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THE ABSORPTION OF AMINO ACID DERIVATIVES OF NITROGEN MUSTARD FROM RAT INTESTINE *IN VITRO*

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Abstract—Certain serine and threonine derivatives of nitrogen mustard are transported across the mucosa of everted sacs of rat small intestine *in vitro*. The rates of transference and the final concentration ratios are comparable both for these derivatives and for the parent amino acids. Histology, water content, and potassium levels suggest little tissue damage in short-term experiments. There is no correlation between the uptake and the biological effect of the compounds against the Walker tumour in rats. Evidently the amino acid moiety is merely acting as a carrier for a cytotoxic group. This evidence is compatible with current theories regarding the mode of action of these carcinostatic agents as biological alkylating agents. The relevance of this study to the uptake of chemotherapeutic amino acid derivatives by tumour cells is discussed.

SOME amino acid derivatives of nitrogen mustard exhibit anti-tumour effects in rats.[1] Investigation of the mode and rate of transport of such compounds into living cells is therefore pertinent. Dosage rates and time schedules will depend upon such data.

Wiseman has shown that many of the natural L-α-amino acids are actively absorbed across the intestinal mucosa of rats[2] and of hamsters.[3] Active absorption was defined as transport against a concentration gradient.In the present study the absorption of the amino acid derivatives of nitrogen mustard was tested using everted sacs of rat intestine *in vitro*.[4] The parent amino acids were similarly tested for comparison.Only compounds containing latent mustard groups are sufficiently stable for an investigation of this type therefore the carbamoyl analogues were chosen for study. Compounds with active mustard groups are too labile for such a tissue experiment and subsequent analysis.

It seems surprising that this convenient technique has apparently been little used as yet in the study of drug absorption from the gut. The anti-tumour agents, 5-fluorouracil and 5-bromouracil, are actively absorbed like uracil, as shown with the everted sac technique.[5] The method has been utilised extensively for investigating transport mechanisms and their specificity with amino acids[3] and sugars.[6]

ANIMALS

Male albino rats of the Wistar strain 200–300 g body weight were used. The rats were starved overnight before the experiment but given drinking water *ad libitum*.

Preparation of the everted intestinal sacs

The rat was killed by a blow on the back of the head and by severing the neck. The small intestine was rapidly dissected out, washed in glucose–saline and everted with a rod.[4] Segments of gut were tied with cotton ligatures to form everted sacs. Each sac was filled with 0·4 ml test solution. Two sacs were immersed in 25 ml test solution in a 100 ml conical flask, gassed briefly with 5% CO_2/95% O_2 and shaken at 37 °C for 1 hr. Only two sacs were taken from a single animal, distal to the duodenum, to obviate any differences in absorption rate in different regions of the small intestine and also to ensure that intact living tissue was used.

Test solutions

Amino acids were from commercial sources and nitrogen mustard derivatives were kindly provided by Dr. R. Wade.[1] Compounds were checked for purity by elementary analysis, filter paper chromatography in several solvents, and determination of the specific optical rotation. Test compounds were dissolved at a concentration of 5 mM in a bicarbonate saline solution containing 0·3% (w/v) glucose[7] but no calcium salts. All solutions were gassed with 5% CO_2/95% O_2 for 10 min.

Analytical method

After incubation, the sac and flask contents were deproteinized by bringing to the isoelectric pH with acetic acid and heating for 5 min in a boiling water bath.[8] The diluted filtrates were analysed for amino nitrogen with ninhydrin–hydrindantin reagent.[9] Appropriate standard solutions were treated similarly.

Treatment of tissue

The physiological state of the tissues was assessed after the experiment from the water content, the potassium content and histological examination.[10] The everted sacs were drained and dried to constant weight at 105 °C overnight. Fat was extracted from the dry material by multiple extractions with ether and petroleum ether.[11] The fat-free dry residues were dissolved in nitric acid, diluted and potassium determined by flame photometry. For histology the sacs were drained, fixed in formol-saline, embedded in paraffin wax and sections stained with haemotoxylin and eosin.

RESULTS

The concentration ratio was calculated as the fraction,

$$\frac{\text{concentration of the compound on the serosal side}}{\text{concentration of the compound on the mucosal side}}$$

i.e. a ratio greater than 1·0 indicates active transport against a concentration gradient.

The results obtained indicate that the nitrogen mustard derivatives of serine and threonine show similar concentration ratios and rates of transference to those of the corresponding unsubstituted amino acids (Table 1). Esterification of the carboxyl group (CB 3178) did not influence transport relative to that of the parent compound (CB 3159). By comparison, glycine and its methyl ester also gave similar transport data. D- and L-forms of the compounds gave similar concentration ratios but the racemic compound gave lower values with some nitrogen mustard derivatives and with serine.

TABLE 1.

Compound	Code	No. of sacs	Concentration ratio Mean	Concentration ratio S.D.	Rate of transference μM/100 mg fat-free dry wt./hr Mean	Rate of transference μM/100 mg fat-free dry wt./hr S.D.	Water content g/100 g fat-free fresh tissue Mean	Potassium content meq./kg fat-free dry tissue Mean
L-Serine	—	6	2·73	± 0·65	6·57	± 1·05	88·6	414
O-[NN-di-(2-chloropropyl)carbamoyl]-L-Serine	CB 3302	6	2·80	± 0·25	9·41	± 1·95	87·8	354
O-[NN-di-(2-chloropropyl)carbamoyl]-DL-Serine	CB 3210	6	1·90	± 0·33	8·95	± 3·71	88·8	365
DL-Serine	—	6	1·71	± 0·19	5·49	± 0·85	88·9	398
O-[NN-di-(2-chloropropyl)carbamoyl]-D-Serine	—	6	2·83	± 0·29	8·15	± 2·87	88·4	368
D-Serine	—	12	2·26	± 0·22	5·77	± 2·14	88·8	350
O-[NN-di-(2-chloroethyl)carbamoyl]-L-Serine	CB 3189	6	1·65	± 0·12	6·40	± 2·54	88·4	409
O-[NN-di-(2-chloroethyl)carbamoyl]-DL-Serine	CB 3159	6	2·42	± 0·31	4·62	± 0·50	89·1	405
O-[NN-di-(2-chloroethyl)carbamoyl]-DL-Serine methyl ester hydrochloride	CB 3178	6	2·38	± 0·16	7·13	± 0·31	89·0	434
Glycine	—	6	1·26	± 0·08	5·68	± 0·56	89·2	317
Glycine methyl ester	—	6	1·25	± 0·10	3·98	± 2·90	89·1	314
DL-Threonine	—	6	2·09	± 0·12	6·06	± 0·72	89·0	341
*O-[NN-di-(2-chloroethyl)carbamoyl]-DL-Threonine	CB 3182	6	1·59	± 0·19	6·02	± 2·23	88·5	326

* Inactive against Walker tumour.[1]

One-dimensional filter paper chromatography in 80% (w/v) phenol–water–ethanol–S.G. 0·88 ammonia (120:40:40:1 by volume) and in *n*-butanol–glacial acetic acid–water (120:30:50 by volume) as solvents was carried out to detect free amino acids leaking out from the tissues. Visual comparisons of these chromatograms revealed that the concentration of endogenous amino acids in the sac contents usually exceeded that in the flask contents. Hence the concentration ratios have no absolute value but will be too high. Although these nitrogen mustard derivatives are labile to hydrolysis at 100 °C,[1] paper chromatography showed that they were stable at pH 7·4 for the duration of the experiments.

From the results recorded here (Table 1) it is clear that there is no direct correlation between active transport and anti-tumour effect. Thus the threonine derivative CB 3182 although inactive biologically shows a similar concentration ratio to CB 3210 which has the greater cytotoxic effect of the series studied.

The water content and potassium content of the sacs after the experiment were remarkably constant (Table 1) suggesting little damage due to manipulation, anoxia or the test compounds. Similarly, the sections of sacs submitted to different treatments were indistinguishable histologically. The tips of villi were cut off in all cases by the eversion procedure but this is expected.

DISCUSSION

Since most somatic and tumour cells possess net negatively charged surfaces[12] it might be expected that those amino acids with a net negative charge at physiological pH would not pass readily across the cell membrane. Thus glutamic acid passes less readily into muscle and liver cells than does glutamine;[13] a phenomenon which might be attributed to a difference in the charges on the molecules. Hence it seemed surprising that one of the nitrogen mustard–serine derivatives (CB 3159) was not transported significantly faster than its corresponding methyl ester (CB 3178) in which the negative charge on the carboxyl group has been lost. The transport of glycine and glycine methyl ester were also similar, both in the concentration ratios achieved and the rates of transference. Glycine was chosen for study as it is a metabolic product of serine in mammalian and other tissues.[14] Thus the nitrogen mustard–serine derivative and its ester behave in a similar manner to an unsubstituted amino acid and its corresponding ester. It has been claimed that the reactive site, in the uptake of amino acids by small intestine, has specific points of attachment for the L-amino group and the L-carboxyl group.[15] Conversely, our results with glycine esters suggest that an ionized carboxyl group does not participate in this process.

Amino acid uptake by Ehrlich ascites tumour cells has been well summarized[16] and is relevant to this discussion. A free amino group appears to be an important structural feature for uptake. Thus L-$\alpha\gamma$-diaminobutyric acid is concentrated more strongly than the corresponding monoaminomonocarboxylic acid by ascites cells.[17] Superficially, intestinal mucosal cells and ascites tumour cells do show some similarities in their characteristics towards amino acid uptake, suggesting similar transport mechanisms.

Formerly it was considered that L-amino acids were actively absorbed and that D-amino acids were merely passively absorbed from intestine.[2] Using solutions of a number of racemic amino acids the L-isomers could be transported against a concentration gradient *in vitro*, but this did not occur with the D-isomers. Each isomer was

determined by a specific method but the use of a racemic mixture as the test substance did not exclude the possibility of competitive effects between the two optical isomers. In fact, competition between D-, and L- isomers of the same amino acid has been shown for methionine. Moreover, D-methionine is actively absorbed, i.e. it is absorbed against a concentration gradient.[18] The uptake of serine and its derivatives in the present study substantiate this hypothesis that D-amino acids can be transferred by an active process. However, it should not be assumed that this is a general rule since D-alanine is not actively transported as is L-alanine.[19]

Since the total amino acid concentration was kept constant in these experiments lower values were observed for the uptake of racemate, in some cases, compared with uptake of either enantiomorph. Thus each isomer was present at only one-half of the total amino acid level and uptake of L-serine is lower at a concentration of 1 mM than it is at 10 mM.[15]

If it can be assumed that the transference of amino acid derivatives across intestinal cells reflects a similar process in malignant cells then a comparison of transport data and the biological effects of these compounds is appropriate. Clearly there is no correlation between the rate of transference and the anti-tumour effect since the threonine derivative which is inactive biologically shows a similar rate of transference to a serine derivative which is active. Since both amino acids occur in proteins and their physical constants are similar, this difference in their biological activity is unexplained.

The data presented do not exclude the incorporation of the test compounds into protein molecules. This possibility cannot be tested directly in the present case, even with radiochemically labelled compounds, due to the lability of the compounds to hydrolysis.[1] However, the protein amino acids valine and methionine are not incorporated into the proteins of intestinal preparations *in vitro*.[20] Therefore, it seems unlikely that non-protein amino acids are incorporated into intestinal preparations in experiments of this type.

Evidently the different anti-tumour activities of the different nitrogen mustard derivatives cannot be attributed to differences in their rates of transport into living cells. It seems that their cytotoxic effects are a measure of their potency as biological alkylating agents, although the series of compounds studied here have only weak activity in this respect. This conclusion accords with current theories concerning the carcinostatic influence of these nitrogen mustard derivatives in which they behave as biological alkylating agents of altered tissue specificity rather than as amino acid analogues.[21]

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REFERENCES

1. F. BERGEL and R. WADE, *J. Chem. Soc.* 941 (1959).
2. G. WISEMAN, *J. Physiol.* **120**, 63 (1953).
3. G. WISEMAN, *J. Physiol.* **133**, 626 (1956).
4. T. H. WILSON and G. WISEMAN, *J. Physiol.* **123**, 116 (1954).
5. L. S. SCHANKER and J. J. JEFFREY, *Nature, Lond.* **190** ,727 (1961).
6. R. K. CRANE, *Physiol. Revs.* **40**, 789 (1960).
7. H. A. KREBS and K. HENSELEIT, *Hoppe-Seyl. Z.* **210**. 33 (1932).

8. G. HUNTER, *Biochem. J.* **62**, 28P (1956).
9. S. MOORE and W. H. STEIN, *J. Biol. Chem.* **211**, 907 (1954).
10. M. W. NEIL, *Biochem. J.* **71**, 118 (1959).
11. A. B. HASTINGS and L. EICHELBERGER, *J. Biol. Chem.* **117**, 73 (1937).
12. E. J. AMBROSE, A. M. JAMES and J. H. B. LOWICK, *Nature, Lond.* **177**, 576 (1956).
13. H. KAMIN and P. HANDLER, *J. Biol. Chem.* **188**, 193 (1951).
14. D. SHEMIN, *J. Biol. Chem.* **162**, 297 (1946).
15. L. R. FINCH and F. J. R. HIRD, *Biochim. Biophys. Acta* **43**, 278 (1960).
16. H. N. CHRISTENSEN, *A Symposium on Amino Acid Metabolism* (Edited by W. D. MCELROY and H. B. GLASS, pp. 63–106. Johns Hopkins Press, Baltimore (1955)).
17. H. N. CHRISTENSEN, T. R. RIGGS, H. FISCHER and I. M. PALATINE, *J. Biol. Chem.* **198**, 1 (1952).
18. E. L. JERVIS and D. H. SMYTH, *J. Physiol.* **151**, 51 (1960).
19. D. F. EVERED and H. G. RANDALL. In preparation.
20. T. SUGAWA, H. AKEDO and M. SUDA, *J. Biochem., Japan* **47**, 131 (1960).
21. W. L. NYHAN, *J. Pharm., Lond.* **130**, 268 (1960).