tion. To 0.5 ml of the solution of the compounds, 0.15 ml of the protamine or quinine solutions were added and the presence of precipitate or turbidity formation was recorded.

Results and discussion. The chemical structures along with the results obtained are shown in the table. The methylsulfone-tetrazole cephalosporins tended to form more abundant precipitate with protamine or quinine than did the ethylaminosulfonic acid compounds. The non-sulfated cephalosporins (cefuroxime, cefamandole, SK&F 82956) formed neither precipitate nor cloudiness. The number of sulfonic acid groups as well as the substituents at the 7-position seems to influence the bulkiness of the precipitate. This complexing or combining phenomenon with precipitate (cloudiness, opalescence) formation may be a visible indication (in contrast to soluble complexes) of the in vivo binding capacity of these cephalosporins. Cefonicid (SK&F 75073)[5] and SK&F 80303[6] are highly serum protein bound with high and prolonged blood levels following parenteral administration[7]. The other cephalosporins studied, which do not form precipitates, have much lower serum protein binding capacity and lower and shorter serum levels. The concentration of compounds in this in vitro test is obviously higher than the obtainable therapeutic serum levels, nevertheless, the combining property of these agents with basic plasma constituents can be simulated in this test model. The complex formed by protamine or quinine with cefonicid or SK&F 80303 is insoluble in water and has biological activity after washing and vacuum-drying. This test model may prove useful in studying mechanisms of the pharmacokinetic and chemotherapeutic behavior of long-acting tetrazole-sulfonic acid cephalosporins and other chemical agents[9].

1 T.Z. Csáky, Cutting's Handbook of Pharmacology, 6th edn, pp. 135, 302, 402. Appleton-Century-Crofts, New York 1979.
2 F. Hawking, in: Advances in Pharmacology and Chemotherapy, vol. 15, p. 289. Academic Press, New York 1978.
3 E. Chargaff and K.B. Olson, J. biol. Chem. *122*, 153 (1937).
4 H.A. Harper, V.W. Rodwell and P.A. Mayes, Review of Physiological Chemistry, 17th edn, p. 109. Lange Medical Publications, Los Altos 1979.
5 P. Actor, J.V. Uri, I. Zajac, J.R. Guarini, L. Phillips, D.H. Pitkin, D.A. Berges, G.L. Dunn, J.R.E. Hoover and J.A. Weisbach, Antimicrob. Agents Chemother. *13*, 784 (1978).
6 J.V. Uri, P. Actor, I. Zajac, D.H. Pitkin, L. Phillips, J.R. Guarini, H.F. Bartus, T.J. Polansky, G.L. Dunn, J.R.E. Hoover and J.A. Weisbach, J. Antibiot. *32*, 1161 (1979).
7 D.H. Pitkin, P. Actor, F. Alexander, J. Dubb, R. Stote and J.A. Weisbach, in: Current Chemotherapy and Infectious Disease, vol. 1, p. 252. Am. Soc. Microbiol., Washington, DC 1980.
8 A. Imada, K. Kitano, K. Kintaka, M. Muroi and M. Asai, Nature *289*, 590 (1981).
9 Since the submission of the manuscript of this communication, additional and basically similar results were obtained with histone (calf thymus type II) and the recently discovered sulfamate group (–N.SO_3H) - containing novel monocyclic β-lactam antibiotic sulfazecin[8].

Cell cycle patterns of thymidylate synthetase and 5,10-methylenetetrahydrofolate polyglutamates in cultured mouse hepatoma cells[1]

D.G. Priest, M.T. Doig and B.E. Ledford

Department of Biochemistry, Medical University of South Carolina, Charleston (South Carolina 29425, USA), 3 April 1981

Summary. The regulation of thymidylate synthetase activity was investigated throughout the first cell cycle after release from an isoleucine block in synchronous cultures of mouse hepatoma (Hepa) cells. Activity in cell extracts increased with the onset of S phase and the increased activity was attributed to a parallel increase in enzyme concentration as determined by titration with tritiated fluorodeoxyuridylate. The polyglutamate chain length of reduced folate cofactors, which could also influence thymidylate synthetase activity, was unchanged.

Thymidylate synthetase (methylenetetrahydrofolate: 2'-deoxyuridine-5'-monophosphate C-methyltransferase; EC 2.1.1.45) catalyzes the reductive methylation of deoxyuridylate (dUMP) to form thymidylate (TMP). The reaction is the one de novo source of dTMP required for DNA synthesis and because the activity of the enzyme is very low in most tissues, it has been suggested that the reaction may be a rate-limiting step in DNA synthesis[2,3]. Due to this pivotal role in DNA synthesis, thymidylate synthetase is a target enzyme in cancer chemotherapy[4] and its regulation during the cell cycle is therefore of considerable interest.

Thymidylate synthetase activity increases dramatically in rapidly proliferating versus stationary cells[5–7] and the specific activity has been shown to increase at the beginning of S phase[8]. Several modes of regulation have been suggested including changes in: a) enzyme synthesis, b) substrate availability, and c) enzyme affinity for the available substrate. Increased specific activity during S phase in mouse 3T6 fibroblasts seems to be controlled at the level of transcription[9]. Neither the concentration of dUMP[10,11] or the monoglutamate form of the folate cofactor[12] appear to limit enzyme activity; however, it has been suggested that changes in the polyglutamate forms of methylenetetrahydrofolate might influence in situ activity[12]. The affinity of thymidylate synthetase is greater for longer chain length polyglutamates[13–17].

The present study involves the stoichiometric titration of thymidylate synthetase in cell extracts[18] and the electrophoretic identification of polyglutamate chain lengths of 5,10-methylenetetrahydrofolate complexes[19] to investigate the influence of enzyme level and polyglutamate chain length on thymidylate synthetase activity during the cell cycle of cultured mouse hepatoma cells.

Materials and methods. All chemicals and materials except those listed below were purchased from Sigma. [6-^{3}H]FdUMP (18 Ci/mmole) and [5-^{3}H]dUMP (18 Ci/mmole) were purchased from Moravek Biochemicals. Folic acid was reduced to tetrahydrofolate by the method of Davis[20] and converted to the methylene derivative by the addition of 75 mM formaldehyde for storage at –70 °C.

The cell line, Hepa, was maintained in culture and synchronized by isoleucine deprivation as previously described[21]. Cell extracts were prepared by a freeze/thaw procedure[22] and aliquots were used for the determination of thymidy-

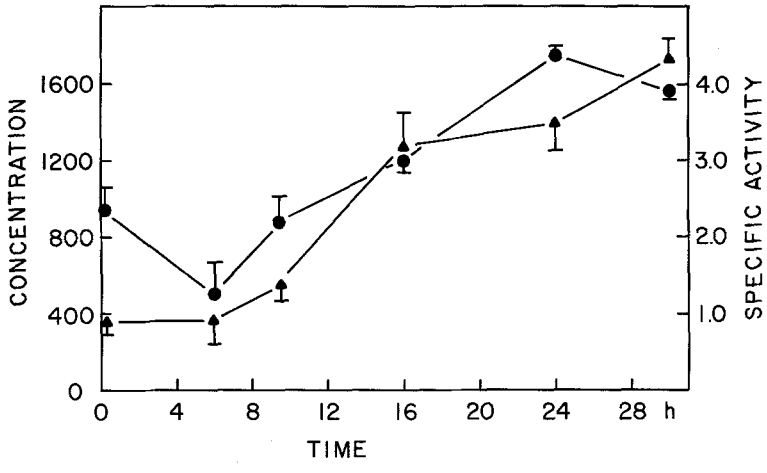

Figure 1. Comparison of thymidylate synthetase specific activity (▲) in μmoles per h per mg protein and concentration (●) in cpm of bound [^{3}H]-FdUMP per mg protein throughout one cell cycle. Error bars represent the range of duplicate assays from a typical experiment.

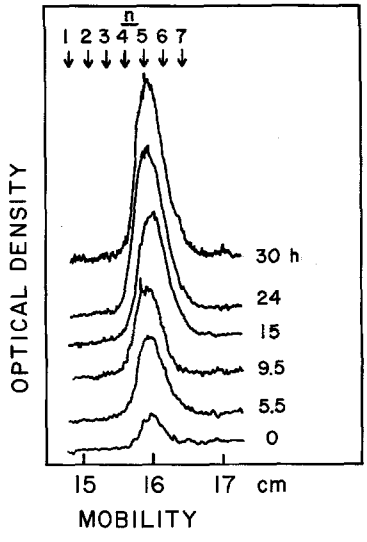

Figure 2. Comparison of folylpolyglutamate chain lengths throughout the Hepa cell cycle. Ternary complexes containing [^{3}H]-FdUMP were prepared from cell extracts at the indicated times in the cell cycle and were co-electrophoresed with complexes containing reference folylpolyglutamates. Fluorographs of these gels were scanned with a microdensitometer. The n indicates the position of bands for standards containing 1-7 glutamate residues attached to the pteroyl moiety.

late synthetase activity[23], thymidylate synthetase concentration[18] and methylenetetrahydrofolate polyglutamate chain length[19]. Protein concentration was estimated by the method of Bradford[24].

Results and discussion. Allen and Ledford[21] have shown that the Hepa cell line used in these studies begins to incorporate thymidine approximately 4 h after release of the isoleucine block. It can be seen in figure 1 that the specific activity of thymidylate synthetase, determined from initial velocity measurements of cell free extracts, increases subsequent to the onset of S phase. The increase in specific activity is paralleled by an increase in the concentration of thymidylate synthetase as determined with the active site titrant, [^{3}H]fluorodeoxyuridylate. Thus, the mode by which the specific activity of thymidylate synthetase is increased is consistent with an increased amount of enzyme.

Increases in thymidylate production could also be influenced by elongation of the polyglutamate portion of the methylenetetrahydrofolate cofactor[12]. Incorporation of endogenous methylenetetrahydrofolate into a complex with excess [^{3}H]fluorodeoxyuridylate and *L. casei* thymidylate synthetase followed by electrophoretic separation based on charge was used to identify the cofactor polyglutamate chain length[19]. It can be seen in figure 2 that no change in the distribution of polyglutamate forms of methylenetetrahydrofolate occur at any point throughout the cell cycle. Thus, the regulation of thymidylate synthetase activity during the cell cycle is not associated with changes in polyglutamate chainlength.

1 Acknowledgments. Supported by grant CA 22754, awarded by the National Cancer Institute, DHEW.
2 P.V. Danenberg, Biochim. biophys. Acta *473*, 73 (1977).
3 R.L. Blakley, in: The Biochemistry of Folic Acid and Related Pteridines, p.231. Ed. A. Neuberger and E.L. Tatum. Elsevier North Holland, Amsterdam 1969.
4 C. Heidelberger, in: Antineoplastic and Immunosuppressive Agents, Handbook of Experimental Pharmacology, vol.38, part I, p.139. Springer, New York 1974.
5 A.H. Conrad and F.H. Ruddle, J. Cell Sci. *10*, 471 (1972).
6 F.I. Haurani, C.G. Kardinal and W.A. Biermann, J. Cell Physiol. *95*, 49 (1978).
7 F. Maley and G.F. Maley, J. biol. Chem. *235*, 2968 (1960).
8 A.H. Conrad, J. biol. Chem. *246*, 1318 (1971).
9 L.G. Navalgund, C. Rossana, A.J. Muench and L.F. Johnson, J. biol. Chem. *255*, 7386 (1980).
10 D.G. Cunningham and R.A. Remo, J. biol. Chem. *248*, 6282 (1973).
11 G. Sander and A.B. Pardee, J. Cell Physiol. *80*, 267 (1972).
12 W. Rode, K.J. Scanlon and J.R. Bertino, in: Chemistry and Biology of Pteridines, p.489. Ed. R.L. Kisliuk and G.M. Brown. Elsevier North Holland, Amsterdam 1979.
13 D.G. Priest and M. Mangum, Archs Biochem. Biophys., in press (1981).
14 B.J. Dolnick and Y.-C. Cheng, J. biol. Chem. *253*, 3563 (1978).
15 R.L. Kisliuk, Y. Gaumont and C.M. Baugh, J. biol. Chem. *249*, 4100 (1974).
16 G.F. Maley, F. Maley and C.M. Baugh, J. biol. Chem. *254*, 7485 (1979).
17 R.L. Kisliuk, Y. Gaumont, E. Lafer, C.M. Baugh and J.A. Montgomery, Biochemistry *20*, 929 (1981).
18 D.G. Priest, C.W. Alford, K.K. Batson and M.T. Doig, Analyt. Biochem. *103*, 51 (1980).
19 D.G. Priest, K.K. Happel and M.T. Doig, J. biochem. biophys. Meth. *3*, 201 (1980).
20 L. Davis, Analyt. Biochem. *26*, 459 (1969).
21 R.P. Allen and B.E. Ledford, Cancer Res. *37*, 696 (1977).
22 D.G. Priest, B.E. Ledford and M.T. Doig, Biochem. Pharmac. *29*, 1549 (1980).
23 D. Roberts, Biochemistry *5*, 3546 (1966).
24 M.M. Bradford, Analyt. Biochem. *72*, 248 (1976).