these enzyme systems by different agents acting on chromosomal functions could be visualized[15].

Zusammenfassung. Isoliertes Rattenleberchromatin baut die endständige Phosphat-Gruppe von (γ-^{32}P) ATP in seine Proteine in Form von Phosphatester von Serin und Threonin ein. Der Einbau ist Mg^{++} und Mn^{++} abhängig und steigt mit Erhöhung des pH des Inkubationsmediums an. Phosphat und Pyrophosphat stimulieren die Phosphorylierung, zyklisches AMP hat keinen Effekt. Die sauren Proteine werden viel stärker als die Histone phosphoryliert. Das eingebaute Phosphat wird sehr schnell freigesetzt, ein Prozess, der von der Anwesenheit von dephosphorylierenden Enzymen abhängt.

E. Schiltz and C. E. Sekeris

Physiologisch-Chemisches Institut der Philipps-Universität, D-355 Marburg, Lahn (Germany), 8 July 1970.

[15] Acknowledgments. We thank Prof. P. Karlson for his encouraging interest in our work and we are grateful to Miss Froehlich, Miss Leyendecker and Mrs. Pfeiffer for their skillfull assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

Effect of Methotrexate on DNA Synthesis and Thymidine Kinase Activity of Human Lymphocytes Stimulated with Phytohaemagglutinin

The folic acid analogue Methotrexate[1] (4-amino-10-methyl pteroyl glutamic acid) is known to inhibit DNA synthesis mainly by blocking the conversion of deoxyuridylate to deoxythymidylate[2-5]. Also associated with the action of MTX are a number of changes in the activities of the enzymes of the DNA synthetic pathway. However, with the exception of the dihydrofolate reductase activity which is generally decreased a short time after MTX[4-7], the activities of other enzymes exhibit a wide range of variability depending on the experimental system employed. In cultures of Chang human liver cells Eker[8] has found an increase of thymidine kinase activity following the addition of MTX. On the contrary Labow, Maley and Maley[9] showed that in regenerating liver MTX completely inhibits the stimulation of deoxycytidylate deaminase, thymidine kinase and thymidylate kinase but increases the activity of thymidylate synthetase.

In PHA stimulated human lymphocytes MTX has been used as a synchronizing agent. It has been shown to reversibly block cells in S phase[10], but the biochemical events related to this block are still unclear.

In the present work we studied the effect of MTX on the incorporation of ^{3}H-thymidine into DNA of stimulated human lymphocytes. MTX induced an increase of incorporation of exogenous thymidine into DNA. For this reason in order to assay the real DNA synthetic activity, ^{32}P incorporation into DNA was evaluated. We also studied the effect of MTX on thymidine kinase activity to rule out the possibility that an increase of the thymidine phosphorylation could be related to the increased thymidine incorporation into DNA.

Methods and material. Human blood was drawn from healthy volunteers of both sexes, mixed with heparin (20 units/ml) and 4 volumes of blood, 1 volume of Plasma gel (Roger Bellon – France) and allowed to sediment at 37 °C for 1–2 h. The supernatant was passed through a nylon wool column (Filtralon) where 90–98% of the pagocytic cells were retained[11]. The lymphocytes were collected by centrifugation, resuspended (1.5×10^6 cells/m^3) in TC 199 containing 20% of autochthonous plasma and distributed in 7 ml aliquots into glass screw-cap flasks of 25 ml total capacity. PHA (Burroughs, Wellcome) was added to a concentration of 10 µl/ml of culture and Methotrexate (Lederle), unless otherwise stated, to a concentration of 5 µg/ml of culture. MTX, when added, was present from the initiation of the cultures. All the cultures were harvested 48 h after the addition of PHA.

In order to study DNA and RNA synthesis ^{3}H-TdR (5 Ci/m*M*, Radiochemical Centre – Amersham) 1 µc/ml and ^{32}P as H_3PO_4 (Sorin – Saluggia) 5 µc/ml were added 1 h and 3 h respectively before harvesting the cultures.

[1] The following abbreviations are used: MTX, methotrexate; PHA, phytohaemagglutinin; TdR, thymidine; dTTP, thymidine triphosphate.

[2] C. A. Nichol and A. D. Welch, Proc. Soc. exp. Biol. Med. *74*, 403 (1950).
[3] M. E. Balis and J. Dancis, Cancer Res. *15*, 603 (1955).
[4] J. R. Bertino, Cancer Res. *23*, 1286 (1963).
[5] W. C. Werkheiser, Cancer Res. *23*, 1277 (1963).
[6] D. Roberts, Cancer *20*, 905 (1967).
[7] J. Borsa and G. F. Whitmore, Molec. Pharmac. *5*, 303 (1969).
[8] P. Eker, J. biol. Chem. *241*, 659 (1966).
[9] R. Labow, G. F. Maley and F. Maley, Cancer Res. *29*, 366 (1969).
[10] J. A. Steffen and W. M. Stolzmann, Expl. Cell Res. *56*, 453 (1969).
[11] J. O. Lamvik, Acta haemat. *35*, 294 (1966).

Table I. Effects of MTX on the percent of cells in S phase, on the incorporation of ^{3}H-TdR and ^{32}P into DNA and on the incorporation of ^{32}P into RNA

Treatment	Labelled cells (%)	^{3}H/µg of DNA (count/min)	^{32}P/µg of DNA (count/min)	^{32}P/µg of RNA (count/min)
Controls (PHA only)	30	1563 ± 438[a] (8)[b]	119.8 ± 16 (6)	1851 ± 285 (3)
Methotrexate (MTX and PHA)	37	5009 ± 1107 (6)	59.3 ± 7.5 (6)	1785 ± 74 (3)

The method of SCOTT, FRACCASTORO and TAFT[12] was adopted for nucleic acids extractions and the amounts of DNA and RNA were determined by UV-spectrophotometry. For RNA determination the two-wavelength correction was used[13]. The radioactivity of the extracts was determined by mixing 0.1 ml aliquots with 10 ml of toluene-absolute ethanol (4:1) scintillator containing 4 g/l Omnifluor (98% 2,5-Diphenyloxazole and 2% *p-bis-(o*-methylstyryl)benzene) and counting in a Nuclear Chicago scintillation spectrometer model Mark I, at an efficiency of 20%.

Standard technique was used in the preparation of autoradiographic smears[14]. Labelling values were determined on at least 1000 cells.

For the determination of thymidine kinase activity about 10^7 cells were homogenized at 4 °C in 0.5 ml of 0.15 *M* KCl, 3 m*M* 2-Mercaptoethanol, 10 m*M* *Tris*-HCl (pH 7.5 at 20 °C) with a motor driven all glass homogenizer. The homogenate was centrifuged at 30,000 × *g* for 15 min at 4 °C and the supernatant fraction was used as the source of enzyme. The incubation mixture contained, in a final volume of 0.3 ml: ATP 9 m*M*, $MgCl_2$ 9 m*M*, NaF 5 m*M*, *Tris*-HCl (pH 7.5 at 20 °C) 100 m*M*, 3-phosphoglycerate 7.5 m*M*, ^{3}H-TdR 2 μ*M* (specific activity 0.5 Ci/m*M*) and 0.15 ml of enzyme. After incubating for 15 min at 37 °C, the reaction was terminated by immersing the tubes in boiling water for 2 min. The amount of TdR phosphorylated was determined by the method of BREITMAN[15]. The disks were dried and counted in Toluene-Omnifluor at an efficiency of 3%.

Results. Morphological observation of the cells after 48 h of culture did not reveal any difference between the controls and the MTX treated cultures, both showing blastoid transformation to the same extent.

Table I shows the effects of MTX on several parameters of DNA synthesis. The results show that the incorporation of exogenous thymidine into DNA is increased more than 3 times after MTX treatment whereas the fraction of cells in S phase, as revealed by autoradiography, was only slightly increased. The results also revealed a decreased incorporation of ^{32}P into DNA and no effect on its incorporation into RNA. The same effect were obtained (not shown) with doses of MTX of 0.5 μg and 25 μg/ml.

Table II shows the effect of MTX on thymidine kinase activity. As expected the lymphocytes stimulated with PHA exhibit a much higher activity than the non stimulated lymphocytes. On the other hand no difference in the ability to phosphorylate thymidine is shown between the lymphocytes treated with PHA alone and those treated with PHA and MTX.

Discussion. The finding that MTX does not inhibit the stimulation of lymphocytes and their entry into DNA synthesis is in agreement with the results of STEFFEN and STOLZMANN[10]. These authors also found that MTX increases the fraction of cells present in the S phase 70 h following PHA stimulation. In contrast, in our experiments after 48 h of culture, the fraction of cells in DNA synthesis, both in control and in MTX treated cultures, were similar. Thus the increased ^{3}H-TdR incorporation into DNA cannot depend on an increased number of cells present in the S phase. Furthermore, the increased ^{3}H-TdR incorporation into DNA induced by MTX did not reflect an increased synthetic rate; in fact ^{32}P incorporation into DNA was depressed by MTX. Under the same conditions ^{32}P incorporation into RNA was not decreased. We concluded, therefore that no ^{32}P pool effect or cellular permeability changes, but true inhibition of DNA synthesis, was responsable for the decreased ^{32}P incorporation into DNA. These results are in agreement with the early reports of WINZLER, WILLIAMS and BEST[16] and of WELLS and WINZLER[17] who found that DNA synthesis was more inhibited by MTX than RNA synthesis.

These data indicate that the higher specific activity of the ^{3}H-thymidine labelled DNA following MTX is due to a decreased size and an increased specific activity of the dTTP pool. This results from the inhibition by MTX of the conversion of deoxyuridylate to deoxythymidylate, a reaction that in most tissues is part of the major pathway leading to dTTP.

Resting lymphocytes possess very little ability to phosphorylate thymidine but this ability increases several fold after PHA stimulation[18]. In our experiments this increase of thymidine kinase activity was not altered by MTX. This is in contrast with the results of both LABOW, MALEY and MALEY[9] in regenerating liver, and EKER[8] in Chang human liver cells, who found respectively an inhibition and an induction of thymidine kinase by MTX[19].

Riassunto. Il methotrexate provoca nei linfociti umani stimolati per 48 con fitoemagglutinina un aumento di incorporazione di timidina ^{3}H nell'ADN senza modificare sensibilmente il numero di cellule in fase S. La sintesi di ADN valutata con l'incorporazione di ^{32}P risulta diminuita, mentre l'attività della timidin kinasi non è modificata dal trattamento con MTX.

L. PEGORARO and G. BENZIO

Section of Haematology, Medical Clinic, University of Turin (Italy), 10 July 1970.

Table II. Effects of MTX on thymidine kinase activity

Treatment	μμmoles of TdR phosphorylated (min/mg of protein)
None	1.2 ± 0.8[a] (9)[b]
PHA	11.2 ± 2.4 (6)
PHA and MTX	11.4 ± 2.8 (6)

[a] Mean values and standard deviations. [b] In parentheses the number of cultures examined.

[12] J. P. SCOTT, A. P. FRACCASTORO and E. B. TAFT, J. Histochem. Cytochem. *4*, 1 (1956).
[13] A. FLEK and N. M. MUNRO, Biochim. biophys. Acta *55*, 571 (1962).
[14] F. GAVOSTO, A. PILERI, C. BACHI and L. PEGORARO, Nature, Lond. *203*, 92 (1964).
[15] R. T. BREITMAN, Biochim. biophys. Acta *67*, 153 (1963).
[16] R. J. WINZLER, A. D. WILLIAMS and W. R. BEST, Cancer Res. *17*, 108 (1957).
[17] W. WELLS and R. J. WINZLER, Cancer Res. *19*, 1086 (1959).
[18] Y. RABINOWITZ, P. WONG and B. A. WILHITE, Blood *35*, 236 (1970).
[19] This work was supported by Consiglio Nazionale delle Ricerche (CNR), Rome.