In the search for the mechanisms which control the onset
and rate of DNA synthesis in the mammalian cell, the
intracellular production of thymidine (Tdn) nucleotides
has received some attention and has been discussed in a
recent review article by O'Brien (31). Increased activity
of the enzymes related to the production of Tdr nucleo-
tides and their incorporation into DNA has been shown
to occur in regenerating rat liver (3, 4, 5, 9, 25) as well as
in other growing tissues (26, 27, 34, 36, 41). Although
these alterations have been interpreted as representing
changes in homeostatic mechanisms supplying the neces-
sary ingredients for DNA synthesis (8), and therefore
being influenced by rather than influencing the rate of DNA
synthesis, some evidence is available to suggest that
alterations in the tissue and/or intracellular levels of Tdr
compounds may have an effect on the rate of DNA syn-
thesis. Greulich et al. (17) found that within 6 hr follow-
ing an i.p. injection of 10 μg of Tdr per adult male mouse
there was a 29% increase in the number of metaphase
mitotic figures in the duodenal epithelium, suggesting
alterations in the mitotic cycle and perhaps in the rate of
DNA synthesis. This leads to the question of the general
significance of Tdr kinase and the possibility of intra-
cellular and/or circulating Tdr in the animal. Relative
to this, the work of Rieke (39) is pertinent. Using adult
mice prelabeled with tritiated Tdr, he showed that host
lymphocytes were able to contribute label to the DNA of
transplanted sarcomas; conversely, prelabeled sarcoma
cells donated label to peripheral monocytes. Because the
biochemical reactions involved in the donation of the
labeled material were not elucidated, it is not known if the

Pyrimidine Metabolism in Tissue Culture Cells Derived
from Rat Hepatomas

II. Thymidine Uptake in Suspension Cultures Derived
from the Novikoff Hepatoma

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SUMMARY

Novikoff tissue culture cells were made totally dependent on thymidine supplied via the medium, by blocking with amethopterin. Radioactive thymidine then was supplied in various molarities, and after incubation periods of varying length the cultures were fractionated into medium, intracellular acid-soluble compounds, and deoxyribonucleic acid (DNA), and the fractions assayed for radioactivity. Several observations were made.

a) The cells rapidly concentrated thymidine from the medium into the acid-soluble fraction, reaching equilibrium in less than 10 min.

b) Radiochromatographic studies showed that the intracellular thymidine was largely (80-90%) in the form of thymidine triphosphate, with some (10-20%) thymidine diphosphate and little, if any, thymidine monophosphate or thymidine.

c) Increasing the concentration of thymidine in the medium caused an increase in the intracellular concentration of thymidine compounds.

d) For cell multiplication, the optimal intracellular concentration of thymidine triphosphate was roughly 5-10 × 10^{-5} M. This was inferred from growth rates in various external concentrations of thymidine, and the relationship of extracellular to intracellular concentrations of thymidine and thymidine compounds.

e) While the over-all rate (but not onset) of DNA synthesis of Novikoff tissue culture cells can be modified by limiting the intracellular thymidine triphosphate concentration, the importance of this mechanism in vivo remains to be demonstrated.
labeled thymidylate (dTMP) in the recipient DNA was degraded to Tdr during the transfer, and if so, whether or not the Tdr was circulating in acid-soluble form. In a somewhat related study, Bryant (6) found that when lymphoid cells labeled in vitro with tritiated Tdr were injected i.p. into partially hepatectomized mice, the radioactivity appeared in the liver parenchymal cell nuclei. Although the mechanisms of transfer again are not known, these works are important because they suggest a possible course of exogenous thymine by demonstrating that cells may under certain conditions synthesize DNA, at least in part utilizing preformed thymine supplied from other parts of the body. Also pertinent to this question is the demonstration of Tdr in rat and in calf thymus (14, 35, 46) and in the Novikoff hepatoma (43) as well as regenerating rat liver (44), which indicates the existence in the mammal of Tdr in the nucleoside form.

Although cells in tissue culture are removed from the controlling influence of the body, their growth can be inhibited by the addition of certain precursors of DNA to the medium. It has been demonstrated for several types of mammalian cells that large concentrations of Tdr in the culture medium (in the range of 10⁻⁴ M) will depress cell growth (28, 29, 30, 48), apparently by producing a deficiency in the intracellular nucleotides of deoxycytidine (Cdr), since this compound added to the culture medium will restore normal cell multiplication (29, 30, 48). It is possible also to produce a specific thymine deficiency by adding amethopterin to the culture medium, provided that suitable levels of adenosine, serine, and glycine are added to relieve other deficiencies induced by the amethopterin (18, 30, 40). Among purines, deoxyguanosine (Gdr) and 2-deoxyadenosine (Adr) block cell growth in cultures of mammalian cells that large concentrations of Tdr in the nucleoside form.

The purpose of the present study was to measure certain parameters of the metabolism of Tdr added to suspension cultures derived originally from the Novikoff rat hepatoma (30). These cells have the desirable characteristics, for a study of this type, of lacking Tdr nucleoside deoxyribosyl transferase (49), and also the ability to catabolize thymine and uracil (30). Among the questions studied are the intracellular form of the Tdr added to the cultures, and the extracellular concentration versus the intracellular concentration of Tdr compounds and the resulting influence on the growth rate. The relationship of the intracellular concentration of Tdr compounds to the metabolism of other pyrimidine nucleosides will be discussed in the third paper of this series (15).

MATERIALS AND METHODS

Novikoff hepatoma-derived suspension cultures were cultivated, and cell concentrations were determined by using haemocytometer as previously described (30). Several cell lines were employed, including the parent line, N1-S1, and a cloned line, N1-S4. No significant differences with respect to Tdr metabolism were noted between the 2 lines, and most of the data to be presented were obtained with the N1-S4 line. The composition of the complete growth medium No. 71, which contains 5% beef serum, and of the buffered basal medium S-77, has been described (30).

Isotopes.—Thymidine-2-¹⁴C, 25 mc/m mole, and thymidine-³H of various initial specific activities were diluted with nonradioactive Tdr to the desired final specific activity. While paper chromatography of the isotopes in Fink’s system No. 8 (12) revealed that the ¹⁴C Tdr did not contain significant impurities, it did show that several batches of ³H Tdr apparently contained moderate amounts of ³H thymine. For this reason, ¹⁴C Tdr was employed exclusively in quantitative experiments, while ³H Tdr was restricted to autoradiography and chromatography of the intracellular acid-soluble compounds. In the latter case, parallel experiments carried out with ¹⁴C Tdr revealed no differences in either the type or the proportion of intracellular Tdr compounds. The final specific activities of the ¹⁴C Tdr solutions were determined as follows: radioactivity was measured in the liquid scintillation counter, and the final concentration of Tdr was calculated from the concentration of the stock solutions of Tdr, which had been assayed by ultraviolet absorption. Although the stocks had been prepared in S-77 which contained phenol red, a 1/1000 dilution of S-77 in 10⁻⁴ M HCl had an absorbance at 267 mμ of less than 0.01, thus permitting an accurate assay on stock solutions of initial Tdr concentration of 10⁻¹ M.

Fractionation of cells.—Except where noted otherwise, the sample material was maintained at a temperature of approximately 5°C.

A. Ten milliliters of cell suspension containing 10⁴–10⁵ cells/ml were placed in a 12-ml centrifuge tube and centrifuged at approximately 1000 × g for 5–10 min. An aliquot of the supernatant medium was diluted 1:10 in H₂O for subsequent assay of radioactivity. The pellet of cells was washed twice in 5 ml of S-77 by resuspension with a vortex mixer and centrifugation as described above. Preliminary experiments showed that these washes did not remove any significant quantities of radioactivity, and the supernatants therefore were discarded.

B. After the 2nd wash, 1 ml of H₂O was added to the pellet, which then was resuspended with the vortex mixer and homogenized at 5°C using an ultrasonic disintegrator (Disontegrator, system forty; Ultrasonic Industries, Inc., Albertson, L.I., N. Y.) operating at a frequency of 90 kc. To the homogenate was added an equal volume (1 ml) of 10% trichloroacetic acid (TCA), and the contents were mixed with the vortex mixer. After centrifugation at approximately 1000 × g for 10–15 min, the supernatant, which contained the intracellular acid-soluble compounds, was decanted and saved for assay of radioactivity.

C. To the pellet 0.5 ml of H₂O was added; it then was resuspended as described above (B). One milliliter of 0.2 N NaOH was added, and the contents were mixed with the vortex mixer, heated in a water bath at 80°C for 20 min, and chilled to 0°C. Two volumes of 10% TCA...
serine, and glycine as described (30). In this situation into medium No. 71 containing amethopterin, adenosine, Tdr deficiency and Tdr excess. Cells were subcultured cold H2O. Stripping film was applied to the slides which were washed twice in ice-cold 5 % TCA and twice in ice-cold 0.4 N perchloric acid and centrifuged. The supernatants were decanted, neutralized with KOH to pH 7.0-8.0 and chromatographed by the system of the Dische reaction (10) was employed, with Adr used. The Burton modification (7) of the Dische reaction (10) was employed, with Adr used as a standard.

Analysis of intracellular acid-soluble compounds.—Aliquots of cell suspensions were chilled quickly to 0°C and washed twice in ice-cold S-77. The pellets were resuspended in ice-cold 0.4 N perchloric acid and centrifuged. The supernatants were decanted, neutralized with KOH to pH 7.0-8.0 and chromatographed by the system of Ives et al. (19).

Autoradiography.—Cells were washed twice in S-77 and smeared on glass slides. After being air-dried, the smears were washed twice in ice-cold 5 % TCA and twice in ice-cold H2O. Stripping film was applied to the slides which then were exposed, developed, and stained as described (24).

RESULTS
Experiments were carried out to determine the effects of Tdr deficiency and Tdr excess. Cells were subcultured into medium No. 71 containing amethopterin, adenosine, serine, and glycine as described (30). In this situation (3.0 ml) were added, and the contents were mixed and centrifuged as described in B. Preliminary experiments revealed that the supernatant, while containing essentially all of the digestion products of the ribonucleic acid (RNA) (13), did not contain a significant amount of radioactivity, or of DNA, as measured by the diphenylamine reaction (10); it therefore was discarded.

D. DNA was extracted essentially according to the method of Schneider (42), as follows. The pellet was resuspended as described in B, in 0.75-2.0 ml of H2O, depending on the pellet volume (usually less than 0.1 ml, in which case the minimum volume was used). An equal volume of 10 % TCA was added, and the contents were mixed with a stirring rod. Stirring continued intermittently during the extraction (20 min at 90°C), after which the contents were cooled and centrifuged as in B. This procedure extracted more than 90 % of the radioactivity in the pellet and represented the optimal time and temperature for extraction of DNA as measured by the diphenylamine reaction.

Estimation of radioactivity.—For liquid scintillation counting, 0.1-ml aliquots of samples were added to glass vials, 10 ml of scintillator solution (naphthalene, 80 gm; 2,5-diphenyloxazole, 5 gm; α-naphthylphenyloxazole, 50 mg; dissolved in 1 liter of a mixture of 5 parts xylene, 5 parts dioxane, and 3 parts ethanol) (21) were added and the sample radioactivity was measured as described (16), using a two-channel liquid scintillation spectrometer (automatic Tri-Carb liquid scintillation spectrometer, Packard Instrument Co., La Grange, Illinois). Preliminary experiments determined that the quenching factors for water and 1:10 dilution of medium No. 71 in water were the same, and that the supernatants containing the acid-soluble compounds, RNA hydrolysate, and extracted DNA (all basically 5 % TCA), all quenched to the same degree but more than water or the 1:10 dilution of medium No. 71 in water. Therefore, in correcting the data for quenching, the appropriate predetermined factor was used.

Diphenylamine reaction.—The Burton modification (7) of the Dische reaction (10) was employed, with Adr used as a standard.

Analysis of intracellular acid-soluble compounds.—Aliquots of cell suspensions were chilled quickly to 0°C and washed twice in ice-cold S-77. The pellets were resuspended in ice-cold 0.4 N perchloric acid and centrifuged. The supernatants were decanted, neutralized with KOH to pH 7.0-8.0 and chromatographed by the system of Ives et al. (19).

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RESULTS
Experiments were carried out to determine the effects of Tdr deficiency and Tdr excess. Cells were subcultured into medium No. 71 containing amethopterin, adenosine, serine, and glycine as described (30). In this situation the endogenous production of dTMP via thymidylate synthetase is blocked, but other cell activities are not hindered except as they depend on dTMP and/or DNA synthesis. Thymidine-2-14C was added to the flasks, and the final concentrations at time of subculture ranged from 10-6 to 10-3 M. The results (Chart 1) show that 10-6 M Tdr was not sufficient to support normal logarithmic growth, and that 10-3 M Tdr was inhibitory. On the other hand, 10-4 and 10-5 M Tdr permitted normal cell multiplication as measured both by diphenylamine and by cell counts. An examination of the curve for 10-5 M Tdr reveals that the cells apparently began to exhaust the supply of Tdr by 24 hr, and by 40 hr could multiply no longer. This is verified in Chart 2, in which the medium curves reflect the concentration of Tdr in the medium, 100 % being equivalent to the initial concentration in each case. The radioactivity in the acid-soluble fractions, though significant, represented a small proportion of the total count in the sample and for this reason is not included in Chart 2.
Intracellular acid-soluble thymidine compounds.—The techniques employed permitted estimates of the size of the intracellular pool of acid-soluble Tdr compounds including Tdr and the various Tdr phosphates, all of which are precursors for DNA-thymine. From Chart 3 it is obvious that when the Tdr concentration in the medium is increased, the intracellular Tdr compounds also are increased. The values were obtained by calculation from the total intracellular acid-soluble radioactivity, the total number of cells in each sample, and the specific activity of the added Tdr. Because the endogenous production of dTMP was blocked as described above, it was assumed that by the time the cells had doubled (first 24 hr), using the Tdr of the medium, the intracellular Tdr compounds would be totally derived from the Tdr of the medium and would have the same specific activity. This would obtain (a) if during a generation-time all the cells doubled their DNA and (b) if the intracellular pools of Tdr compounds were small relative to the total amount of thymine in the DNA. The first condition was demonstrated by autoradiography and will be described in a subsequent paragraph. That the second condition applies may be inferred from the following calculations. Based on the data in Chart 1, the average DNA content of 10^6 cells is approximately 7.6 μg. Using the thymine content of rat DNA, roughly 28 moles % (47), a value of 2.1 μg or 7 nanomoles (nmoles) of DNA-thymine/10^6 cells is obtained. This is more than twice the pool size found in the highest intracellular concentration of 4 X 10^-3 M, which in addition, if the cells ordinarily had a large reserve pool of Tdr compounds, the addition of amethopterin would not shut off DNA synthesis abruptly, whereas preliminary experiments indicated that in less than an hour after addition of amethopterin all DNA synthetic activity had stopped (unpublished data).

It was desirable to obtain an estimate of the intracellular molar concentration of the Tdr compounds and to compare the effects on cell growth of alterations in this level (Chart 4). The % increases in cell DNA/ml were obtained from the data in Chart 1, and the intracellular concentration to intracellular concentration of Tdr compounds in amethopterin-blocked N1-S4 cells. Amethopterin, adenosine, serine, and glycine added as described in Chart 1, at time of subculture. All values are averages of duplicate flasks. Experiment 1. nM = nanomoles = 10^-9 moles.

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The nature of the intracellular Tdr compounds after the administration of radioactive (H or 14C) Tdr to the suspensions was determined. The experiments were carried out as described in “Materials and Methods”. It was found, over a wide range of concentrations (10^{-6}-10^{-3} \text{ M}) of added Tdr, that whatever the amount, the Tdr taken into the cells was converted quite rapidly into dTTP (80-90\%) and dTDP (10-20\%). It was not possible to detect significant intracellular amounts of dTMP or Tdr in any of these experiments. An indication of the rapidity of attainment of a stable intracellular concentration of Tdr compounds, after addition of radioactive Tdr to the suspensions, can be obtained from an examination of Chart 5. These results are similar to those reported for Ehrlich ascites cells (20) and for a strain of Lactobacillus acidophilus (32); in both cases a stable equilibrium was obtained in less than 10 min. In addition, preliminary experiments (Chart 6) revealed that even in cells incubated at 4°C Tdr was taken into the acid-soluble fraction, and that by 1 hr the intracellular level of Tdr compounds was already almost one-half that of cells incubated at 37°C. Apparently DNA synthesis was shut off, inasmuch as no isotope was incorporated during this time. While amethopterin was not included in these experiments (Charts 5 and 6), a comparison of the data with those of Chart 3 will reveal similar levels of intracellular acid-soluble Tdr compounds from 10 min to 56 hr for a given concentration of Tdr in the medium. By the latter time, however, the Tdr concentration in the medium had dropped significantly in the 10^6 and 10^7 M flasks (Chart 2), and the intracellular levels therefore were somewhat lower. The relationship of the negative feedback of dTTP on Tdr kinase (19) to the rapid achievement of a stable intracellular level of Tdr nucleotides, even in the presence of large concentrations of Tdr in the medium, is not clear. Because adenosine triphosphate (ATP) reverses this feedback inhibition (19) knowledge of the intracellular levels of ATP will be required before any conclusions can be made.

**Autoradiography.**—Autoradiography was carried out with 3HTdr, as described, to obtain an estimate of the homogeneity of the cell population with respect to DNA synthesis during logarithmic growth. As anticipated, only some of the cells (approximately 50\%) were synthesizing DNA (as evidenced by labeling) during a 1-hr exposure to the isotope. When the exposure was increased to a large fraction of the generation time (10 hr), most of the cells were labeled and, after a 25-hr exposure, essentially all were labeled heavily. These findings support the assumption that during logarithmic growth all the cells undergo synthesis of DNA during one generation time.

Dependence of amethopterin-blocked cells on exogenously supplied thymidine.—That the cells blocked by amethopterin were dependent on Tdr supplied in the medium for a thymine source was demonstrated by the failure of such cells to grow if Tdr was not added (30). Furthermore, if the quantity of added Tdr was low (10^{-6} \text{ M}), the cells were able to grow until the Tdr was exhausted (Charts 1 and 2) after which growth stopped, indicating that growth in the presence of amethopterin depended on a continuous sup-
ply of exogenous Tdr. When the quantity of thymine incorporated into DNA over a specific interval was calculated from radioactivity data, however, the value usually was somewhat lower (10—30% less) than that calculated from measurements made on aliquots of the same samples with the diphenylamine reaction, assuming a thymine content of 28 moles % the value for rat DNA (47). The reasons for this discrepancy are not clear; however, because of the apparent complete dependence of the blocked cells on exogenous Tdr, and because the discrepancy was variable, we feel that it probably is an artifact. In any case, the data do show that the bulk of DNA-thymine (measured by diphenylamine and calculation) incorporated by amethopterin-blocked cells was derived from the medium.

5-Methyldeoxycytidine as a thymine source.—Because amethopterin interferes with the methylation of deoxyuridine (Udr) monophosphate (31), 5-methyldeoxycytidine (5-MeCdr) was tried as a possible precursor of DNA-thymine; this compound being chosen because the methyl group is already attached to position of the pyrimidine ring. It was found that the amethopterin block could be relieved completely by the addition of this compound at 10^{-4} M (Chart 7). It is of interest to note that 10^{-4} M 5-MeCdr produced a lower growth rate that nevertheless was logarithmic; at 10^{-3} M some growth took place for 24 hr, but after that the cell population appeared to decline. As anticipated, neither Udr nor Cdr at 10^{-4} M would support the growth of amethopterin blocked cells.

**DISCUSSION**

**Intracellular concentration of thymidine compounds.**—It may be seen from the data in Chart 4 that large changes in the intracellular quantities of Tdr compounds have an effect on cell multiplication. If too little be present, the cells are starved for thymine and cannot synthesize DNA; in the presence of too much, growth is depressed, apparently by starvation for deoxythymidylate (dCMP) (29, 30). Similar findings were reported by Eagle et al. (11) for certain amino acids (valine, lysine, and threonine) essential to the growth of HeLa cells. These workers found that minimal intracellular concentrations of these compounds were required, ranging from 1 to 5 × 10^{-4} M, depending on the particular amino acid. It was found further that the cells concentrated the amino acids from the medium; that is, the intracellular concentrations were higher than the extracellular concentrations. It can be seen from an examination of Chart 4 that with respect to Tdr a minimal intracellular concentration of Tdr compounds also is required for growth, and, surprisingly, that this concentration is in the same range as those reported by Eagle et al. (11) for the essential amino acids. Although the mechanisms are not clear, another similarity was found: the Novikoff cells apparently could not exhaust the medium Tdr completely (Chart 2, 10^{-5} M), nor could the HeLa cells utilize all the essential amino acids when the concentrations in the medium were too low. The Novikoff cells also concentrated the Tdr from the medium (4- to 5-fold at 10^{-4} M in the medium) but not as much as HeLa cells concentrated the amino acids. At the higher internal concentrations of Tdr compounds growth was depressed (Chart 4); but in the midranges the cells grew as well as did the unblocked control cells. These and the other data already presented imply that the normal range of concentration of intracellular dTTP in logarithmically growing Novikoff tissue culture cells is approximately 5·10^{-4} × 10^{-4} M, although from the shapes of the 2 curves in Chart 4 this obviously is only a rough estimate. It is of interest, however, to compare these concentrations with those reported by Reichard (37) to be inhibitory for the production of dCMP from cytidylate by extracts of chick embryos. It was found that, at about 10^{-4} M, dTTP was 50% inhibitory, with 90% inhibition occurring at approximately 8·10^{-4} M. These figures are remarkably similar to the intracellular concentrations of dTTP which begin to inhibit the growth of the Novikoff cells, probably by a similar mechanism, although the estimation of these concentrations in the latter case is admittedly indirect and inferential.

**Effect on cell multiplication of limiting thymidine triphosphate production.**—Because cell growth is apparently related to the maintenance of the intracellular pools of dTTP at minimal levels, the possibility should be considered of depressing the rate of DNA synthesis by controlling the rate of production of dTTP. Because Tdr added to the medium is concentrated so rapidly by the cells (Charts 5 and 6), it would be difficult to maintain a stable but restricted level of this compound in the medium. When, however, 5-MeCdr is utilized to relieve the thymine deficiency (Chart 7), there apparently are some rate-limiting steps in its conversion to dTTP, possibly at the deamination step, although it is not known whether this occurs at the nucleoside or nucleotide level in this system. In any case, a much higher concentration in the medium (10^{-4} M) is required to restore normal growth than would be necessary with Tdr (10^{-5}·10^{-4} M), and further, at an intermediate level (10^{-4} M) growth does occur but at a lower rate (Chart 7). Although the over-all rate of DNA
synthesis is thus lowered by the apparent restriction of the production of dTTP, it is not known if the increased generation time results from a longer period for interphase DNA synthesis, or from a lengthening of telophase + G-1, as suggested by Siiskin and Kinosita (45).

When the rate of production of dTTP is too slow, which appears to be the case with 10⁻⁴ M 5-MeCdr (Chart 7), cell growth is retarded greatly and the cultures stop growing after a relatively short time. When the cells do not have any source of thymine (Chart 7, Cdr and Udr) they maintain for approximately a generation time and then begin to disintegrate. This suggests that, although variations in the intracellular levels of dTTP can influence the overall rate of DNA synthesis, once the onset of DNA synthesis has occurred, a sufficient supply of thymine must be maintained. Further, it has been shown (unpublished data) that in amethopterin-induced dTMP starvation of mammalian cells (40) the onset of DNA synthesis occurs on schedule; thus it appears unlikely that the production of dTTP, it is not known if the increased production of dTTP, or from a lengthening of telophase + G-1, as suggested by Siiskin and Kinosita (45).

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Pyrimidine Metabolism in Tissue Culture Cells Derived from Rat Hepatomas: II. Thymidine Uptake in Suspension Cultures Derived from the Novikoff Hepatoma

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