Active Transport of Choline Sulfate by Barley Roots [1, 2]

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Choline sulfate is a major product of sulfate metabolism in plants (9, 13). This sulfate ester accounts for up to one-third of the radioactivity in the soluble organic compounds formed after uptake of radiosulfate by plant roots. The analogous choline phosphate occurs in higher plants and is involved in phosphate transport (8). No similar role for choline sulfate as a carrier for sulfate could be detected, but the uptake of choline sulfate was found to be the result of active transport.

Studies on the active transport of choline sulfate by excised barley roots are described in this paper. Barley roots have been widely used in active transport studies and form considerable amounts of choline sulfate. The uptake of this zwitterion was studied over a wide range of concentrations in an attempt to elucidate the nature of its dual uptake mechanism. A number of structural analogues of choline sulfate were synthesized and tested as competitive inhibitors of the active transport of choline sulfate. These inhibitor studies were designed to utilize some of the concepts developed in the course of the intensive investigation of the active surface of acetylcholinesterase (6, 18). A preliminary report has appeared (10).

Materials and Methods

Barley seeds (*Hordeum vulgare* L.) were germinated and seedlings grown in aerated solutions of 2×10^{-4} M $Ca(NO_3)_2$ under the conditions described by Epstein (3). After 7 days the roots were excised, rinsed, and used in the uptake experiments. Samples of roots weighing 1 to 2 g (fresh weight) were transferred to test tubes with 50 ml of aerated experimental solutions containing 5×10^{-4} M $Ca(NO_3)_2$. After 3 hours the experiment was terminated by decanting the experimental solutions and rinsing twice with distilled water. The roots were then placed in a desorption solution of 5×10^{-4} M $Ca(NO_3)_2$ for 1 hour (7). For assay of the radioactivity in the roots we developed a procedure for liquid scintillation counting of the intact, dried roots (11).

"Carrier-free" choline sulfate-S^{35} was synthesized according to the method of Segel and Johnson (12). For the uptake experiments sufficient activity of the "carrier-free" choline sulfate was added to solutions of nonradioactive choline sulfate to give less than 3 % counting error in the final assay of the roots. Choline sulfate and its sulfate ester analogues were made by esterification of the corresponding amino alcohols with sulfuric acid (1, 14). The esters were recrystallized from aqueous ethanol. The infrared spectra corresponded to spectra of other monoalkyl sulfates. The other inhibitors used were obtained commercially.

Results

The uptake of choline sulfate was first studied over a range of concentrations, from 10^{-7} M to 10^{-1} M. The results for the range 3×10^{-6} M to 10^{-1} M are shown in figure 1. Choline sulfate is apparently taken up by 2 mechanisms. One mechanism accounts for the uptake of choline sulfate at low substrate concentrations, while the other dominates at higher concentrations. The kinetics of the 2 mechanisms were resolved by the methods used by Epstein et al. (4) for potassium absorption by barley roots. A Lineweaver-Burk plot was made for the low-range mechanism (fig 2). The straight line was determined by the method of least squares, omitting the point corresponding to 3×10^{-4} M because some uptake by the high-range mechanism occurs at this concentration. From this plot the values for V_{max_1} and K_{M_1} were calculated and inserted into the first term of the Michaelis-Menten equation:

$$v = \frac{V_{max_1}[S]}{K_{M_1} + [S]} + \frac{V_{max_2}[S]}{K_{M_2} + [S]}$$

This gives the theoretical uptake rates for the low-range uptake mechanism, represented by the solid line at low concentrations and continued by the broken line at higher concentrations. In other experiments the Lineweaver-Burk plot has been shown to be linear down to 10^{-7} M, the lowest concentration studied. The values of K_{M_1}, as determined in a number of experiments, range from 5×10^{-6} M to 2×10^{-5} M. The maximum uptake rate of 19.5 mμ mole/g hr is very low, only about 5 % of that of potassium uptake.

The Lineweaver-Burk plot for the high-range

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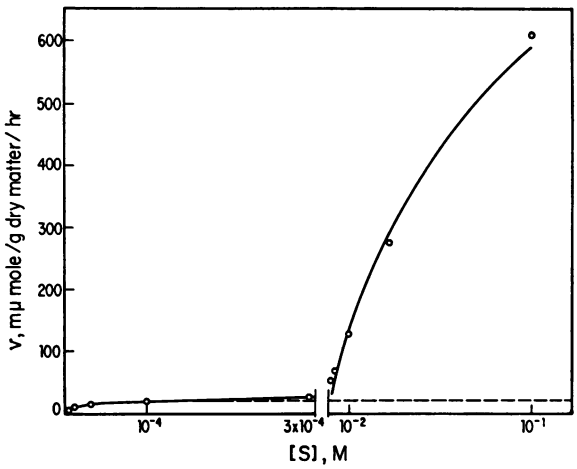
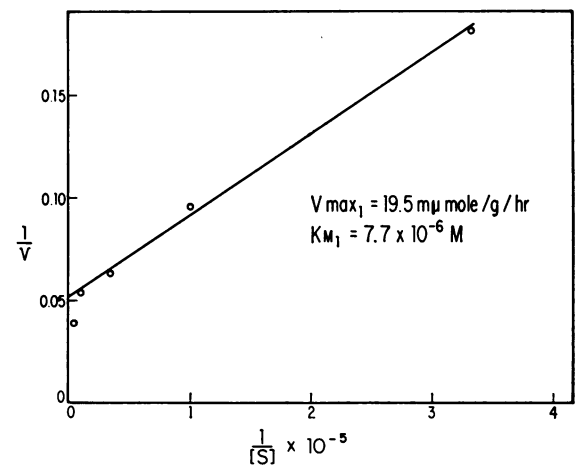


FIG. 1. Uptake of choline sulfate as a function of concentration. The horizontal scale is changed between 3×10^{-4} M and 10^{-3} M. The points represent means of 2 replicates from a single experiment.



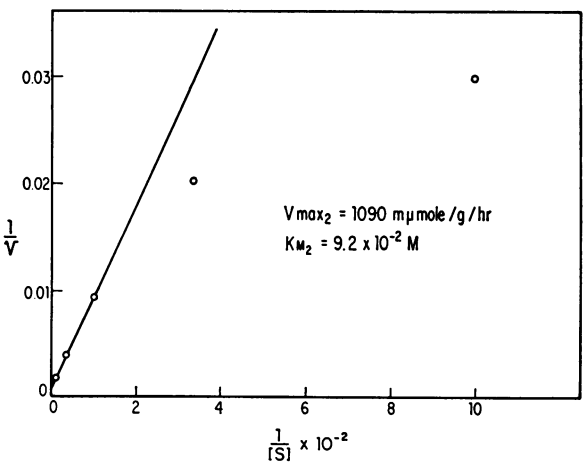


FIG. 2 (*upper*). Lineweaver-Burk plot for the low-range mechanism.

FIG. 3 (*lower*). Lineweaver-Burk plot for the high-range mechanism.

mechanism (fig 3) was then obtained after subtracting the theoretical uptake rates for the low-range mechanism from the experimental data. The straight line was determined using the points corresponding to 10^{-2} M, 3×10^{-2} M and 10^{-1} M choline sulfate. The values for V_{max_2} and K_{M_2} were calculated and inserted into the second term of the Michaelis-Menten equation. This gives the theoretical uptake rates for the high-range mechanism and the total uptake rate v represented by the solid line in figure 1. At choline sulfate concentrations of 10^{-3} M and 3×10^{-3} M the uptake rates were higher than would be expected from Michaelis-Menten kinetics.

When nitrogen was bubbled through the experimental solutions for 2 hours prior to, and during the experiment, the uptake of choline sulfate by the low-range mechanism decreased by more than 90 %, while the uptake by the high-range mechanism only decreased by some 50 %. Placing the roots in a 5×10^{-4} M solution of unlabeled choline sulfate for 1 hour after the experiment did not result in any significant decrease of activity in the roots, ruling out diffusion exchange as the mode of transport, at least for the low-range mechanism.

A number of analogues of choline sulfate were tested as inhibitors of active transport by the low-range mechanism (table I). Preliminary experiments showed that these analogues functioned as competitive inhibitors. The K_i values were determined graphically by the method of Dixon (2), using a choline sulfate concentration of 10^{-5} M and inhibitor concentrations of 10^{-5} M, 3×10^{-5} M, 6×10^{-5} M and 10^{-4} M. These concentrations are well below the region of the high-range mechanism. The Dixon plots tended to level off when the ratio of the inhibitor to choline sulfate exceeded 10, sometimes even above a ratio of 6. This means that the uptake of choline sulfate did not continue to decrease as the inhibitor to substrate ratio increased. The reason for this is not clear. This limits the accuracy of the determinations of K_i, especially of the higher K_i values. Table I shows the relative K_i values for the inhibitors. The K_i values are expressed relative to the K_i values for 2-aminoethyl sulfate (K'_i), which was included as a standard of comparison with each group of inhibitors. The relative K_i values given are the averages of 2 determinations which differed by not more than 25 %. The absolute value of K_i for 2-aminoethyl sulfate varied between 3×10^{-5} M and 6×10^{-5} M. Removal of 1 or 2 methyl groups from choline sulfate or substitution of the methyl groups with 1 ethyl group doubles the K_i value or decreases the inhibitory strength to one-half. 2-Aminoethyl sulfate is a decidedly weaker inhibitor than the monoethyl or the methylated analogues. Extension of the carbon chain, especially beyond C_3, results in a marked decrease of the inhibitory strength of the analogues. The sulfonic acid taurine is a slightly better inhibitor than the sulfate ester, while the thiosulfate and especially the sulfite analogue are weak inhibitors. Acetylcholine and choline phosphate inhibit very weakly

Table I. *Relative K_i Values for Inhibitors of Active Transport of Choline Sulfate by Barley Roots*
The K_i values are expressed relative to the value for 2-aminoethyl sulfate.

	R = $-CH_2CH_2OSO_3^-$	K_i/K_i'
2-Aminoethyl sulfate	H_3N^+-R	1.0
2-Methylaminoethyl sulfate	$CH_3N^+H_2$-R	0.6
2-Dimethylaminoethyl sulfate	$(CH_3)_2N^+H$-R	0.6
Choline sulfate	$(CH_3)_3N^+$-R	0.3
2-Ethylaminoethyl sulfate	$C_2H_5N^+H_2$-R	0.7
	$H_3N^+(CH_2)_nOSO_3^-$	
2-Aminoethyl sulfate	n = 2	1.0
3-Amino-1-propyl sulfate	n = 3	1.2
4-Amino-1-butyl sulfate	n = 4	1.6
5-Amino-1-pentyl sulfate	n = 5	3.2
	R = $H_3N^+CH_2CH_2^-$	
2-Aminoethyl sulfate	R-OSO_3^-	1.0
Taurine	R-SO_3^-	0.8
2-Aminoethyl thiosulfate	R-SSO_3^-	1.6
2-Aminoethyl sulfate	R-OSO_2^-	High*
Acetylcholine	$(CH_3)_3N^+CH_2CH_2$-OOC•CH_3	Very high**
Choline phosphate	$(CH_3)_3N^+CH_2CH_2$-OPO_3H^-	Very high
2-Aminoethyl sulfate	$H_3N^+CH_2CH_2OSO_3^-$	1.0
1-Amino-2-propyl sulfate	$H_3N^+CH_2CH(CH_3)OSO_3^-$	1.5
2-Amino-2-methyl-1-propyl sulfate	$H_3N^+C(CH_3)_2CH_2OSO_3^-$	1.3
2-Dimethylamino-2-methyl-1-propyl sulfate	$(CH_3)_2N^+HC(CH_3)_2CH_2OSO_3^-$	High
2-Aminoethyl sulfate	$H_3N^+CH_2CH_2OSO_3^-$	1.0
Choline	$(CH_3)_3N^+CH_2CH_2OH$	Very high
Sulfate	SO_4^{--}	Very high

* Relative K_i: 4–8
** Relative $K_i > 8$

if at all. Branching of the carbon chain has little effect. The high K_i of 2-dimethylamino-2-methyl-1-propyl sulfate may be due to steric hindrance caused by the addition of 2 methyl groups. Finally it is shown that choline and sulfate ions inhibit very weakly if at all.

Discussion

The uptake of choline sulfate by the low-range mechanism is undoubtedly the result of active transport. More experiments are needed, however, to determine the nature of the high-range mechanism. The discrepancies between the data and the expected values of v at the lower end of this range and the incomplete inhibition under anaerobic conditions indicate that the high-range mechanism may not comply with all criteria for active transport.

The fact that 2 such diverse ionic species as choline sulfate and potassium ion (4) both have 2 discrete uptake mechanisms, and that the high-range mechanisms start functioning at the same concentration, suggests that dual uptake mechanisms may be a widespread phenomenon in roots.

On the basis of the inhibitor studies we propose, in analogy with Wilson's (17) early model of the acetylcholine-acetylcholinesterase complex, that the carrier for choline sulfate has 2 sites: An anionic site which binds the quaternary nitrogen and a cationic site which binds the sulfate group (fig 4). This model can explain the effects of the inhibitors studied. A lengthening of the carbon chain will result in a poorer fit between the inhibitor and the carrier, decreasing the inhibition. A branching of the carbon chain will result in little loss of inhibitory strength since no sites on the carrier are directely involved. Changes in substituents on the quaternary nitrogen and in the sulfate group will result in changes in the binding forces involved. Both sites are important for the binding of inhibitors to the carrier; choline and sulfate alone are poor inhibitors. According to this 2-site model of the carrier, the uptake of choline sulfate by the low-range mechanism should decrease

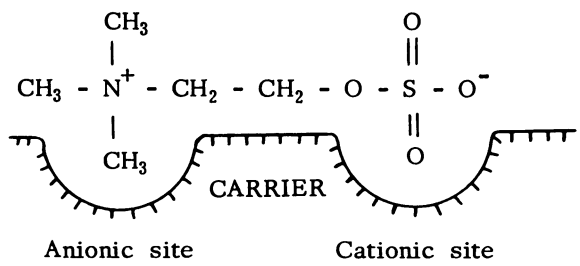


FIG. 4. Model for the choline sulfate carrier.

at high choline sulfate concentrations (15). Unfortunately this cannot be checked since any decrease would be masked by the uptake of choline sulfate by the high-range mechanism.

It is of interest to compare the effects of inhibitors of the active transport of choline sulfate with the effects of inhibitors of the hydrolysis of acetylcholine by acetylcholinesterase. Removal of the methyl groups of acetylcholine decreases the inhibitory strength by a factor of 50 (16), while for choline sulfate the factor is only 3 to 4. Branching of the carbon chain of acetylcholine decreases the inhibition by a factor of 100 to 1000 (5), while the effect on the active transport of choline sulfate is slight. There are thus obvious differences between the binding forces of the acetylcholine-acetylcholinesterase complex and those functioning in the active transport of choline sulfate.

The physiological significance of the dual mechanisms of choline sulfate uptake by barley roots is not known. The very low rates of choline sulfate uptake by the low-range mechanism may suggest that the ability of the roots to transport choline sulfate actively is of very limited physiological significance under normal conditions.

Further studies of the similarities and differences between the active transport of choline sulfate, the hydrolysis of acetylcholine by acetylcholinesterase, and the enzymic sulfatation of choline analogues should prove useful for a better understanding of the carriers and binding forces involved in active transport.

Note added in proof:

Comparisons may also be made between the active transport of sulfate ester analogues of choline sulfate, and the enzymatic sulfatation of the corresponding amino alcohols. The value of K_M for the sulfatation of 2-dimethylaminoethanol was twice that of choline, the same ratio found for the active transport of the sulfate esters (Orsi, B. A. and B. Spencer. 1962. Choline sulphokinase. Biochem. J. 85: 19 P.).

Summary

Barley roots (*Hordeum vulgare* L.) take up choline sulfate by 2 mechanisms: A low-range mechanism which is the result of active transport and operates at half maximal velocity at a choline sulfate concentration of approximately 10^{-5} M, and a high-range mechanism which becomes apparent at about 2×10^{-4} M.

A 2-site carrier for choline sulfate with an anionic site which binds the quaternary nitrogen and a cationic site which binds the sulfate group is proposed.

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