Biochemical Pharmacology, Vol. 17, pp. 2228–2229. Pergamon Press. 1968. Printed in Great Britain

# The presence of a substance with hydrogen transporting properties in some samples of methotrexate

(*Received* 16 *April* 1968; *accepted* 10 *May* 1968)

METHOTREXATE (4 Amino $N^{10}$ methyl pteroylglutamic acid), an analogue of folic acid, is widely used in the chemotherapy of leukaemias. Its effectiveness has been assumed to depend on its ability to inhibit enzymic reactions involving folate or folate derivatives, particularly dihydrofolate reductase (dihydrofolate + $NADPH_2 \rightarrow$tetrahydrofolate + NADP).[1] Reports have been published, however, of its ability to affect processes in which the reduction of folate is not involved. Perhaps the most interesting of these is the observation that methotrexate can inhibit mitosis of cells, resulting in "metaphase arrest" *in vitro*.[2, 3] The fact that this "metaphase arrest" can be relieved by leucovorin has led to the speculation that the "metaphase arrest" may be due to the ability of methotrexate to bring about changes in the hydrogen bonding between chromosomes.[4] This finding is clearly of the greatest interest in connection with the chemotherapeutic use of methotrexate in the treatment of leukaemia.

In the work described below, we have found that several parenteral, freeze-dried samples of methotrexate which had been stored at 4° for 1–2 yr, contained a substance which is capable of transferring hydrogen from $NADPH_2$ or $NADH_2$ to the tetrazolium salt MTT. Samples more recently obtained have been found not to contain this substance. We do not know whether this substance was present in the older samples originally, or has been formed as a result of prolonged storage.

During the course of a study on the levels of dihydrofolate reductase activity in rat tissues at different times after injection of methotrexate, we have developed a colorimetric system for the detection and estimation of the enzymic activity in intact cells or fresh frozen tissue sections.[5] Using this assay system, which depends on the reduction by tetrahydrofolate of the tetrazolium salt, MTT, to formazan, the following was observed: Addition of low concentrations of the stored parenteral, sodium methotrexate to the assay system, consisting of 20 $\mu$M $NADPH_2$, 100 $\mu$g MTT, 20 $\mu$M dihydrofolate, dihydrofolate reductase, and 0·1 M Tris pH 7·5 in a total volume of 2 ml, led to the expected decrease in formazan production, ($10^{-6}$ M methotrexate is routinely used to inhibit completely dihydrofolate reductase). However, addition of solutions of this methotrexate at concentrations $\geq 10^{-2}$ M exhibited increased rates of formazan production. It appeared that high concentrations of these samples of methotrexate were able to mediate the transfer of hydrogen from $NADPH_2$ or $NADH_2$ to MTT. (Fig. 1). Methyl paraben and propyl paraben, which are present in the parenteral sodium methotrexate as preservatives, were found to be inactive with respect to the hydrogen transferring reaction. Solutions of the parenteral, freeze-dried, sodium methotrexate were examined for the presence of contaminants by chromatography on Whatman 3 MM paper, using 0·1 M '$PO_4$ pH 6·9 as the developing solvent. The solutions of parenteral methotrexate were found to contain a variety of compounds which fluoresced under u.v. light (Hanovia Chromatolite) and which separated from methotrexate on the chromatograms. When strips of the paper chromatograms were placed in a solution containing 500 $\mu$g MTT, 500 $\mu$g $NADH_2$ and Tris 0·1 M pH 7·5 in a total volume of 5 ml it was seen that formazan production was almost entirely associated with a yellow band which was located on the starting lines of the chromatograms. No formazan production was observed at the RF occupied by methotrexate. The areas of the chromatograms associated with formazan production were washed with 6 N acetic acid. A yellow eluate was obtained which, at neutral pH, had a single absorption peak in the u.v. at 259 m$\mu$ compared with the two peaks, one at 259 m$\mu$ and the other at 304 m$\mu$ exhibited by solutions obtained by elution of the methotrexate zone

of the chromatograms. The solution obtained by elution of the starting line of the chromatograms was found to be effective in inhibiting dihydrofolic reductase, and also catalysed the hydrogen-transferring reaction in the spectrophotometer, yielding similar results to those given in Fig. 1.

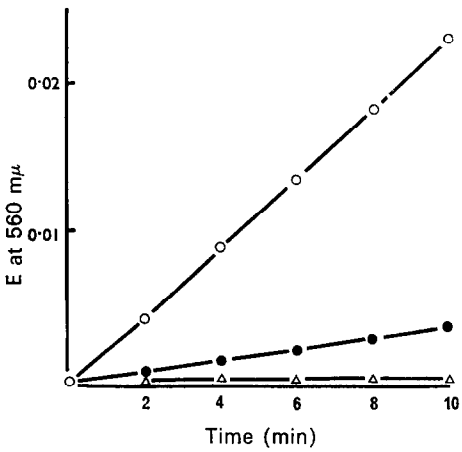


FIG. 1. ○———○ complete system $NADH_2$ or $NADPH_2$ 20 μM, methotrexate 10 mM, Tris pH 7·5 0·05 M, MTT 100 μg. Total vol. 2 ml; ●———● less $NADH_2$ or $NADPH_2$; △———△ less MTX.

Although the concentrations of methotrexate used in this study to induce the hydrogen transfer reaction are much higher than those normally employed, in view of the known protein-binding capacity of pteridines[6] it appears possible that even using much lower concentrations in incubation media, locally high concentrations could be produced by absorption onto proteins. It would therefore appear to be desirable to examine samples of methotrexate to be used in mitotic arrest studies for the presence of such a contaminant before use.

*Acknowledgement*—We are indebted to Lederle Co., Great Britain, for the generous gifts of the methotrexate used in this study.

*The Research Department,*
*The Marie Curie Memorial Foundation,*
*Caterham, Surrey*

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