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THE RELATION BETWEEN L-METHIONINE UPTAKE AND SODIUM IN RAT SMALL INTESTINE *IN VITRO*

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SUMMARY

1. Uptakes of L-methionine and mannitol by rat jejunum *in vitro* were measured over test periods from 5 to 120 sec after 30 min pre-test periods in the presence or absence of Na.

2. The initial stage in methionine uptake was dependent on the presence of Na^+ and to a lesser extent on the K^+ concentration. In contrast mannitol uptake was independent of Na and K.

3. The initial stage in methionine uptake can be reactivated 30–60% within 5 sec by replacing an Na-deficient intestine into an Na-containing medium.

4. Initial methionine uptake was greater with a normal intracellular and low medium Na concentration than with a high medium and low intracellular Na concentration. It is suggested that the intracellular Na concentration is a critical factor, more important than the Na gradient, in determining the rate of amino acid transfer across the luminal membrane.

INTRODUCTION

The involvement of Na in the intestinal transfer of non-electrolytes is well established, but in spite of various interesting hypotheses, the precise role of sodium is still unknown. Recently Schultz, Curran, Chez & Fuisz (1967) have approached the problem by measuring amino acid entry into intestinal mucosa over very short periods in an attempt to study the influx of amino acid across the luminal membrane. The present experiments are fundamentally an extension of this work, but involve the use of a different technique, different animal species, a different amino acid, different part of the intestine, and, perhaps more important, much shorter periods of influx, the shortest being as low as 5 sec. Since the object is to study amino

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acid influx unaffected by subsequent movement of the amino acid, the shorter the time interval the better. These studies have in some ways confirmed the observations of Schultz *et al.* (1967), but in other ways differ and our interpretation of the results is fundamentally different.

METHODS

Rationale

The rationale of the experiments was to produce different Na gradients across the luminal membrane of the epithelial cell, and to see how this affected influx of methionine. The gradient across the luminal membrane depends on the concentration of Na in the surrounding medium and the concentration of Na inside the cell. The former can be changed quantitatively as desired. The intracellular Na concentration is not under accurate control but can be changed by incubating the tissue for a preliminary period in solutions with different Na concentrations. The assumption is made that a piece of gut incubated in saline with 143 m-equiv Na/l. will maintain its internal Na concentration, while a piece of gut incubated in Na free saline will lose Na, thus reducing the intracellular concentration. Evidence for this has been obtained by Bosakova & Crane (1965) and by Schultz, Fuisz & Curran (1966). Hence the experiments involved incubating sacs of everted intestine for two periods, a pre-test period of 30 min without methionine, and a test period during which methionine influx was measured, and which varied from 5 to 120 sec. If the Na concentration was 143 m-equiv/l. in the medium in both the pre-test and test periods the Na gradient during the test periods was considered to be 'normal'. If the Na concentration was zero in the pre-test period and 143 m-equiv/l. in the test period, it was considered that during the test period the Na concentration would be greater in the medium than in the cell and this is regarded as a downhill gradient of Na across the luminal membrane (an exaggeration of the normal Na gradient). If the Na concentration was 143 m-equiv/l. in the pre-test period and zero in the test period, the Na concentration in the test period would be smaller in the medium than in the cell, and this is regarded as an uphill gradient of Na across the luminal membrane (a reversal of the normal Na gradient). The terms downhill Na gradient and uphill Na gradient are used in these senses in subsequent discussion.

Technique

Unfasted male white rats of the Sheffield strain weighing between 230 and 270 g were used. In all experiments everted sacs of the middle fifth of the small intestine were used 17 cm in length and of weight 1–1·5 g. In both pre-test and test periods the sacs were shaken at 80 c/min at 38° C and with a gas phase of 95 % O_2/5 % CO_2. The saline was bicarbonate saline (Krebs & Henseleit, 1932) modified as described below.

In the pre-test period the sacs were incubated in flasks containing 25 ml. saline and contained initially 1 ml. of the same saline. At the end of the period, the sacs were removed quickly, touched on dry filter paper to remove adhering medium, and immediately immersed in saline for the test period. The time elapsing from withdrawal of the sac from the pre-test solution to immersing in the test solution was about 3 sec. The test solution consisted of 25 ml. saline of composition as described below and in addition either [*Me*-^{14}C]L-methionine or [1-^{14}C]D-mannitol in a concentration of 1 mM. L-methionine was used as an example of an amino acid known to involve a transport system which has been much studied. Mannitol was used as a substance which does not involve any specific transport mechanism. The test solutions were contained in beakers to allow rapid and easy access in the very short

periods used, and ligatures attached to the intestine enabled rapid and precise immersion into and removal from the test solution to be made. The sacs were left in the test solution for pre-determined time intervals ranging from 5 to 120 sec after which they were quickly removed and washed by immersing thrice in 100 ml. isotonic mannitol (300 mM) solution at 4–6° C. Washing occupied about 5 sec, and also served to retard any subsequent reaction. The serosal fluid was then drained off and the sacs after weighing to determine fluid uptake, were homogenized with 5 ml. each of 0·6 N-H_2SO_4 and 10% sodium tungstate and 15 ml. hot water. The homogenates were filtered and aliquots of the filtrates were added to scintillation fluid (Bray, 1960) and the samples counted by liquid scintillation. Uptakes as counts per minute per sac were converted to μ-mole per sac using an aliquot of the test solution itself as a standard.

Chemical. The [*Me*-^{14}C]L-methionine and [1-^{14}C]D-mannitol were obtained from the Radiochemical Centre, Amersham.

Saline. The electrolyte composition of the pre-test and test solutions varied in different experiments. The main object of the work was to study the effect of the Na gradient, and for this purpose Na was replaced with either choline or K, so that Na was either completely absent or present in a concentration of 143 m-equiv/l. A subsidiary interest was to see whether the effects of Na on amino acid transfer was affected by the concentration of K; and the concentration of K was either 0, 6 or 31 m-equiv/l. The composition of the different salines is shown in Table 1. At the highest concentration of K (31 m-equiv/l.) it is not possible to raise the concentration of K to this level and yet maintain a normal Na concentration and osmolarity. Accordingly, some of the experiments with this high concentration of K are complicated by the fact that the concentration of K is not the same in the pre-test and test period. They do nevertheless serve to show that the effect of Na gradient on the active uptake of methionine could be influenced by the concentration of K.

TABLE 1. Composition of saline used. Five different salines designated A, B, C, D and E

	A (mM)	B (mM)	C (mM)	D (mM)	E (mM)
NaCl	118	0	0	118	0
$NaHCO_3$	25	0	0	25	0
NaH_2PO_4	0	0	0	1·2	0
KCl	4·7	4·7	4·7	0	0
$KHCO_3$	0	0	25	0	0
KH_2PO_4	1·2	1·2	1·2	0	0
Choline-Cl	0	118	118	0	118
Choline-HCO_3	0	25	0	0	25
$MgSO_4$	1·2	1·2	1·2	1·2	1·2
$CaCl_2$	2·5	2·5	2·5	2·5	2·5
Total Na^+, m-equiv/l.	143	0	0	144	0
Total K^+, m-equiv/l.	6	6	31	0	0

RESULTS

Fig. 1 shows the uptake of L-methionine and of mannitol for various test periods in the presence and absence of Na. When pre-test and test solutions both contain Na (143 m-equiv/l.) and K (6 m-equiv/l.) (solution A,

Table 1) L-methionine uptake exceeded mannitol uptake by an increasing proportion as the test period was increased. In test periods of only 5 sec methionine uptake exceeded mannitol uptake by approximately twofold and this increased to approximately sevenfold with a test period of 120 sec. Fig. 1 also contains data from experiments in which sodium was absent from both pre-test and test solutions (solution B in Table 1). It is seen that mannitol uptakes are almost identical in the presence and absence of Na suggesting that mannitol uptake is not Na dependent. In contrast, the

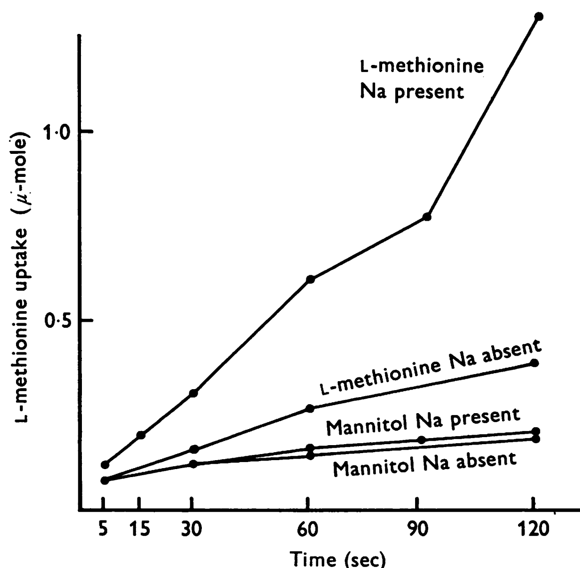

Fig. 1. Uptake of L-methionine or D-mannitol from 1·0 mM concentration by sacs of everted intestine. In all cases [K] was 6 m-equiv/l. and the absence or presence of Na in the pre-test and test periods was as indicated.

L-methionine uptakes were greatly reduced in the absence of Na. It is thus possible to divide the L-methionine uptake into two fractions, a Na dependent and a Na independent fraction. While the Na independent fraction of methionine uptake is much smaller than the Na dependent fraction, it is, nevertheless, somewhat greater than the mannitol uptake. This could be due to some small residual activity of the selective methionine transfer mechanism in the absence of Na from the external medium or alternatively, there is a possibility that methionine distributes passively in a larger space than does mannitol.

The results indicate that in the test periods used we are dealing with a process which has characteristics similar to those well established for

transport measured over much longer periods (30–60 min), e.g. the L-methionine uptake is much greater than the mannitol uptake, and unlike mannitol transfer it is Na dependent. Part of this uptake could be described as contamination of the surface of the gut with the test solution. This part is non-specific and does not involve any transport mechanism. In addition there can be an additional uptake or entry, which is related to the specific transport mechanism. In the case of methionine total uptake is measured, but it is only the specific entry or transport process which is of interest. This can be obtained from the total uptake in two ways. If uptake in the absence of Na is subtracted from the uptake in the presence of Na, a value is obtained which can be described as the Na-dependent methionine uptake. Another way is to subtract the mannitol uptake from the methionine uptake, and this gives a value which can be described as selective methionine uptake. Both of these methods have been used.

Effect of Na gradient on L-methionine uptake

In these experiments the uptake of L-methionine was investigated in conditions in which the Na gradient across the luminal membrane was changed as described in the Methods section, and the results are seen in Table 2. The test periods selected were 5, 30, 60 and 120 sec. Four different kinds of experiments were done: (1) normal Na concentration was present in pre-test and test periods; (2) Na was present in the pre-test period and absent in the test period; (3) Na was absent in the pre-test period but present in the test period; (4) Na was absent in both pre-test and test periods. These four types of experiments can be referred to briefly as (1) Na:Na, (2) Na:no Na, (3) no Na:Na and (4) no Na:no Na. In setting out the results the initial Na and K concentrations in the pre-test and test periods are shown. Reference to Table 1 will show the combinations of the different salines which were used. Three different concentrations of K were used, 0, 6 and 31 m-equiv/l. With 0 and 6 m-equiv/l. the initial K concentration was the same in the pre-test and test periods. As explained in the methods section this is not possible with the 31 m-equiv K/l.

A comparison of the effects of uphill and downhill Na gradients on the entry of methionine can be derived from Table 2 in the following way. The Na-dependent methionine uptake is obtained by subtracting the uptake in the absence of Na from that in the presence of Na. Thus, if we used the terminology suggested above, the 'no Na:no Na' values subtracted from the 'Na:Na' values give the Na-dependent uptake of methionine, when the Na gradient across the intestine is normal. The 'no Na:no Na' values subtracted from the 'Na:no Na' values give the Na-dependent uptake of methionine for an uphill Na gradient, and the 'no Na:no Na' values subtracted from the 'no Na:Na' values give the Na-dependent uptake of

TABLE 2. L-methionine uptake by sacs of everted intestine under different conditions of Na gradient. The figures given are the means ± S.E. expressed in μ-mole with the number of experiments in brackets

K concn. (m-equiv/l.)		Na concn. (m-equiv/l.)		Test period (sec)			
Pre-test	Test	Pre-test	Test	5	30	60	120
0	0	144	144	0·131 ± 0·003 (9)	—	0·700 ± 0·010 (2)	1·677 ± 0·044 (8)
0	0	144	0	0·122 ± 0·006 (5)	—	0·643 ± 0·077 (3)	1·185 ± 0·078 (8)
0	0	0	144	0·093 ± 0·003 (8)	—	0·485 ± 0·055 (2)	0·982 ± 0·057 (6)
0	0	0	0	0·069 ± 0·005 (4)	—	0·260 (1)	0·423 ± 0·017 (4)
6	6	143	143	0·114 ± 0·003 (20)	0·315 ± 0·009 (13)	0·622 ± 0·025 (19)	1·325 ± 0·064 (8)
6	6	143	0	0·111 ± 0·005 (6)	0·317 ± 0·009 (3)	0·488 ± 0·004 (4)	0·878 ± 0·031 (9)
6	6	0	143	0·093 ± 0·003 (15)	0·269 ± 0·015 (3)	0·507 ± 0·049 (3)	0·868 ± 0·050 (9)
6	6	0	0	0·061 ± 0·003 (8)	0·163 ± 0·021 (2)	0·258 ± 0·004 (2)	0·382 ± 0·010 (4)
6	31	143	0	0·099 ± 0·004 (10)	0·268 ± 0·008 (7)	0·438 ± 0·028 (4)	0·694 ± 0·030 (5)
31	6	0	143	0·077 ± 0·002 (8)	0·239 ± 0·011 (8)	0·445 ± 0·023 (6)	0·933 ± 0·050 (6)
31	31	0	0	0·058 ± 0·002 (6)	0·157 ± 0·006 (6)	—	0·341 ± 0·017 (2)

methionine for a downhill gradient. In making this correction the values used for Na-independent uptake at each period were the pooled values at different potassium concentrations. The values for Na-dependent methionine uptake calculated in this way are given in Tables 4, 5 and 6, which show the actual transfers with the normal gradient, the uphill gradient and the downhill gradient and also the latter two as a percentage of the transfer with the normal gradient.

TABLE 3. Mannitol uptake by sacs of everted intestine. The results are the means ± S.E. of all the mannitol experiments with the number of experiments in brackets

Test period (sec)	5	30	60	120
Mannitol uptake (μ-mole)	0·060 ± 0·001 (29)	0·112 ± 0·004 (15)	0·151 ± 0·001 (7)	0·185 ± 0·004 (25)

TABLE 4. Na-dependent and selective uptakes of L-methionine from 1·0 mM concentrations by sacs of everted intestine. The uptake is expressed as the mean ± S.E. and for convenience the percentage of the control values is also given. The initial K concentration was zero in all cases. For further explanation see text

		L-methionine uptake			
		Na-dependent		Selective	
Direction of Na gradient	Experimental period (sec)	μ-mole	% normal	μ-mole	% normal
Normal	5	0·070 ± 0·004	—	0·071 ± 0·003	—
Uphill	5	0·061 ± 0·006	87	0·062 ± 0·006	87
Downhill	5	0·032 ± 0·004	46	0·033 ± 0·003	46
Normal	60	0·442 ± 0·010	—	0·549 ± 0·010	—
Uphill	60	0·385 ± 0·077	87	0·492 ± 0·077	90
Downhill	60	0·227 ± 0·055	51	0·334 ± 0·055	61
Normal	120	1·288 ± 0·046	—	1·492 ± 0·044	—
Uphill	120	0·796 ± 0·079	62	1·000 ± 0·078	67
Downhill	120	0·593 ± 0·058	46	0·797 ± 0·057	53

The results can also be expressed as selective methionine uptake by subtracting the values for mannitol uptake from those for methionine uptake under the same conditions. In fact, as mannitol uptake varies very little with the changes in Na or K concentration it was considered reasonable to use the mean values shown in Table 3, for correction of the methionine uptake. The values for selective methionine uptake are also included in Tables 4, 5 and 6, and again the actual values are given, together with the transfers expressed as a percentage of the transfer with the normal gradient.

The results expressed as selective methionine uptake and Na-dependent

TABLE 5. Na-dependent and selective uptakes of L-methionine from 1·0 mM concentrations by sacs of everted intestine. The uptake is expressed as the mean ± S.E. and for convenience the percentage of the control values is also given. The initial potassium concentration was 6 m-equiv/l. in all cases. For further explanation see text

Direction of Na gradient	Experimental period (sec)	L-methionine uptake: Na-dependent μ-mole	Na-dependent % normal	Selective μ-mole	Selective % normal
Normal	5	0·053 ± 0·004	—	0·054 ± 0·003	—
Uphill	5	0·050 ± 0·006	94	0·051 ± 0·005	94
Downhill	5	0·032 ± 0·004	60	0·033 ± 0·003	61
Normal	30	0·157 ± 0·011	—	0·203 ± 0·010	—
Uphill	30	0·159 ± 0·011	101	0·205 ± 0·010	101
Downhill	30	0·111 ± 0·016	71	0·157 ± 0·015	77
Normal	60	0·364 ± 0·025	—	0·471 ± 0·025	—
Uphill	60	0·230 ± 0·004	63	0·337 ± 0·004	72
Downhill	60	0·249 ± 0·049	68	0·356 ± 0·049	76
Normal	120	0·936 ± 0·065	—	1·140 ± 0·064	—
Uphill	120	0·489 ± 0·033	52	0·693 ± 0·031	61
Downhill	120	0·479 ± 0·051	51	0·683 ± 0·050	60

TABLE 6. Na-dependent and selective uptakes of L-methionine from 1·0 mM concentrations by sacs of everted intestine. The uptake is expressed as the mean ± S.E. and for convenience the percentage of the control values is also given. The initial K-concentration was as indicated in the last three lines of Table 2. For further explanation see text

Direction of Na gradient	Experimental period (sec)	L-methionine uptake: Na-dependent μ-mole	Na-dependent % normal	Selective μ-mole	Selective % normal
Normal	5	0·053 ± 0·004	—	0·054 ± 0·003	—
Uphill	5	0·038 ± 0·004	72	0·039 ± 0·004	72
Downhill	5	0·016 ± 0·003	30	0·017 ± 0·002	31
Normal	30	0·157 ± 0·011	—	0·203 ± 0·009	—
Uphill	30	0·110 ± 0·010	70	0·156 ± 0·009	77
Downhill	30	0·081 ± 0·013	52	0·127 ± 0·012	63
Normal	60	0·364 ± 0·025	—	0·471 ± 0·025	—
Uphill	60	0·180 ± 0·028	49	0·287 ± 0·028	61
Downhill	60	0·187 ± 0·023	51	0·294 ± 0·023	62
Normal	120	0·936 ± 0·065	—	1·140 ± 0·064	—
Uphill	120	0·305 ± 0·032	33	0·509 ± 0·030	45
Downhill	120	0·544 ± 0·051	58	0·748 ± 0·050	66

uptake agree well, and only show small quantitative differences. At zero concentration of K, the selective methionine uptake and Na-dependent methionine uptake are greater with an uphill gradient than with a downhill gradient at all test periods. At K concentrations 6 and 31 m-equiv/l. the methionine uptake was greater with 5 sec and 30 sec periods, but less at 60 sec and variable at 120 sec periods. Thus at the 5 sec period in all cases there was greater Na-dependent methionine uptake or selective methionine uptake when the Na gradient was uphill than when it was downhill.

Another important feature is the initial very rapid recovery of the Na-deficient gut when immersed in Na saline. In the 5 sec period the recovery is already 30–60 % depending on the K concentration. The initial rate of recovery is not maintained and the methionine uptake has not reached the control value within the experimental period used.

DISCUSSION

These results confirm those of Schultz *et al.* (1967) in showing that the initial stage of entry of amino acids into the intestine is sodium sensitive. They go further by showing that even in the short period of 5 sec sensitivity to sodium can be detected. Incidentally, they also show that it is possible to get reproducible data using an interval for measurement as low as 5 sec.

The essential question defined by Schultz *et al.* (1967) pertains to the relative importance of extracellular and intracellular Na. They discussed this at some length and pointed out that the dependence of amino acid transfer on extracellular Na concentration would support the theory of Crane (1962), while dependence on a high intracellular Na concentration would support the views of Csaky (1963). The results of Schultz *et al.* (1967) with 60 sec periods were interpreted in favour of the importance of extracellular Na. In contrast, we interpret our results as favouring the importance of intracellular Na.

The essential criterion in coming to this conclusion is whether methionine movement is greater with a downhill Na gradient or an uphill Na gradient across the luminal membrane. Of all the test periods used, the 5 sec period probably reflects best the effects of the Na gradient. Our results with 5 sec intervals show in all cases that methionine movement is greater with an uphill Na gradient. In other words, provided a normal intracellular Na concentration is maintained the cell has the capacity to take up methionine even though Na is absent from the medium during the test. Conversely, if intracellular Na concentration is reduced the cell responds with a much diminished ability to take up methionine, even

though a high Na concentration is available outside the cell membrane. This indicates that the intracellular Na concentration is relatively more important than a downhill Na gradient for entry of methionine into the cell.

As mentioned the same interpretation can be made at all three K concentrations studied, although there appears to be certain quantitative differences arising at different K concentrations. This applies particularly to (*a*) the recovery of intestine in Na saline after pre-incubation in Na-free saline, and (*b*) the gradual loss of activity in Na-free saline after pre-incubation in Na saline. These changes may be related to the cellular turnover of Na being dependent on the K level, or the level of activity of the amino acid transfer mechanism being dependent on the precise relationships between the cellular Na and K concentration at the transfer site. The latter point is particularly relevant if an Na-K-dependent ATPase is involved in the entry process. Other workers have observed the significance of K in the over-all intestinal transport of non-electrolytes (Rummel & Stupp, 1960; Larralde, Bello & Fernandez-Otero, 1962; Crane, Forstner & Eichholz, 1965; Newey, Sanford & Smyth, 1968), and our results indicate that the involvement of K may be at the entry process.

In general our results indicate the importance of cellular Na, but we would stress two reservations about this conclusion. It is possible that after pre-incubating intestine in Na saline, enough Na remains on the surface of the cell to create a downhill gradient even after immersion in Na-free saline. It should be remembered, however, that excess fluid was blotted from the surface before the test period and also that any remaining Na would be quickly diluted by immersion of the gut in the Na-free saline. The other difficulty is that pre-incubation of intestine in Na-free saline could cause serious damage to the cell as found by Robinson & Felber (1966), and hence the subsequent capacity for methionine transfer in Na saline might have been reduced. Damage to the cell does in fact seem to take place since there is not complete recovery in methionine uptake from 60 to 120 sec. Our findings differ from those of Schultz *et al.* (1967) in this respect, since they found complete recovery of amino acid transfer after 60 sec. Later experiments by these workers (Goldner, Schultz & Curran, 1969), however, indicated incomplete recovery of sugar influx in these conditions. Regarding this point it is remarkable, and in this case we are in agreement with Schultz *et al.* (1967) that such a large recovery of amino acid transfer can occur within a short time of returning the Na-depleted intestine to a Na-containing medium. In our experiments about 50% recovery of amino acid uptake occurs within 5 sec indicating that in this short time enough Na enters to contact the Na-sensitive mechanism. This might be taken as evidence that the Na-sensitive mechanism is close

to the luminal boundary of the cell and could well be the brush border ATPases (for reference see Eichholz (1967), Berg & Szekerczes (1966), Rosenberg & Rosenberg (1968) and Richardson (1968)).

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