 1–6 h led to large increases in the transport not only of α -aminoisobutyric acid

Fig. 2. Effect of length of preincubation period on the uptake of α -aminoisobutyric acid by the uterus

Uptake of α -aminoisobutyric acid from a 1 mM solution in normal Krebs-Ringer bicarbonate buffer, to which the uteri were added after the preincubation period, was measured for 1 h. Oestradiol-primed tissues were removed from rats injected with 1 μ g of the hormone 3 h earlier. Four to eight values were averaged for each point. Δ , $\circ$, Choline chloride and choline bicarbonate replaced NaCl and NaHCO₃ in preincubation media. $\blacktriangle$, $\bullet$, Preincubation in buffer containing 143 mequiv. of Na⁺/l. $\triangle$, $\blacktriangle$, Oestradiol-primed; $\circ$, $\bullet$, controls.

(upper two lines of Fig. 2) but also of L-proline, L-alanine and L-aminocyclopentanecarboxylic acid (Table 1). The effect was shown in the 3 h oestradiol-primed tissues as well as in the controls. The maximum effect on uptake of α -aminoisobutyric acid was given after preincubation for 3 h (Fig. 2). Uptake of L-valine, L-phenylalanine or L-leucine, in contrast, was not increased by the preincubation (Table 1). In fact, the distribution ratio for leucine was below normal in these experiments.

Effects of the composition of the preincubation medium on subsequent uptake of α -aminoisobutyric acid

In the experiments described below, changes were made only in the composition of the buffer used for the preliminary incubation. Unless otherwise stated, uptake of α -aminoisobutyric acid was measured at 37°C in a normal Krebs-Ringer bicarbonate medium, with O₂+CO₂ (95:5, v/v) as the gas phase.

Ionic composition and tonicity of the preincubation medium. The uptake of α -aminoisobutyric acid was not increased if Na⁺ was omitted from the preincubation medium (Fig. 2), the optimum concentration of Na⁺ being in the range 200–300 mequiv./l (Fig. 3).

Table 1. *Effect of prior incubation of the immature rat uterus on the subsequent uptake of various amino acids*

Both the initial and final incubations were carried out at 37°C in Krebs–Ringer bicarbonate buffer with O₂+CO₂ atmosphere. Amino acid (1 mM) was present in the final incubation period only. In oestradiol-primed samples, the hormone was injected into the rats 1 h before uteri were removed, except with phenylalanine, where 3 h was the period used. Values are averages (two to 14 per group). D.R., Distribution ratio. **P*<0.01, and ***P*<0.05, for change due to preincubation.

Amino acid	Time of preincubation (h)	Time with amino acid (h)	No oestradiol		With oestradiol	
			D.R.	Change due to preincubation (%)	D.R.	Change due to preincubation (%)
L-Proline	4	1	6.8	+298*	8.2	+136*
L-Proline	6	1	9.4	+450*	9.6	+174*
L-Alanine	4	1	12.73	+83*	—	—
1-Aminocyclopentane-carboxylic acid	1	2	3.24	+76*	5.06	+87*
	4	2	5.54	+201*	7.43	+174*
L-Valine	5	1	1.21	+6	—	—
L-Phenylalanine	3	1	1.47	+5	1.50	+10
L-Leucine	4	4	0.51	-63**	—	—

Fig. 3. *Effect of varying the Na⁺ concentration of the preincubation medium on the subsequent uptake of α -aminoisobutyric acid by the immature rat uterus*

Uteri were preincubated for 4 h at 37°C in Krebs–Ringer bicarbonate medium, which had been modified to have the [Na⁺] shown on the abscissa. The uteri were then incubated for 2 h at 37°C in fresh, normal Krebs–Ringer bicarbonate medium containing 1 mM- α -aminoisobutyric acid. Choline chloride (118 mM) replaced NaCl in the media containing 25 mequiv. of Na⁺/l. Uptakes are shown for uteri from rats injected 1 h earlier with 1 μ g of oestradiol (▲) and for uteri not pretreated with oestradiol (●). Four to ten values were averaged for each point.

Preincubating the uteri for 3 or 4 h in normal medium containing 143 mequiv. of Na⁺/l resulted in increases in uptake even though the subsequent transport was

measured from a medium containing only 4 mequiv. of Na⁺/l (1 h distribution ratios of 3.82 ± 0.06 versus 1.60 ± 0.05 , *P*<0.001, for control tissues; 5.09 ± 0.25 versus 2.15 ± 0.06 , *P*<0.001, for 3 h oestradiol-primed samples, with and without a 4 h preincubation respectively; cf. Table 4).

Uptake of α -aminoisobutyric acid is higher than normal when hyperosmotic media are used (T. R. Riggs, M. W. Pan & H. W. Feng, unpublished work). We therefore examined the effect of osmoticity during the preincubation period to determine the extent to which the increases shown at the high Na⁺ values of Fig. 3 were due to hyperosmoticity. The data of Table 2 suggests that both Na⁺ and Cl⁻ concentration influenced the subsequent transport, but that hyperosmoticity, as such, probably had no effect. A hyperosmoticity produced by sucrose did not change subsequent uptake significantly, whether the Na⁺ and Cl⁻ concentrations were normal or increased (Table 2, lines 4 and 5 versus line 3; line 9 versus line 8). Raising [Cl⁻] independently of [Na⁺] led to increased uptake (compare lines 1 and 2; lines 5 and 7). The maximum effect was apparently given at about 190 mequiv. of Cl⁻/l, since 250 mequiv./l did not result in a further increase (line 9 versus line 11). On the other hand, raising [Na⁺] from 143 to 203 mequiv./l at a constant [Cl⁻] did not increase uptake (line 7 versus line 11), and increasing [Na⁺] from 203 to 264 mequiv./l gave a highly significant elevation (line 11 versus line 12). It should be pointed out, in addition, that the effects of raising [Na⁺] from 0 to 143 mequiv./l (Figs. 2 and 3) cannot be attributed to differences in [Cl⁻], since the latter was held constant in these experiments.

The results in Table 2 suggest that Li⁺ was inhibitory when it was added during the preincubation

Table 2. *Effect of hyperosmoticity of the preincubation medium on subsequent uptake of α -aminoisobutyric acid*

Preincubation was for 4 h at the Na^+ and Cl^- concentrations and osmoticities indicated. Uptake of α -aminoisobutyric acid was measured in each case from a 1 mM solution in a 2 h incubation in normal Krebs-Ringer bicarbonate medium with 143 mequiv. of Na^+/l . Distribution ratios are averages $\pm$ s.e.m. (four to ten samples in each of the different groups). n.s., Not significant ($P > 0.05$).

Expt. no.	$[\text{Na}^+]$ (mequiv./l)	$[\text{Cl}^-]$ (mequiv./l)	Osmoticity (mosm)	Hyperosmotic agent	Distribution ratio	P
1	25	10	550	Sucrose (478 mm)	2.99 ± 0.18	—
2	25	249	550	LiCl (239 mm)	7.99 ± 0.50	<0.001 vs. Expt. 1
3	143	128	308	None	10.42 ± 0.98	—
4	143	128	428	Sucrose (120 mm)	11.54 ± 1.10	n.s. vs. Expt. 3
5	143	128	550	Sucrose (242 mm)	8.22 ± 0.78	n.s. vs. Expt. 3
6	143	248	550	LiCl (120 mm)	10.38 ± 0.56	n.s. vs. Expt. 5
7	143	248	550	Choline chloride (120 mm)	13.94 ± 0.62	{ <0.001 vs. Expt. 5 <0.001 vs. Expt. 6
8	203	188	428	NaCl	16.81 ± 1.03	—
9	203	188	550	NaCl + sucrose (129 mm)	14.28 ± 0.76	n.s. vs. Expt. 8
10	203	252	550	NaCl + LiCl (64 mm)	8.74 ± 0.37	<0.001 vs. Expt. 9
11	203	248	550	NaCl + choline chloride (60 mm)	13.88 ± 0.51	{ n.s. vs. Expt. 9 <0.001 vs. Expt. 10
12	264	249	550	NaCl	23.07 ± 0.96	<0.001 vs. Expt. 11

Fig. 4. *Effect of type and pH of buffer used during the preincubation period on the uptake of α -aminoisobutyric acid*

Uteri were preincubated for 4 h at 37°C with Krebs-Ringer media buffered with 0.01 M-sodium phosphate (○), HCO_3^- - CO_2 (●) or 0.1 M-tris (△), and then incubated for 2 h in fresh Krebs-Ringer bicarbonate buffer, pH 7.4, containing 1 mM- α -aminoisobutyric acid. ---, Distribution ratio given by untreated uteri incubated for 2 h in Krebs-Ringer bicarbonate medium, pH 7.4. Four to ten values were averaged for each point.

period. In no case was uptake of α -aminoisobutyric acid as great after preincubation of the tissues in LiCl as when an equivalent amount of choline chloride was

used (Table 2). Nearly the same distribution ratio was given when Li^+ was added at any of the Na^+ concentration tested (25, 143 or 203 mequiv./l), even though $[\text{Li}^+]$ varied inversely with $[\text{Na}^+]$.

Ca^{2+} and Mg^{2+} were also found necessary to produce the maximum effect of preincubation (Table 3, line 12). In addition, phosphate, acetate and bromide were respectively two-thirds, one-half and one-sixth as effective as chloride when Na^+ was added to a concentration of 264 mequiv./l.

Type and pH of the buffer in the preincubation medium. Fig. 4 shows the effect of preincubation in each of three different buffers and over the pH range 6.2–8.6. At pH 7.4, 0.1 M-tris led to the greatest increase, but at pH 8.0 bicarbonate was superior. A 0.01 M-tris buffer was without effect at pH 7.4, and even 0.1 M-tris was much less effective at pH values above and below 7.4. This latter result may have been due to an inhibition by the tris; when 0.1 M-tris was added to the normal bicarbonate buffer at pH 8.0, the uptake was not significantly greater than that found in fresh tissues. A 0.01 M-sodium phosphate buffer was least effective of the three used at all pH values (Fig. 4). However, when 0.01 M-sodium phosphate was added to bicarbonate at pH 6.8, the result was a greater increase in uptake than given by either one alone (distribution ratios of 11.18 ± 0.69 and 8.31 ± 0.63 respectively in bicarbonate buffer with and without phosphate; $P < 0.01$). Air also served as well as 100% O_2 with the phosphate buffer, especially if glucose was added to the medium. Preincubation in 0.1 M-sodium phosphate buffer led to an α -aminoisobutyric acid distribution ratio of 18 in a subsequent 2 h incubation, but this high value could

Table 3. *Effect of preincubation under various conditions on uptake of α -aminoisobutyric acid*

All uteri, except in Expt. 1, were preincubated for the indicated time in normal Krebs–Ringer bicarbonate or sodium phosphate buffer (0.01 M), pH 7.4, under the conditions indicated and, unless otherwise stated, at 37°C under O₂. They were then removed, blotted lightly, added to fresh Krebs–Ringer bicarbonate buffer containing 1 mM- α -aminoisobutyric acid, and incubated for 2 h. For the control (Expt. 1), fresh uteri were incubated for 2 h with α -aminoisobutyric acid. Distribution ratios are averages $\pm$ S.E.M. (three to 22 samples in each group). n.s., Not significant.

Expt. no.	Preincubation conditions		Distribution ratio	P (vs. Expt. no. 1)	P
	Time (h)	Buffer Other			
1	0	—	5.68 $\pm$ 0.33	—	—
2	4	HCO ₃ ⁻ -CO ₂ O ₂ , 37°C	10.42 $\pm$ 0.98	<0.001	
3	4	HCO ₃ ⁻ -CO ₂ 0.5 mM-Dinitrophenol	3.38 $\pm$ 0.22	<0.01	
4	4	HCO ₃ ⁻ -CO ₂ 5 mM-CN ⁻	1.25 $\pm$ 0.29	<0.001	
5	1	Phosphate N ₂ , 37°C	7.17 $\pm$ 0.49	n.s.	
6	2	Phosphate N ₂ , 37°C	4.88 $\pm$ 0.37	n.s.	
7	4	Phosphate N ₂ , 37°C	1.24 $\pm$ 0.09	<0.001	
8	4	Phosphate N ₂ + glucose	1.99 $\pm$ 0.03	<0.001	<0.001 vs. Expt. 7
9	2	HCO ₃ ⁻ -CO ₂ N ₂ , 37°C	6.36 $\pm$ 0.44	n.s.	<0.05 vs. Expt. 6
10	4	Phosphate N ₂ , 1°C	6.29 $\pm$ 0.23	n.s.	<0.001 vs. Expt. 7
11	4	HCO ₃ ⁻ -CO ₂ O ₂ , 1°C	5.04 $\pm$ 0.32	n.s.	<0.001 vs. Expt. 2
12	4	HCO ₃ ⁻ -CO ₂ Ca ²⁺ -Mg ²⁺ -free	7.75 $\pm$ 0.44	<0.01	<0.05 vs. Expt. 2

be attributed to the effect of the increased [Na⁺] in the solution.

Effects of other agents and conditions during the preincubation period

Cyanide, 2,4-dinitrophenol or anaerobiosis during the preincubation period led to a great decrease in the uptake of α -aminoisobutyric acid (Table 3). The inhibiting agents were added only to the preincubation media and not to the buffers from which uptakes were measured. The loss of uptake was dependent upon the length of the preincubation period, as shown in the experiments in which nitrogen was used. Bicarbonate was better than phosphate when the preincubation was anaerobic; and the addition of 10 mM-glucose gave slight but statistically significant protection against loss of activity. When the preincubation was carried out at 1°C, the subsequent uptake of α -aminoisobutyric acid was the same as in fresh tissues, whether the gas phase during the preincubation period was O₂ or N₂. Separate experiments showed that incorporation of ¹⁴C from L-leucine into protein over a 2 h period after a 4 h preincubation under N₂ at 37°C was one-twentieth of that when the preincubation was carried out at 1°C.

Tests of the possibilities that tissue damage or the removal of toxic materials might account for the increased uptake in preincubated uteri

Peckham & Knobil (1962) have reported that uptake of α -aminoisobutyric acid by isolated rat

diaphragm could be increased markedly by cutting the tissue or by crushing it with a stirring rod. We therefore tested the possibility that the increased uptake of amino acids found after preincubation was a result of degeneration of the uterus. Six uteri were crushed to varying degrees with a stirring rod, and uptake was then measured by incubating them for 2 h with 1 mM- α -aminoisobutyric acid. Uptake was decreased in all cases, the distribution ratios varying from 0.93 to 4.44 in the six samples, with an average of 2.59 $\pm$ 0.47. This value was significantly below ($P < 0.001$) that given by uncrushed tissues (Table 3).

The possibility was also examined that the preincubation fluid had removed material from the uterus that normally inhibited amino acid uptake. The usual procedure, that of placing the preincubated uterus in fresh buffer to measure amino acid uptake, was therefore modified to test the effect of the preincubation fluid on subsequent uptake. In one set of experiments, uteri were preincubated for 3 h in Krebs–Ringer bicarbonate buffer, and this preincubation fluid was then used to measure uptake of α -aminoisobutyric acid into fresh uteri for 1 h. Distribution ratios averaged 2.40 and 2.89 for control and 3 h oestradiol-primed uteri respectively (two values each). These values compare with averages of 3.47 and 5.22 for uptake into untreated uteri with fresh buffer. In a second test, uteri were preincubated for 4 or 5 h in buffer, and α -aminoisobutyric acid was then added to the same flasks for measurement of uptake. Distribution ratios after 1 or 2 h in these tissues were two- to three-fold the values in comparable ones incubated in fresh medium without the preincubation.

Comparison of the effects of puromycin added during and after the preincubation period

Puromycin (0.2mM) prevented the increase in subsequent uptake of α -aminoisobutyric acid when added during preincubation in normal medium, but it had no effect when added only during the uptake period (Table 4). The effect was seen whether the final incubation was carried out at 143 or 4 mequiv. of Na^+ /l (Table 4). The small decrease in uptake found after preincubation in low- Na^+ buffer (Fig. 2 and Table 4: line 1 versus line 4 in column 3) was prevented by puromycin (Table 4). The values shown in Table 4 were obtained with unprimed uteri. The same qualitative effects were given with tissues primed with oestradiol for 3h.

The data of Table 4 show also that uptake of α -aminoisobutyric acid by the preincubated uterus was greatly depressed when it was measured at a medium $[\text{Na}^+]$ of 4 mequiv./l, whether the preincubation was carried out in the absence or presence of puromycin (compare lines 2 and 6, columns 3 and 4 respectively).

Additional characteristics of the uptake of α -aminoisobutyric acid by preincubated uteri

The above results showed that preincubated uteri had several of the characteristics of uptake of α -aminoisobutyric acid shown by fresh uteri: the uptake was decreased when a low- Na^+ buffer was used; oestradiol-priming increased uptakes of the same amino acids; uptake of α -aminoisobutyric acid was little affected by a 1h incubation with puromycin.

Only the shape of the time-curve was altered. A number of other agents and conditions were therefore used to alter uptake of α -aminoisobutyric acid by preincubated tissues, so as to compare their effects with those found in fresh uteri. This comparison is shown in the last two columns of Table 5. Essentially the same effects were given by all conditions except the 2h incubations with glycine, valine or phenylalanine. In the fresh uterus, the inhibition of uptake of α -aminoisobutyrate produced by these three amino acids was much greater after a 2h incubation than after 1h (Riggs *et al.*, 1968). In the preincubated uterus, however, the percentage inhibition was the same at both times (Table 5). This difference accounts for the lack of correspondence between the inhibition by these amino acids in the 2h incubation when preincubated uteri are compared with fresh uteri. The inhibition produced in 1h was the same in the two types of tissue (Table 5).

Kinetic parameters of uptake of α -aminoisobutyric acid

Uptake into both control and oestradiol-primed preincubated uteri was measured at concentrations of α -aminoisobutyric acid ranging from 0.5 to 10mM and the results were compared with those found for fresh uteri. The experiment was repeated with 0.2mM-puromycin in the preincubation medium, and a third series was run in which the puromycin was added during the uptake of α -aminoisobutyric acid by fresh uteri. The non-saturable entry, K_m and V_{max} , were calculated in each case by using the methods of Akedo & Christensen (1962). The results (Table 6)

Table 4. *Inhibition of the preincubation effect by puromycin*

Uteri were preincubated as described in columns 2 and 3, and then incubated for 1h with 1mM- α -aminoisobutyric acid at the Na^+ concentration shown in column 1. The concentration of puromycin was 0.2mM. Values are averages (four to eight for each group, except where noted in parentheses).

[Na ⁺] in incubation (mequiv./l)	Preincubation time (h)	[Na ⁺] in preincubation (mequiv./l)	Distribution ratio in flask			
			Puromycin absent	Puromycin present		Throughout
				At preincubation only	At incubation only	
143	0	No preincubation	3.47 ± 0.16	—	2.93 ± 0.27	—
143	3	143	7.68 ± 1.17	3.08 ± 0.06*	9.37 ± 0.45	—
143	4	143	7.68 ± 0.81	—	—	3.26 ± 0.17*
143	3	Na ⁺ -free	2.45 ± 0.17	4.00 ± 0.14*	—	—
4	0	No preincubation	1.60 ± 0.05	—	1.48 ± 0.15	—
4	3	143	2.50 ± 0.11 (2)	1.51 ± 0.05*	—	—
4	4	Na ⁺ -free	1.75 ± 0.10	—	—	1.80 ± 0.08

* Difference from control (column 3) is statistically significant ($P < 0.01$).

Table 5. *Effects of various amino acids, K^+ and other conditions on uptake of α -aminoisobutyric acid in uteri previously incubated for 4 h in normal Krebs-Ringer bicarbonate buffer*

The inhibitor or altered condition was present only during the incubation with 1 mM- α -aminoisobutyric acid, which followed the preincubation. The competing amino acid was present at 20 mM. The percentage inhibition of uptake of α -aminoisobutyric acid by the various agents or conditions in 1 or 2 h without the preincubation period is given by Riggs *et al.* (1968). Values are averages of four for each group.

Condition or agent present	Incubation time ...	Percentage change in distribution ratio in preincubated uterus		Relative percentage change in preincubated uterus vs. control†	
		1 h	2 h	1 h	2 h
L-Methionine	—	—	-95	—	96
α -Aminoisobutyric acid	—	—	-89	—	102
L-Proline	—	—	-81	—	101
Glycine	—	-51	-49	111	67
L-Valine	—	-31	-39	97	57
L-Phenylalanine	—	+2	-8	97	19
L-Leucine	—	—	+9	—	‡
L-Lysine	—	—	+6	—	‡
K^+ (124 mequiv./l)	—	-75	-89	95	—
Phosphate buffer, pH 7.4	—	—	-19	—	90
Phosphate buffer, pH 6.8	—	—	-24*	—	89
Phosphate buffer, pH 5.4	—	—	-70*	—	104
0.5 mM-Dinitrophenol	—	—	-72	—	122
Incubation at 23°C	—	—	-85	—	88

* Relative to value given in phosphate buffer, pH 7.4.

† $\left(\frac{\% \text{ change in distribution ratio in preincubated uterus}}{\% \text{ change in distribution ratio in fresh uterus}} \right) \times 100$

‡ Compare values in column 3 with -27% for L-leucine and +37% for L-lysine in fresh uterus.

Table 6. *Kinetics of uptake of α -aminoisobutyric acid into preincubated uteri*

Preincubated samples were treated for 3 h at 37°C in Krebs-Ringer bicarbonate buffer with or without 0.2 mM-puromycin. They were then incubated for 1 h in fresh buffer, without puromycin and with α -aminoisobutyric acid over the range 0.5–10 mM. Oestradiol-primed tissues were taken from animals that had been injected with 1 μ g of the hormone 3 h earlier. 24–30 values were used in the calculations of K_m and V_{max} under the various conditions. The oestradiol by itself had no significant effect on K_m under any of the conditions ($P > 0.05$). Non-saturable entry was statistically the same in all eight cases.

Condition	Oestradiol	Non-saturable entry (per h)	K_m (mM)	V_{max} (mmol/h per kg of cell water)	P (for change in V_{max})
No preincubation	—	0.4	1.0	6.5	<0.001
No preincubation	+	0.3	1.1	10.8	
Preincubation	—	0.3	0.7	13.4*	<0.001
Preincubation	+	0.2	1.2	27.2*	
Preincubation with puromycin	—	0.3	0.5	5.8†	<0.02
Preincubation with puromycin	+	0.4	0.5†	7.2†	
No preincubation; incubation with puromycin	—	0.4	0.3†	2.8†	<0.001
	+	0.3	0.5†	7.2†	

* Significantly greater than the value obtained without the preincubation.

† Significantly less than the comparable value obtained in the absence of puromycin.

show that the preincubation led to an increased $V_{\max}$ of uptake ($P < 0.001$) without a change in K_m ($P > 0.3$). Puromycin added during the preincubation period prevented the rise in $V_{\max}$. The results also show that puromycin produced a significant decrease in $V_{\max}$ ($P < 0.01$) and K_m ($P < 0.05$) to below values found in fresh uteri (compare especially lines 1 and 2 with lines 7 and 8, Table 6).

Discussion

The change in transport capacity of the uterus during incubation can explain the time-lag in the uptake curve shown in Fig. 1 (also apparent in Fig. 3 of Riggs *et al.*, 1968) and in the time-curve for amino acid uptake by the uterus reported by Roskoski & Steiner (1967). The time-curve of uptake swings upward over approximately the first 2 h because the rate of entry of amino acid is increased as the incubation proceeds. It is clear that the capacity for uptake of affected amino acids will depend upon the length of the incubation period. For this reason, the true transport kinetics of the uterus, as removed freshly from the animal, cannot be determined *in vitro* for α -aminoisobutyric acid and related amino acids unless some agent such as puromycin is added to prevent the rapid increase in rate of transport. The uptakes of the various amino acids likewise cannot be compared quantitatively under other conditions, since they vary greatly in their sensitivities to the incubation (see Table 1).

Several possible explanations for the phenomenon need to be considered. First is whether the preincubation increases the uptake by washing substances out of the tissue. The possible removal of inhibitory material is suggested by the fact that addition of preincubation medium to fresh uteri produced some inhibition of uptake, especially in oestrogen-primed tissues. In contrast with this result, however, is the finding that uptake into the preincubated uterus was increased as much when it was measured from the preincubation medium as when fresh buffer was used. In addition, the rate of uptake of amino acid increased with time of incubation of uteri in the same buffer, as is demonstrated by the results in Fig. 1. It seems more likely that the inhibition produced by the preincubation medium was due to other factors, e.g. accumulation of metabolites such as lactate. Nor does it seem probable that the increased uptake is a result of washing endogenous free amino acids from the cells. The removal of free amino acids would be expected, if anything, to decrease, rather than increase, the uptake of an added amino acid, since it would decrease the possibility of exchange diffusion. Furthermore, it is unlikely that the release of either inhibitors or amino acids from the uterus would depend upon all of the conditions and factors needed to produce the increased uptake.

Tews *et al.* (1970) have proposed that an increase in tissue concentrations of cyclic AMP (adenosine 3':5'-cyclic monophosphate) may be responsible for the elevated uptake of α -aminoisobutyric acid they observed after preincubation of rat liver slices. The nucleotide was found to stimulate uptake of the amino acid in both fresh and preincubated slices. A similar explanation is possible for the increased transport in the uterus, especially in view of the finding of Griffin & Szego (1968) that cyclic AMP can increase uterine uptake of leucine and lysine *in vitro*. However, we were unable to find an increase in leucine uptake after preincubation (Table 1).

Whether or not cyclic AMP is involved, our results suggest that metabolic reactions are necessary for the increased transport and that these reactions lead to an increase in either the efficiency or amount of an amino acid carrier system in the uterus. The requirement for metabolism is suggested both by the high sensitivity of the transport change to composition and pH of the preincubation medium and by the elimination of the effect when the preincubation was carried out at 1°C or in the presence of metabolic inhibitors. The buffer concentrations of Na^+ , Cl^- and Ca^{2+} - Mg^{2+} during the preincubation period all influenced subsequent amino acid transport. The need for Na^+ during the preincubation, on the other hand, appeared to be independent of its requirement for maximum uptake of α -aminoisobutyric acid. Uptake was low from a low- Na^+ buffer even after tissues had been preincubated in media with a normal $[\text{Na}^+]$ (Table 4).

When cyanide, dinitrophenol or nitrogen was used, uptake of α -aminoisobutyric acid was less than that given by uteri not preincubated, even though the agent was added only during the preincubation period. After anaerobiosis, there would be no carry-over of the inhibitory condition into the incubation medium, as would be expected with cyanide or dinitrophenol, to inhibit the transport directly (see Riggs *et al.*, 1968). The results especially with nitrogen therefore suggest destruction of one or more components of the transport system present in the fresh tissue. The destroyed substance may itself be a protein, or it may be a non-protein factor needed for synthesis of protein as well as for uptake of α -aminoisobutyric acid. Resynthesis of the protein or other factor can occur under oxygen, but it cannot take place after incubation without oxygen, as shown by the great decrease in incorporation of $[^{14}\text{C}]$ -leucine into protein after a 4 h preincubation under nitrogen at 37°C. Breakdown of the factor does not occur if the anaerobic preincubation is carried out at 1°C, however, since uptake is normal and incorporation is high after this treatment. Metabolism is apparently needed for the breakdown as well as for the synthesis, and the former but not the latter can occur anaerobically. Once the substance is broken down during the long incubation under nitrogen at

37°C, very little is re-formed even during a subsequent hour under oxygen, as shown by the low uptake of α -aminoisobutyric acid and greatly decreased leucine incorporation under these conditions.

The results obtained in the presence of puromycin would at first seem to rule out the possibility that a protein component of the transport system was being destroyed under anaerobiosis. Preincubation of uteri for up to 4 h with puromycin did not change uptake of α -aminoisobutyric acid from that given by fresh tissues. The puromycin was used at a concentration that we had shown to inhibit incorporation of leucine into protein by 95% (Riggs *et al.*, 1968). These facts suggest that protein synthesis is not needed over 4 h for normal uptake, and therefore that no breakdown of transport protein has occurred. The kinetic data of Table 6 show, however, that puromycin decreased the V_{max} of uptake of α -aminoisobutyric acid significantly. The distribution ratio was essentially unchanged, because the K_m was also lowered. If the V_{max} can be used as an index of the amount of carrier present, as we have suggested (Riggs *et al.*, 1968), we may conclude that the transport system originally present in fresh uteri had been lost to some extent during the preincubation and that puromycin had prevented its recovery. The results suggest a turnover of transport protein in the uterus at a rate similar to that reported for comparable protein in rat diaphragm (Kostyo & Redmond, 1966; Elsas *et al.*, 1967).

On the other hand, the results with puromycin show that protein synthesis was required if the preincubation were to lead to an increased transport capacity. The preincubation elevated the V_{max} of uptake of α -aminoisobutyric acid without changing the K_m , and puromycin could prevent this rise in V_{max} . These results provide good evidence that one or more protein components of a carrier system are formed during the preincubation period, to account for the increased transport.

The change in transport capacity during preincubation also showed a specificity typical of metabolism, in that the uptake was increased for only some of the amino acids tested. The results suggest that the increase in carrier protein altered amino acid transport by only the 'A system' (Oxender & Christensen, 1963). This conclusion is based, first, on the fact that the affected amino acids are all short-chain, more-polar, ones; all inhibit uptake of α -aminoisobutyric acid (Riggs *et al.*, 1968) and all require buffer Na^+ for maximum uptake (T. R. Riggs, M. W. Pan & H. W. Feng, unpublished work). The 'A system' has been designated largely on the

basis of these characteristics. In addition, the characteristics of uptake of α -aminoisobutyric acid were found to be essentially the same in the preincubated as in the fresh tissue. Finally, preincubated tissues showed greater absolute response in uptake to injected oestradiol, and the preincubation affected uptakes of only those amino acids whose transports were oestradiol-sensitive (Riggs *et al.*, 1968). These changes would not be expected together unless the preincubation-induced transport was by way of a system similar to one already present, and which was used extensively by α -aminoisobutyric acid. In particular, the responses to altered $[Na^+]$, pH and oestradiol suggest that the system in question is like the 'A system' found in fresh uteri.

These results may also explain the fact that oestradiol can stimulate uptake of the 'L-site' amino acids, L-valine and DL-norleucine, in a 4 h incubation, even though the evidence suggests that the hormone affects transport by only the 'A system' (Riggs *et al.*, 1968). Nearly all of the neutral amino acids can apparently use both 'A' and 'L sites' (see Christensen, 1966, for example); in fact, it has been difficult to discover an analogue with absolute specificity for one or the other. If a component of the 'A system' is increased during the incubation period, after 4 h the amount of valine and norleucine transported by the 'A system' may be large enough for a significant response to oestradiol to be detected.

This work was supported in part by Grant AM-03709 from the National Institutes of Health, U.S. Public Health Service.

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