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FOLIC ACID ANTAGONISTS (THE DIHYDROFOLATE REDUCTASE INHIBITORS): APPLICATION OF TETRAZOLIUM BIOAUTOGRAPHY ON DETERMINATION OF SERUM AND URINE METHOTREXATE

by

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SUMMARY.

The inhibition effects of methotrexate upon the growth of microorganisms used in the microbiological assays of folate were studied. Small degree of inhibition effects were seen with S.faecalis and P.cerevisiae. Total inhibition of the growth of L.casei was seen when the concentration of methotrexate in the growing culture is as low as 0.01 ng/ml. The application of L.casei-tetrazolium bioautography with unwashed culture led to a method for the determination of antifolate. Studies on the oral absorption of 10 mg methotrexate in psoriasis patients indicated that a peak level of about 200 ng/ml was reached within 1 hour after doses and declined to zero level within 48 hours.

INTRODUCTION

The folic acid antagonists are known to have anti-leukemia activity (Farber et al 1948). The 4-amino-4-deoxy-10-methylpteroylglutamic acid (methotrexate) one of the folate antagonists has been shown to have the most effective curing effect in cancer chemotherapy and known to have a biochemical role as dihydrofolate reductase inhibitor (Condit, 1960; Condit and Eliel, 1960; Wright et al 1960; Hertz et al 1961; Hustu et al 1973; Frei et al 1975) and this property is used as a method for its detection (Bertino and Fischer, 1964). Since folate antagonists had a competitive action with folic acid and its derivatives thus we would expect to see growth inhibition of folate dependent microorganisms. Using this principle and

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the tetrazolium bioautographic techniques we can see the inhibition zone or zones and thus detection the inhibitor.

MATERIALS AND METHODS.

The 4-amino-4-deoxy-10-methylpteroylglutamic acid (methotrexate) tablets or parenteral doses were obtained from Lederle laboratories devision, American Cyanamid Company. They were used for oral absorption studies and also used for the inhibition studies. Microbiological assays with Lactobacilus casei, Streptococcus faecalis and Pediococcus cerevisiae were carried out using aseptic technique (Herbert, 1966) and a semiautomated method (Leeming and Portman-Graham, 1973)

Pteroylglutamic acid (Folic Acid) was purchased from Koch-Light Laboratories, Colnbrook, Buckinghamshire, UK. 5-formyltetrahydropteroylglutamic acid (Folinic Acid) was a gift from Laderle Laboratories. 10-formylpteroylglutamic acid was prepared according to Blakley (1959), 5-methyltetrahydropteroylglutamic acid was prepared according to the method of Blair and Saunders (1970) and Dihydropteroylglutamic acid was prepared by the method modified from Futterman (1963).

Triphenyltetrazolium (chloride) was purchased from BDH Chemicals Ltd; Poole, England. Thin layer chromatographic plates were Art. 5716 DC-Fertigplatten cellulose (Pre-coated TLC plates without fluorescent indicator) dimension 0.1 mm x 20 mm x 20 cm and purchased from Merck (U.K.). After 2 to 5 ul of samples were applied onto the thin layer chromatographic plates with sterilized micropipettes they were developed with 3% aqeous ammonium chloride containing 1% ascorbic acid (W/V). The distance from the application points to the solvent front is adjusted for 15 cm. The developed plates were left to half dried in refrigerator at 6°C before putting on the tray (culture trays Code Number H 43/1 purchased from Jencons Scientific Ltd, England.) of settled sterilized plain agar (Oxoid Ionagar No. 2, purchased from Oxoid, England or this can be substituted by Noble, Agar special of Dilfco, U.S.A.).

The plain agar was prepared by dissolving 3 to 5 g of the agar powder in 250 ml distilled water and sterilized by autoclaving at 121°C for 15 minutes. The agar medium was prepared by adding

appropriate assay media, 3 to 5 g of agar powder, 0.5 g ascorbic acid, 30 ml 0.1M phosphate buffer pH 6.1, 5 ml of 2% sterilized tetrazolium solution (W/V), 10 ml of diluted appropriate microorganism in the final volume of 300 ml adjusted by distilled water. The agar media were prepared in such a manner that the media were sterilized by autoclaving at 121°C for 15 minutes and cooled to 45°C before the tetrazolium solution and diluted microorganism solutions were added. The cooled agar media (45°C) were poured to cover the developed thin layer chromatographic plates and let settle before covering with thin film of plastic sheet and covered. The prepared plates were incubated in the air-ventilated incubator at 37°C for 24 hours. The inhibition zone or zones could be seen at the various Rf values of the inhibitors detected. By varying the amount of standard methotrexate applied onto the chromatographic plates this can be used as standard curve and thus unknown could be determined.

The inhibition effects of various concentrations of methotrexate in the microbiological assays with L. casei, S. faecalis and P. cerevisiae were set out as shown in Table I.

Patients with psoriasis on treatment with methotrexate were used in the studies of serum methotrexate levels. These subjects had oral doses of 10 mg of methotrexate after the first venous blood samples were taken and then at 1,2,3, 4,24 and 48 hours after doses.

Table I Schedule of the studies of inhibition of methotrexate on microbiological asssay of folates with L. casei, S. faecatis and P. cerevisiae.

Concentration of methotrexate (ng/ml)	Concentration of added folate* (ng/ml)					
	1	2	3	4	5	6
0	0	2	5	10	20	30
0.5	0	2	5	10	20	30
5.0	0	2	5	10	20	30
50.0	0	2	5	10	20	30
500.0	0	2	5	10	20	30

*indicates pteroylglutamic acid, 7,8-dihydropteroylglutamic acid,5-methyltetrahydropteroylglutamic acid, 10-formylpteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid were used in the studies with L. casei. Pteroylglutamic acid, 7-8-dihydropteroylglutamic acid and 5-formyltetrahydropteroylglutamic acid were used with P. cerevisiae. Concentrations of the appropriate folates in each assay media were similar to those of the assay media without methotrexate and as shown in the results section.

RESULTS.

The inhibition effects of methotrexate were studied in the growing cultures of L. casei, S. faecalis and P. cerevisire. Various degrees of inhibition effects were seen in all three cultures. With L. casei total inhibition was seen at a concentration as low as 10 pg/ml of methotrexate in the growing cultures as shown in Table II. With S. faecalis and P. cerevisiae some inhibition effects were seen as shown in Tables III and IV, respectively.

The bioautography of standard methotrexate and serum methotrexate were shown in Plates I and II. The serum methotrexate levels from psoriasis subjects after 10 mg of methotrexate orally were shown in Table V.

TABLE II The inhibition of methotrexate on the microbiological assay of folates with L. casei.

Concentration of methotrexate (ng/ml)	Concentration of folate (ng/ml)*					
	1	2	3	4	5	6
0	0	2	5	10	20	30
0.5	0	0	0	0	0	0
5.0	0	0	0	0	0	0
50.0	0	0	0	0	0	0
500.0	0	0	0	0	0	0

*detected results from all folates as shown in Table I.

TABLE III The inhibition effect of methotrexate on the growing cultures of S. faecalis.

Concentration of methotrexate (ng/ml)		Concentration of folate (ng/ml)					
		1	2	3	4	5	6
A	0.00	0	2.0 ± 0.0	5.0 ± 0.0	10.0 ± 0	20.0 ± 0	30 ± 0.0
A	0.50	0	1.9 ± 0.1	4.8 ± 0.2	9.6 ± 0.5	19 ± 1	27 ± 2.0
A	5.00	0	1.0 ± 0.1	3.2 ± 0.3	6.0 ± 1.0	11 ± 2	20 ± 1.0
A	50.00	0	0	0	0	1.0 ± 0	2.0 ± 0
A	500.00	0	0	0	0	0	0
B	0.00	0	2.0 ± 0.0	3.0 ± 0.0	5.0 ± 0.0	8.0 ± 0.0	10 ± 0
B	0.50	0	1.8 ± 0.2	2.8 ± 0.2	4.8 ± 0.2	7.5 ± 0.5	9.7 ± 0.3
B	5.00	0	1.0 ± 0.2	2.0 ± 0.3	3.0 ± 0.5	5.0 ± 0.5	6.0 ± 1.0
B	50.00	0	0	0	0	0	0
B	500.00	0	0	0	0	0	0
C	0.00	0	1.5 ± 0.1	3.0 ± 0.1	5.0 ± 0.5	8.0 ± 1.0	13.0 ± 1.0
C	0.50	0	1.5 ± 0.1	3.0 ± 0.1	4.9 ± 0.5	7.9 ± 1.0	12.8 ± 1.0
C	5.00	0	1.5 ± 0.1	3.0 ± 0.1	4.5 ± 0.5	6.0 ± 1.0	11.0 ± 1.0
C	50.00	0	0	0	0	2.5 ± 0.5	5.0 ± 0.5
C	500.00	0	0	0	0	0	0

A = Studies when pteroylglutamic acid is used.
B = Studies when dihydropteroylglutamic acid is used.
C = Studies when 5-formyltetrahydropteroylglutamic acid is used.

TABLE IV. The inhibition effect of methotrexate on the growing cultures of P. cerevisiae.

Concentration of methotrexate (ng/ml)	Concentration of folate* (ng/ml)					
	1	2	3	4	5	6
0.0	0	1.0 ± 0.0	3.0±0.5	5.0 ± 0.5	8.0 ± 0.5	13.0±0.5
0.5	0	1.0 ± 0.0	3.0±0.5	5.0 ± 0.5	8.0 ± 0.5	13.0±0.5
5.0	0	1.0 ± 0.0	3.0±0.0	4.8 ± 0.5	6.5 ± 0.5	11.0±0.5
50.0	0	0.9 ± 0.1	2.9±0.1	4.8 ± 0.5	7.0 ± 1.0	8.5±0.5
500.0	0	0.5 ± 0.0	2.6±0.2	4.2 ± 0.5	5.0 ± 0.5	5.7±0.2

* 5-formyltetrahydropteroylglutamic acid is used in the studies.

TABLE V The serum methotrexate from 2 subjects after 10 mg of orally administered methotrexate.

Time after doses (h)	Methotrexafe concentration (ng/ml)
0	0
1	200±25
2	100±0.0
3	70±5.0
4	50±0.0
24	20±5.0
40	0

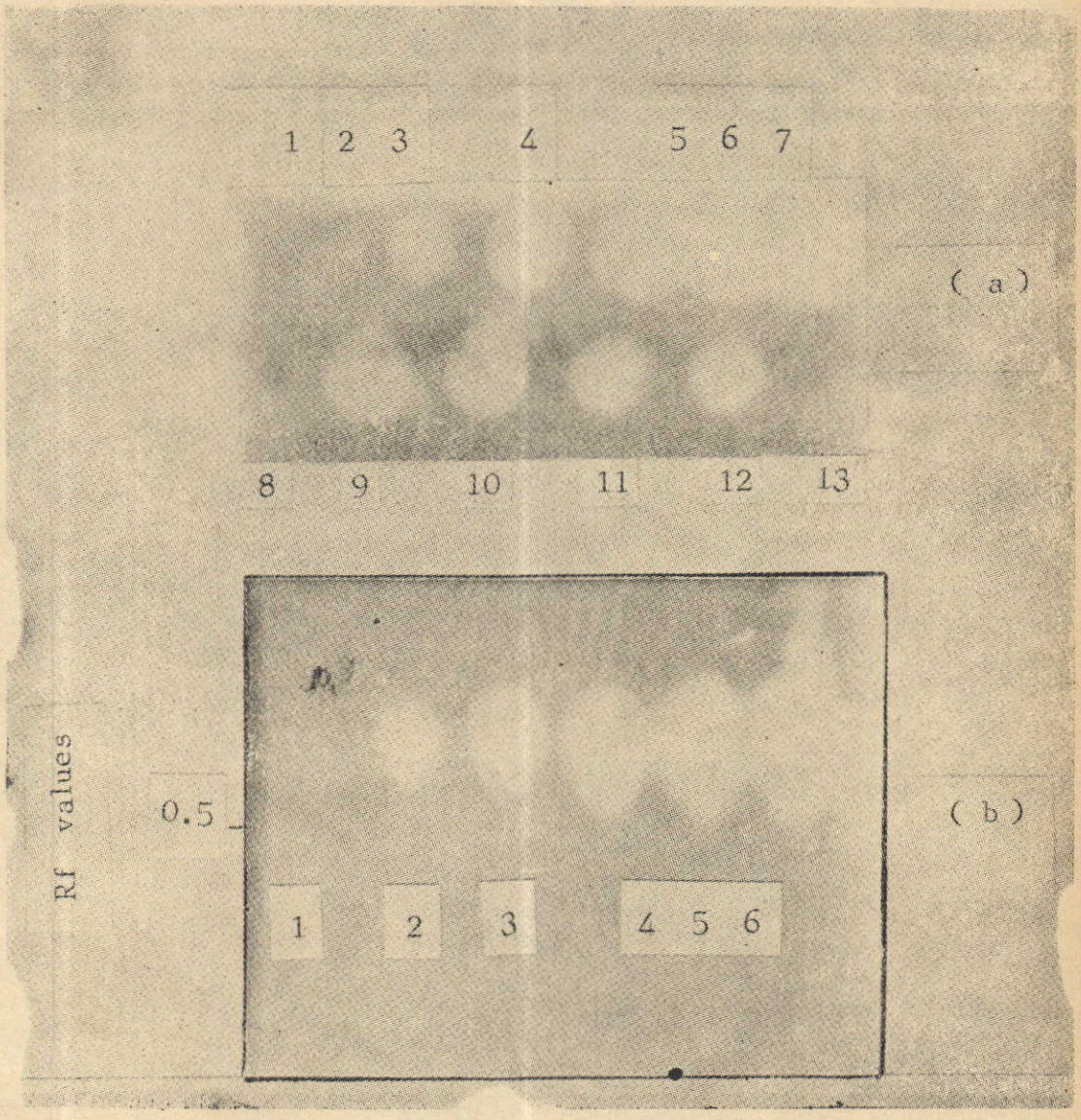

PLATE I: The inhibition zones of methotrexate on the bioautography with L. casei. Plate Ia was developed by using diffusion effect and the methotrexate concentrations (ng/ml) were 0, 5, 50, 100, 200, 300, 500 for spots 1 to 7, respectively and spots 8 to 13 were unknown from sera of a patient after 10 mg of methotrexate orally. Plate I b was developed by running a vertical chromatography of standards in 3% aqeous ammonium chloride solvent and the concentrations (ng/ml) were 5, 50, 100, 200, 300, 500 sespectively.

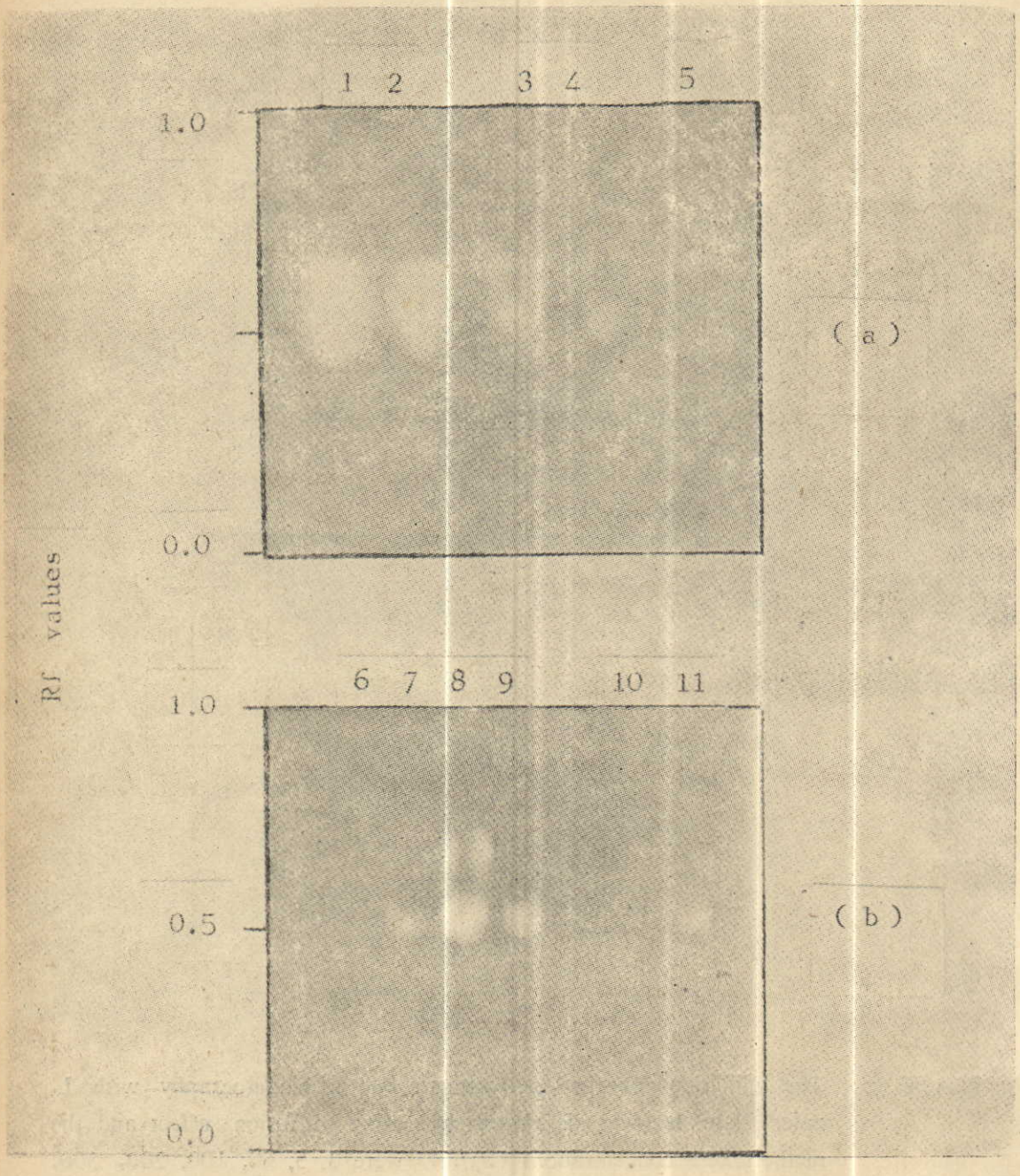

PLATE II : Bioautography of samples from subjects after 10 mg of methotrexate orally. Plate II a, at time 1, 2, 3, 4, 48 hours after doses and Plate I Ib at time 48, 24, 4, 24, 48, 24 hours respectively.

DISCUSSION.

The inhibition effects of methotrexate on the growing of L. casei, S. faecalis and P. cerevisiae are different. With S. faccalis and P. cerevisiae only partial inhibition were seen (Tables III & IV) and thus these two microorganisms are not of much use in the tetrazolium bioautography of antifolates. The very high sensitivity of inhibition of methotrexate to L. casei (Table II) made the organism a very useful for the application with bioautographic technique. The 2 techniques of plates preparation are of some usefulness, the first one is useful for the determination and characterization of the unknown and the second one is useful for the quantitative analysis of the unknown. Eventhough some enzymatic techniques are available for the determination of dihydrofolate reductase inhibitors (Werkheiser et al 1961; Bertino and Fischer, 1964) it is useful for only the quantitative determination. The technique presented here have advantages over the enzymatic techniques in the way that it could be used for both qualiative and quantitative analyses and required no complicated instrumentation. The plates used in this technique can also be prepared by using paper chromatography and thin layer chromotography as described here.

Studies on serum methotrexate levels in psoriasis patients after 10 mg of orally administered methotrexate (Table V) indicated that it is quickly absorbed and presented in the serum unmetabolized as shown in plates I and II. Since determination of serum methotrexate is possible it is reasonable to assume that the determination of urine methotrexate could be equally achieved. Eventhough methotrexate is quickly absorbed it is also quickly excreted into the urine (Henderson et al 1965; Goodman and Gilman, and Gilman, 1970; Huffman et al 1973). Small amount of methotrexate was left circulating in the blood 24 hours after doses (Plate II b). It is not clear whether the absorbed methotrexate is totally excreted or partially excreted into urine after doses. Evidences from many sources (Henderson et al 1965; Goodman and Gilman, 1970: Huffman et al 1973) and the pharmacokinetics analysis of the data presented here indicated that the absorbed methotrexate is partially retained by the body tissues. This is supported by the fact that without rescuring agent the treatment of patients with methotrexate led to a lethal effect (Sullivan et al 1959: Goldin et al 1953; Duff et al 1961;) Blair and Searle, 1970).

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ย่อความ

ผู้ทำการวิจัยได้ศึกษาถึง ความสามารถการขัดขวางการเจริญเติบโตของจุลอินทรีย์ ที่ใช้ในการ ตรวจหาปริมาณของ Folic acid โดย Methotrexate การศึกษาพบว่า Methotrexate มีผลไม่มากนักต่อการขัดขวางการเจริญเติบโต ของ S. faecalis และ P. cerevisiae แต่มีผลอย่างมาก ต่อการเจริญเติบโตของ L. casei จากการค้นพบนี้เมื่อใช้ L. casei ใน tetrazolium bioautography จะสามารถใช้ตรวจหาปริมาณของสารพวกที่เป็น antifolate ได้ การศึกษาระดับของ serum methotrexate ในคนไข้ psoriasis พบว่าหลังจากการกิน 10 มก. Methotrexate (tablets) ระดับของ Methotrexate ที่ปรากฏในเลือดสูงขึ้นถึงจุดสูงสุด (200 ng/ml) ภายใน 1 ชม. และ จะค่อย ๆ ลดลงจนหมดไปภายในเวลา 48 ชม. Methotrexafe ที่ปรากฏในเลือดจะเป็นแบบเดียวกับที่ให้กินเข้าไป

REFERENCES.

1. Bertino, J.R., and Fischer, G.A. (1964): Techniques for study of resistance to folic acid antagonists.. Meth. med. Res. 10 297-307.

2. Blair, J.A., and Saunders, K.J. (1970): A convenient method for the preparation of dl-5-methyltetrahydrofolic acid (dl-5-methyl-5,6,7,8-tetrahydropteroyl-L-monoglutamic acid). Anal. Biochem. 34 376-381.

3. Blair, J.A., and Searle, C.E. (1970): Reversal of methotrexate toxicity in mice by 5-methyltetrahydrofolic acid. Brit. J. canc. 24 603-906.

4. Blakley, R.L. (1959): The reaction of tetrahydropteroylglutamic acid and related hydropteridines with formaldehyde. Biochem. J. 82 707-715.

5. Condit, P.T. (1960): Studies on the folic acid vitamins. III. The duration of the effects of folic acid antagonists in man. Cancer 13 229-235.

6. Condit, P.T., and Eliel, L.P. (1960): Effects of large infrequent doses of A-methopterin on acute leukemia in children. J. Am. med Assoc. 172 105/451-107/453.

7. Duff, J.K., Sullivan, R.D., Miller, E., Ulm, A.H., Clarkson, B.D., and Clifford, P. (1961) Antimetabolite-metabolite cancer chemotherapy using continuous intraarterial methotrexate with intermittent citrovorum factor. Method of Therapy. Cancer 14 744-752.

8. Farber, S., Diamond, L.K., Mercer, R.D., Sylvester, R.F. Jr., and Wolff, J.A. (1948): Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-amino pteroylglutamic acid (Aminopterin). New. Engl. J. Med. 238 787-793.

9. Futterman, S. (1963): Preparation and properties of dihydro folic acid. In 'Methods in enzymology' 6 801-802. (S.P. Colowick and N.O.Kaplan, editors), Academic press.

10. Goldin, A., Mantel, N., Greenhouse, S.W., Venditti, J.M., and Humphreys, S.R. (1953): Estimation of the antileukemic potency of the antimetabolite aminopterin, administered alone and in combination with citrovorum factor or folic acid. Canc. Res. 13 843-850

11. Goodman, L.S., and Gilman, A. (1970): The pharmacological basis of therapeutics. pp 1360-1395. Macmillan.

12. Hertz, R., Lewis, J. Jr., Lipsett, M B. (1961): Five years experience with the chemotherapy of metastatic choriocarcinoma and related trophoblastic tumors in women. Am. J. Obstet. Gynec. 82 631-640

13. Henderson, E.S., Adamson, R.H., Denham, C., Oliverio, V,T. (1965): The metabolic fate of tritiated methotrexate. I. Absorption, excretion and distribution in mice, rats, dogs and monkeys. Canc. Res. 25 1008-1017.

14. Herbert, V. (1966): Aseptic addition method for Lactobacillus casei assay of folate activity in human serum. J. clin. Path. 19 12-16.

15, Huffman, D.H., Wan, S.H., Azarnoff, D.L., and Hoogstraten, B. (1973): Pharmacokinetics of methotrexate. Clin. Pharm. Ther. 14 572-579.

16, Hustu, H.O., AurR.J.A., Verzosa, M.S., Simone, J.V., and Pinkel, D. (1973): Prevention of central nervous system leukemia by irradiation. Cancer N.Y. 32 585-597.

17. Leeming, R.J., and Portman-Graham, H. (1973): An automated method for microbiological assays. Med. Lab Tech. 30 21-25.

18. Sullivan, R.D., Miller, E., and Sikes, M.P. (1959): Antimetabolite - metabolite combination cancer therapy. Effects of intra-arterial methotrexate-intra muscular citrovorum factor therapy in human cancer. Cancer 12 1248-1262.

19. Werkheiser, W.C. (1961): Specific binding of 4-aminofolic acid analogues by folic acid reductase. J. biol. Chen, 236 888-893.

20. Wright, J.C., Gumport, S.L., and Golomb, F.M. (1960): Remissions produced with the use of methotrexate in patients with mycosis fungoides. Canc. Chemother. Pep. 9 11-20.