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FOLIC ACID-DISPLACEMENT IN MAN*

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Abstract—Seven compounds, either closely related in structure to folic acid or fragments of the folic acid molecule, were tested in man for the ability to displace a dose of 5 μg of tritium-labeled folic acid/kg (20 μc) administered 24 hr previously. The compounds were administered intravenously at a dosage of 1 μmole/kg, and their ability to displace intracellular folic acid was assessed by measurement of folic acid radioactivity appearing in the urine in the next 6 hr. Those compounds in which the structural integrity of the pteridine moiety of folic acid was unimpaired were effective folic acid-displacing agents; compounds lacking the pteridine moiety were unable to displace folic acid; compounds having a modified pteridine moiety showed only weak displacing ability, despite the very high affinity *in vitro* of one of them (methotrexate) for the enzyme dihydrofolate reductase.

The results suggest that in man, a structurally intact pteridine moiety is necessary for effective displacement of folic acid from cells. Folic acid-displacing ability cannot be explained solely on the basis of relative affinity for dihydrofolate reductase; it is postulated that an additional factor in folic acid-displacing ability is relative affinity for a folic acid transport mechanism.

IF LARGE doses of unlabeled folic acid are administered intravenously to normal human subjects who have previously received tracer amounts of tritium-labeled folic acid, the labeled folic acid is displaced and can be recovered from the plasma and urine.[1] The displacement effect can be demonstrated even after a considerable interval; in a typical experiment, almost half of an initial labeled dose of 15 μg/kg could be displaced and recovered in the urine when a flushing dose was given 3 days later.[2] It is therefore evident that folic acid in man can be retained as such within the cells for considerable periods and that its retention does not necessarily result in its immediate conversion to biologically active coenzyme forms.

Little is known regarding the mechanism by which this intracellular accumulation of folic acid takes place; it is probable, however, that such an accumulation process must at some stage involve binding of the folic acid molecule either to a carrier site at the cell membrane or to a fixed site within the cell itself.[3] Furthermore, it is likely that such binding would involve only a limited number of groups and a limited area of the folic acid molecule; other molecules with similar patterns of electron density also should show affinity for the folic acid-binding site. In the experiments reported here, a series of compounds closely related in structure to folic acid were tested for the ability to displace tracer doses of labeled folic acid from cells—i.e. for their ability to compete for the folic acid-binding site or sites.

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MATERIALS AND METHODS

Subjects were healthy adult males ranging in age from 24 to 32 years and in weight from 62 to 80 kg. All were in a good state of nutrition. Tritium-labeled folic acid (The Radiochemical Centre, Amersham, England), of activity 524 μc/mg, was purified by chromatography on DEAE-cellulose as previously described;[2] the specific activity after chromatography was 420 μc/mg. The labeled folic acid was diluted with unlabeled folic acid so that subjects received no more than 20 μc tritium radioactivity in a dose of 5 μg folic acid/kg. The labeled folic acid was administered intravenously in normal saline to which 0·2% sodium bicarbonate had been added. Urine was collected and pooled for 23 hr (urine radioactivity usually returned almost to background levels within 8 hr after the initial dose). A 1-hr preflush blank urine was then collected in order to obtain the level of background radioactivity and, 24 hr after the initial dose of labeled folic acid, the compound whose folic acid-displacing ability was to be tested

TABLE 1. DISPLACEMENT OF TRITIUM-LABELED FOLIC ACID

Subject	Test agent	Total radioactivity excreted 0–6 hr after administration of flushing agent (% of retained dose*)	Folic acid radioactivity isolated by column chromatography (% of total displaced radioactivity)
1		2·1	11·6
2		1·0	
3	Pteroylglutamate	73·5	69·0
4	Pteroylglutamate	68·4	72·0
5	Pteroyltriglutamate	66·8	71·4
6	Pteroyltriglutamate	62·4	64·2
7	Pteroic acid	25·1	77·4
8	Pteroic acid	18·9	68·1
9	4-Amino-10-methylpteroylglutamate (methotrexate)	11·9	50·2
10		10·9	61·7
11	5-Formyltetrahydropteroylglutamate (folinic acid)	10·0	39·8
12		8·4	45·2
13	*p*-Aminobenzoylglutamate	1·1	
14	*p*-Aminobenzoylglutamate	0·7	13·0
15	Glutamate	1·4	5·9
16	Glutamate	1·1	9·4

* Retained dose: Administered dose (5 μg/kg; 20 μc) − radioactivity excreted prior to the flushing dose. Retained dose ranged from 82 to 90% of the administered dose.

was administered intravenously in saline–bicarbonate† at a dosage level of 1 μmole/kg.‡ Each compound was tested in two subjects. No untoward effects were noted except in the case of pteroic acid: both the subjects receiving this compound complained of transitory flushing, sweating, and weakness. Urines were collected for 6 hr, and 0·5-ml aliquots were counted in a Packard Tri-Carb liquid scintillation counter. Folic acid

† Pteroic acid was not readily soluble in saline–bicarbonate; 0·1 N sodium hydroxide solution was added until the compound dissolved and the pH of the solution was then adjusted to 8·5 with dilute hydrochloric acid.

‡ Folic acid derivatives were generously supplied by Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, N.Y. *p*-Aminobenzoylglutamate was obtained from H. M. Chemical Co. Ltd., Santa Monica, Calif.

Racemic calcium leucovorin was used at a dosage level of 2 μmoles/kg, equivalent to 1 μmole of the biologically active *l*, L-isomer/kg.

radioactivity was isolated by chromatography of whole urine samples on DEAE-cellulose as previously described[2] and was determined separately. Thirty mg folic acid was administered intravenously to each subject at the conclusion of the experiment in order to displace any remaining radioactivity. The results are summarized in Table 1.

RESULTS AND DISCUSSION

It was found (see Table 1) that those compounds in which the structural integrity of the pteridine moiety of folic acid was unimpaired (pteroyltriglutamate and pteroic acid) were effective folic acid-displacing agents; compounds lacking the pteridine moiety were unable to displace folic acid; compounds having a modified pteridine moiety showed only weak displacing ability despite the very high affinity *in vitro* of one of them [4-amino-10-methylpteroylglutamate (methotrexate)] for the enzyme dihydrofolate reductase.[4] Thus, in man, a structurally intact pteridine moiety is necessary for effective displacement of folic acid from cells.

Interpretation of these results is rendered difficult by the fact that little is known to date regarding the mechanism for retention of folic acid within cells. This retention could be attributed to adsorption of folic acid at specific fixed sites within the cell but can equally well be explained as being brought about by an active transport system able to accumulate folic acid against a concentration gradient. It is highly unlikely that a molecule as polar and lipid-insoluble as pteroylglutamate would be able to enter cells as readily as it does *without* the mediation of a membrane carrier system; moreover, the uptake of folic acid by cells in man exhibits a number of characteristics of active transport: a high degree of specificity (e.g. the closely related compound methotrexate penetrates only very slowly[5, 6]), saturation kinetics, uphill transport,[2] competition for entry with other substrates[7, 8] and, as shown here, displacement phenomena.

Methotrexate

A probable relationship exists between the inability of methotrexate to enter cells readily and its inability to act as an effective folic acid-flushing agent. It may be that methotrexate is unable to make use of existing carrier mechanisms and thus that such small amounts of methotrexate succeed in penetrating the cells that the intracellular concentration of the compound remains too low for effective displacement of folic acid from binding sites. Alternatively, a considerable portion of the intracellular folic acid may be bound to sites for which methotrexate has little affinity (i.e. to sites other than dihydrofolate reductase). A third possibility also exists: that methotrexate has a high affinity for the folic acid-transport system, but that little of the methotrexate carrier complex is dissociated at the inner surface of the cell membrane, so that not only does little methotrexate enter the cell but few carrier sites are available for folic acid to leave the cell. In this connection it should be recalled that a high affinity for a carrier system does not necessarily result in a high rate of transport into cells; Finch and Hird[9] have observed that, among certain groups of amino acids capable of sharing the same carrier, those with a high affinity for the carrier are transported more slowly than those with a low affinity when the rates of entry are independently compared at concentrations high enough to give carrier saturation. Additional work will be required to distinguish between these possibilities, but it should be noted that other

evidence is available suggesting that methotrexate, despite the fact that it is poorly transported, may have considerable affinity for the folic acid carrier. We have observed[7] that, in man, preloading with methotrexate decreases the rate of entry of folic acid into cells when the compound is administered at fairly high dose-levels; this effect can most easily be explained by competition for a common carrier. A similar competition was noted between 4-aminopteroylglutamate (aminopterin) and folate by Condit and Grob;[8] preloading with aminopterin was found to reduce greatly the rate of extrarenal uptake of intravenously administered folic acid.

Pteroic acid

Franklin and co-workers[10] noted some years ago that intravenous administration of pteroic acid to human subjects resulted in the excretion of small amounts of pteroylglutamate activity in the urine. At the time, this observation was interpreted as representing a partial conversion of pteroic acid to pteroylglutamate; such a conversion appears unlikely, however, in view of the fact that pteroic acid lacks hematopoietic properties.[11] In the light of the results reported here it appears probable that the folic acid excretion observed by these workers after the administration of pteroic acid represented the displacement of endogenous folic acid or closely related folic acid-active compounds, as a result of competition for a limited number of carrier or binding sites.

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