STUDIES ON THE INDUCTION OF THYMINE DEFICIENCY AND ON THE EFFECTS OF THYMINE AND THYMIDINE ANALOGUES IN *ESCHERICHIA COLI*[1]

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We have shown that a thymine-requiring strain of *Escherichia coli* strain 15_{T^-} loses the power to multiply, or "dies" when it is permitted to metabolize and grow in the absence of exogenous thymine (Barner and Cohen, 1954). The effect is ascribed to a kind of "unbalanced growth," in this instance continuing cell syntheses in the absence of a balanced synthesis of normal deoxyribose nucleic acid (DNA). Evidence has been summarized to suggest that many types of bactericidal treatments produce their effects in this way (Barner and Cohen, 1956) and more particularly we have demonstrated that some lethal effects of ultraviolet radiation are consequences of unbalanced growth (Cohen and Barner, 1954).

In this paper are presented the results of studies on the use of inhibitors in the blocking of thymine synthesis and utilization. The phenomenon of thymineless death is of considerable chemotherapeutic interest if it can be extended to organisms other than 15_{T^-}. Therefore, experiments were designed to determine whether unbalanced growth and death could be induced in bacteria which do not normally have an exogenous thymine requirement. It also appeared desirable to determine whether thymine analogues would prevent normal DNA synthesis in the presence of exogenous thymine.

We have previously found that bacterial strain (15_{T^-}) specifically blocked with respect to nuclear synthesis as a result of thymine deficiency can afford very useful experimental materials. For example, it has been shown with this system that the induction of enzyme biosynthesis may proceed in the absence of normal DNA synthesis (Cohen and Barner, 1955). Also it is possible to synchronize bacterial division in mass cultures of 15_{T^-} by withholding thymine and later returning it to the complete medium (Barner and Cohen, 1955). It was evident that the extension of such phenomena to other organisms would be important in the development of experimental biological systems.

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MATERIALS AND METHODS

Bacterial strains. Four strains of *Escherichia coli* were employed. These included the thymine-requiring mutant, strain 15_{T^-}, and its nonauxotrophic parent, strain 15, as well as a cytosine- or uracil-requiring strain W_{C^-}, and strain B. The cultivation and properties of these organisms in broth and in some synthetic media have been described (Barner and Cohen, 1954).

Estimation of growth and division. Growth was followed turbidimetrically (Cohen and Barner, 1955). Viable count was determined by spreading small aliquots of diluted cultures on broth agar plates.

In all experiments employing antagonists, strain 15_{T^-} was grown to the appropriate cell concentration in the presence of thymine. The culture was then chilled and centrifuged and the sediment was washed in the absence of thymine. Finally the bacteria were resuspended and incubated in media containing the compounds under examination.

Compounds. Thymidine was prepared in this laboratory and was found to be identical with a sample obtained from the California Biochemical Foundation. Thymine, uracil, xanthine, and hypoxanthine were products of Schwarz, Inc. Orotic acid was kindly given to us by Dr. D. Wright Wilson of the University of Pennsylvania. 5-Bromouracil was obtained from the Krishell Co. Azathymine, 5-nitrouracil, 5-hydroxyuracil, and thymine ribofuranoside were generously supplied by Dr. Jack Fox of the Sloan-Kettering

Institute for Cancer Research; 5-hydroxyuracil deoxyriboside and 5-bromouracil deoxyriboside were kindly given to us by Dr. Donald Visser of the University of Southern California, and spongothymidine by Dr. Werner Bergmann of Yale University. Amino acids and vitamins were obtained from Nutritional Biochemicals.

Growth in sulfanilamide-containing media. Sulfanilamide inhibits the growth and multiplication of *E. coli* by preventing the incorporation of *p*-aminobenzoic acid into folic acid. A folic acid deficiency prevents the bacterium from synthesizing a large number of essential metabolites which contain the one-carbon fragments normally handled by the folic acid-containing coenzymes. It was shown that a number of these metabolites (serine, methionine, valine, xanthine and thymine) added to the medium containing sulfanilamide will permit the growth and multiplication of *E. coli* (Rutten, Winkler, and DeHaan, 1950). The sole pyrimidine which appeared to be required under these conditions was thymine. The medium of Rutten *et al.* was modified as follows: a double strength mineral medium (Cohen and Arbogast, 1950) containing 4 mg sulfanilamide per ml was autoclaved and, on cooling, was supplemented with glucose and the other essential metabolites. The final solution was adjusted to the normal mineral medium concentration containing 2 mg sulfanilamide per ml. The final concentrations of the metabolites are given in table 1.

TABLE 1

Supplements to sulfanilamide medium (SM)

Compound	Final Concentration *μg. per ml*
DL-Methionine	30
DL-Serine	30
DL-Valine	30
L-Histidine	30
Hypoxanthine	30
Xanthine	30
Ca Pantothenate	1
Pyridoxine	1
Thiamin	1
Thymine	30
Glucose	1000

Strain B was grown overnight in mineral medium containing 1 mg glucose per ml. The glucose was exhausted from the medium and viable count was *ca.* 10^9 per ml. The sulfanilamide-metabolite medium (SM) was inoculated at a 1:50 dilution and incubated overnight with aeration to a viable count approaching 10^9 per ml. On dilution into SM medium, after a 20- to 40-minute lag, the division time, determined by viable count, was 2.5 to 3 hours and the mass doubling time, determined turbidimetrically, tended to be slightly longer. Dilution into the sulfanilamide medium without metabolites other than thymine and glucose resulted in a very long lag and a very slow increase in viable count.

Thus the division time in a complete SM medium, although markedly improved by the presence of metabolites, is several times longer than in the absence of sulfanilamide. It can be concluded therefore that the supplement does not fulfill the complete requirement of strain B for essential metabolites containing one-carbon fragments or provides these metabolites in a form which is not optimal for their utilization in the formation of polymers.

Omission of thymine from the SM medium resulted in a rapid decline of viable count. Omission of any one or all of the other metabolites in the presence of thymine inhibited multiplication but did not result in death. A typical experiment is presented below:

The overnight culture from SM was centrifuged, diluted in SM and permitted to go through one division. At this point the culture was washed once with mineral medium containing 2 mg sulfanilamide per ml and resuspended in SM medium lacking thymine, hypoxanthine, and xanthine. In figure 1*b* can be seen the increase of turbidity in cultures to which have been added either thymine, the purines, or both. The data show a considerably greater and more rapid effect in turbidity increase as a result of the absence of purine than that due to omission of thymine.

Figure 1*a* presents the viable counts of the same cultures. In the absence of exogenous purine, the viable count increases slightly and rapidly reaches a plateau. In the presence of all metabolites with the single exception of thymine, the viable count was constant for about 40 minutes and then fell off rapidly. In all comparable experiments the viable counts fell to about 10 per cent of the original in the time necessary for one division, a rate of killing comparable to that observed with thymine-deficient 15_{T^-}[2]. A comparison of figures

[2] With 15_{T^-}, extended incubation under conditions of thymineless death permits the survival of one bacterium per 10^4 cells. Survival of strain B in

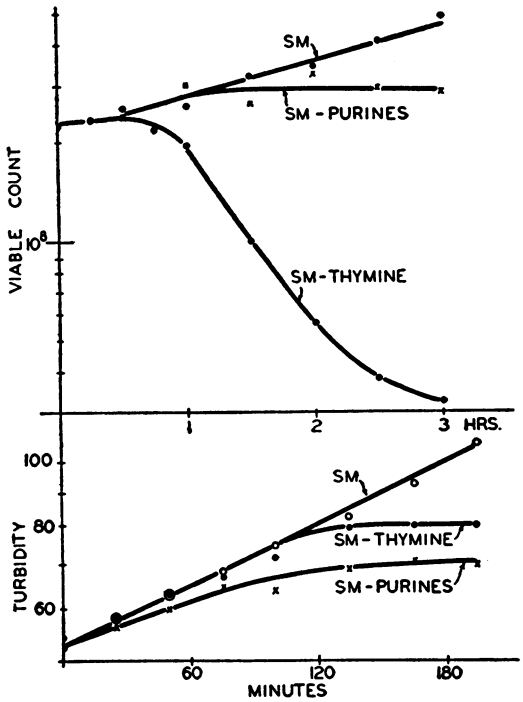

Figure 1. Growth of strain B in complete sulfanilamide-containing medium (SM) and its response upon deletions of purines or thymine. *a* (top): viable counts. *b* (bottom): turbidity data.

1*a* and 1*b* demonstrates that the phenomenon of thymineless death in this organism is also accompanied by cell growth, as indicated by an increase in turbidity of the culture comparable to that observed with cultures of 15_{T-} dying in the absence of thymine.

If an amino acid such as methionine, as well as thymine, was omitted from the SM medium, it was observed that only about half the cells were killed in one hour and no death occurred thereafter. If methionine alone was omitted from the SM medium, a very slow rate of division was observed. Thus the imposition of even an incomplete amino acid deficiency upon the thymine deficiency markedly reduced the killing observed. A marked reduction of the rate of killing in thymine deficiency with strain 15_{T-} has also been observed by the use of the tryptophan analogue, 5-methyl tryptophan.

Growth in the presence of excretion products. One hypothesis suggests that thymineless death may be due to the accumulation of a soluble toxic product. To test this hypothesis, strain 15_{T-} was grown in thymine-containing media until thymine was exhausted at 10^9 bacteria per ml. When the cells were removed from the exhausted medium, it was found that the medium containing products excreted during growth did not kill 15_{T-} but instead protected 15_{T-} from death in the absence of thymine (Barner and Cohen, 1954). The nature of this protective effect is not clear but it is evident that the hypothesis of a toxic product cannot be tested easily in these circumstances.

It has been shown that 15_{T-} excretes a variety of metabolic products during utilization of glucose. These include uracil, as a major ultraviolet absorbing component, and orotic acid and hypoxanthine as lesser components (Cohen and Barner, 1954). When 15_{T-} is incubated for two hours with 10^9 bacteria per ml in a glucose medium lacking thymine, the viable count falls in two hours to 1 per cent of the original value. In this interval uracil is excreted to give about 4 μg per ml and orotic acid to about 1 μg per ml. The addition of these metabolites to a glucose-thymine medium at 10 times these concentrations does not affect the division time of 15_{T-} nor do these excretion products significantly increase the rate of dying of these bacteria on incubation in the absence of thymine.

When uracil is added at 100 μg per ml to 15_{T-} in media containing thymine at 2 μg per ml, the division time was observed to increase from 45 to 55 minutes. At 1 μg of thymine, 100 μg of uracil increased the division time by 60 to 75 per cent and prevented more than a doubling of the bacteria. Despite this inhibition of multiplication the turbidity of such cultures increased about 7- to 8-fold in a 3-hour interval. The presence of 100 μg uracil did not affect the rate of dying of 15_{T-} in the absence of thymine. Uracil thus appears to act as a thymine competitor at a sufficiently high uracil-to-thymine ratio.

The effects of high concentration of uracil are superficially similar to those obtained with

SM lacking thymine was of the order of 100 to 1000 times that observed with 15_{T-}. Analysis of the survivors in the latter instance have indicated that these bacteria are still thymineless, but grow at a much slower rate than the bulk of the original culture. It appears reasonable to suppose that the proportion of very slow growing B is considerably increased under conditions of growth in the SM medium, as compared to the rate of growth in media lacking sulfanilamide. This would limit the extent of killing in SM medium devoid of thymine.

5-bromouracil (BrU) whose properties will be discussed in greater detail below. At this point we may present some data comparing the effects of uracil and 5-bromouracil on 15_{T-} in the presence of thymine. In figure 2 it can be seen that 100 μg uracil is somewhat less active than 10 μg of 5-bromouracil as a thymine analogue and that each compound appears to potentiate the other's activity.

The effect of thymine analogues. Azathymine, 5-nitrouracil, and 5-bromouracil (BrU) were tested as thymine analogues with 15_{T-}. Azathymine and 5-nitrouracil at 20 μg per ml had no effect on division in the presence of 0.4 μg of thymine or on the rate of dying in the absence of thymine. 5-Bromouracil is an effective thymine analogue (Hitchings *et al.*, 1950*a* and 1950*b*) and appears to replace thymine in the synthesis of DNA (Dunn and Smith, 1954; Zamenhof and Griboff, 1954).

When 15_{T-} was incubated without thymine in the presence of 3 μg bromouracil per ml, the turbidity of the culture usually increased five- to sixfold before growth ceased. Nucleic acids were determined (Schneider, 1945) and both DNA and ribose nucleic acid (RNA) were found to increase in proportion to the turbidimetric increase. Despite this apparent growth, the viable count of the culture was observed to double very slowly and then fell off as in thymineless death, albeit at a somewhat slower rate. A typical experiment is presented in figure 3. In many experiments with 5-bromouracil, viable count never increased more than twofold.

In the presence of thymine, 5-bromouracil was also effective in provoking cell death, as determined by the reduction of viable count. Turbidity increase was extensive, despite the loss of the power to multiply and examination of the culture revealed tremendously long bacteria. The lack of relation of turbidity to viable count is clearly demonstrated in figure 4. In this figure, a weight ratio of 5-bromouracil to thymine of 5 to 10:1 is seen to permit a single division, while a larger ratio did not permit even this. At a ratio of 10 of Br to thymine the bacteria were killed after a single division. Larger ratios increased the death rate. In order to completely overcome the inhibitory action of 5-bromouracil at 10 μg per ml, it was necessary to add 200 μg of thymine per ml of culture of 15_{T-}. In contrast to the extraordinary sensitivity of this organism to 5-bromouracil,

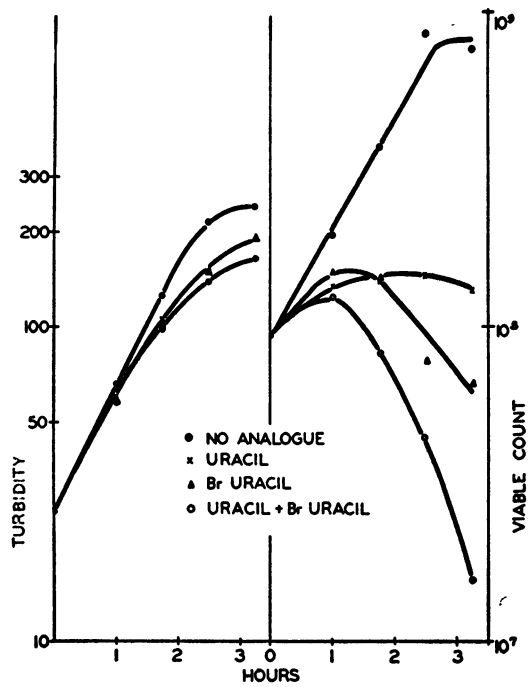

Figure 2. The eflect of thymine analogues on strain 15_{T-} in the presence of limiting thymine. Medium contained 1 μg/ml thymine, 10 μg 5-bromouracil, and 100 g uracil added as indicated.

amounts up to 500 μg per ml were inactive in reducing the division time of the parent strain 15, strain B, and strain W_{C-}.

Thymidine analogues in the presence of thymine. Bromouracil is far less effective in killing cells in

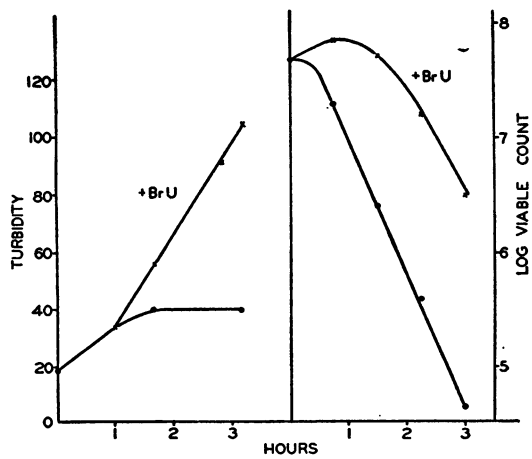

Figure 3. The response of strain 15_{T-} to 5-bromouracil (BrU) as sole pyrimidine source in synthetic medium. Control contained no pyrimidine.

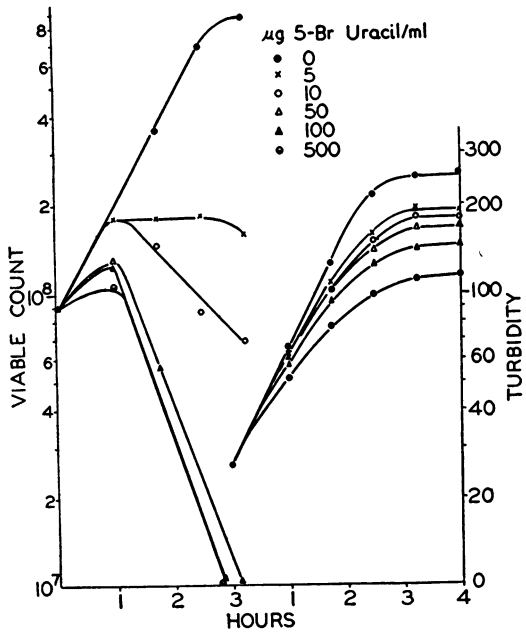

Figure 4. The effect of 5-bromouracil (BrU) on strain 15_T- in synthetic medium containing 1 μg thymine/ml.

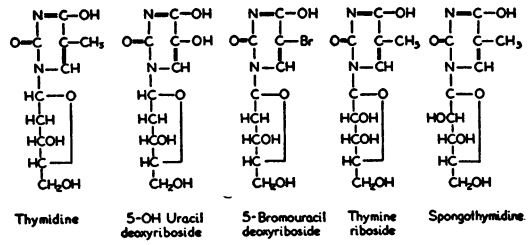

Figure 5. The structures of various thymidine analogues tested.

the presence of thymidine. This result was expected since free pyrimidines are rarely used to a significant extent in bacteria and animals, which do however appear to incorporate pyrimidine nucleosides. Although there are some indications that plants can incorporate pyrimidines, even here thymidine appears to be used to a greater extent than is thymine (McQuade *et al.*, 1955). Therefore it would be desirable to have thymidine analogues since the more ready utilization of the deoxyriboside suggests that competition with this metabolite would be potentially more effective in inhibiting division than is competition with the free pyrimidine. Until very recently preparative methods were inadequate to supply compounds of this type but a number have now become available. Thymidine analogues were obtained in which the pyrimidine was altered at the 5 position without alteration of the deoxyribosyl moiety. These were 5-hydroxyuracil deoxyriboside and 5-bromouracil deoxyriboside (Beltz and Visser, 1955). Two others were obtained in which thymine was present but in which the sugar moiety was altered. These were thymine ribofuranoside and spongothymidine (Bergman and Feeney, 1951). The latter is a natural product isolated from sponges, in which the sugar is reported to be D-arabinose. The structures of these compounds are given in figure 5.

Strain 15_T- was incubated in media containing 7.9×10^{-6} M thymine and 5-bromouracil, 5-hydroxyuracil deoxyriboside, or 5-bromouracil deoxyriboside at a molar ratio of inhibitor to thymine of 6.6. In figure 6, it can be seen that the hydroxyuracil deoxyriboside had no effect on the division time of 15_T-. A tenfold increment of this compound similarly had no effect on the growth and division of 15_T-. 5-Bromouracil at this concentration permitted one division, which was then followed by a slow decline in viable count. However, the deoxyriboside of 5-bromouracil produced a precipitous killing of 85 per cent of the cells,

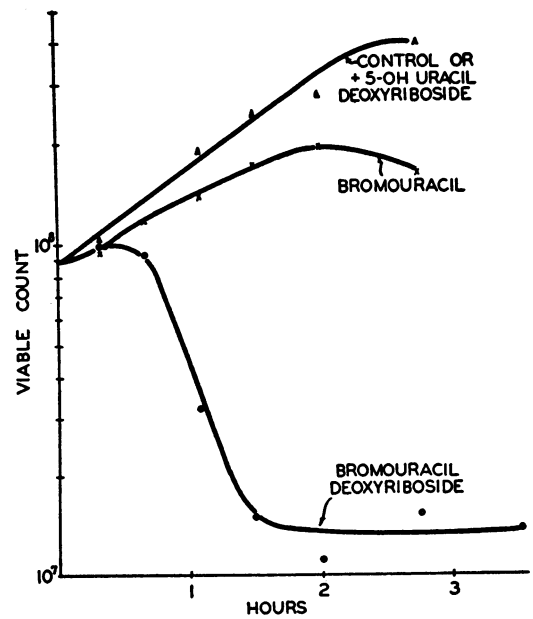

Figure 6. The effect of various analogues on strain 15_T- in medium containing 1 μg thymine/ml. All inhibitors tested at a molar ratio of 6.6 to thymine.

followed by a bacteriostasis of the rest of the population.

When the three compounds were tested at these concentrations on strains 15, W_{C-}, and B grown in the absence of exogenous thymine, no effect was obtained on the growth or division of these organisms.

Strain 15_{T-} was incubated in media lacking free thymine but containing thymine ribofuranoside or spongothymidine. It was observed that the former was slowly able to yield thymine for growth. However, death occurred with spongothymidine, albeit less rapidly than in the absence of the compound. These results are presented in figure 7.

In the presence of thymine in the medium, thymine ribofuranoside nevertheless inhibited growth, apparently until such a time as it was decomposed. Spongothymidine, however, was only slightly effective in the presence of exogenous thymine.

Thymidine analogues in presence of thymidine. Strain 15_{T-} was grown in thymine, centrifuged, resuspended and incubated in 7.9×10^{-6} M thymidine and various concentrations of the

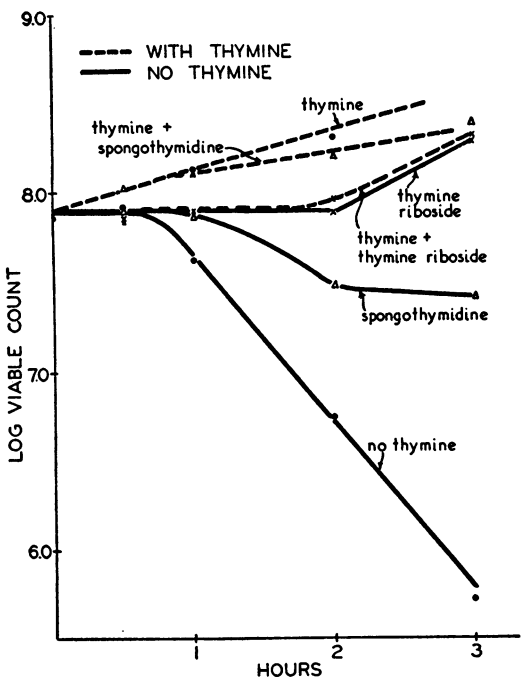

Figure 7. The response of strain 15_{T-} to thymidine analogues in media with or without thymine. Molar ratio of inhibitors to thymine (when present) 6.6.

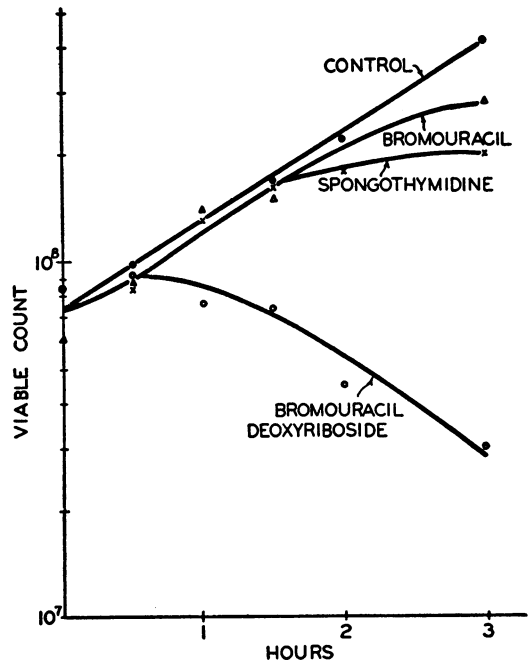

Figure 8. The effect of various analogues on strain 15_{T-} in medium containing 1.9 μg thymidine/ml as pyrimidine source. Molar ratio of inhibitors to thymidine 26.4.

analogues effective in the presence of thymine. Thymine ribofuranoside was essentially ineffective at a molar ratio of 33. Spongothymidine inhibited division significantly in three hours. Molar ratios of inhibitor to thymidine of 6.6, 33, and 66 permitted 63, 42, and 36 per cent, respectively, of the control viable count. At comparable molar concentrations, the inhibition afforded by spongothymidine is only slightly greater than that produced by 5-bromouracil in the presence of thymidine.

In contrast to the relative lack of effectiveness of 5-bromouracil, the bromouracil deoxyriboside is fairly active in inducing death of 15_{T-} in the presence of thymidine. These results are presented in figure 8.

DISCUSSION

It had been shown that a multiple deficiency including that for thymine can be imposed on non-thymine-requiring strains of bacteria, e.g., *E. coli* strain B, by growth in the presence of sulfanilamide (Rutten *et al.*, 1950). The experiments presented above demonstrate by means of this technique that thymineless death can be

induced in strain B as well as in 15_{T^-}. Also we have shown that a single deficiency with respect to compounds other than thymine, e.g., purine or methionine, induces bacteriostasis even as observed in most auxotrophic mutants of *E. coli*. Furthermore, multiple deficiencies which include a thymine requirement tend to protect the organism against thymineless death. These results are consistent with the theory of "death by unbalanced growth," since a deficiency other than that for thymine will usually prevent both nuclear and cytoplasmic growth. For example, purine is organized into both RNA and DNA, and its lack produces an inhibition of cell synthesis in general. A similar effect is noted under methionine deficiency and in the absence of methionine and thymine, the cell is unable to synthesize cytoplasm in excess of its normal nuclear complement.

It has been customary to distinguish the action of sulfanilamide from that of the antibiotics, the former being classed as "bacteriostatic" in contrast to the "bactericidal" penicillin or streptomycin. The effect of sulfanilamide is clearly to prevent both cytoplasmic and nuclear syntheses as a result of the multiple deficiencies produced. These experiments illustrate that the bacteriostatic sulfanilamide can become bactericidal if the deficiency for metabolites induced by this drug is made specific for a nuclear constituent, thymine.

Contrariwise, bactericidal antibiotics, e.g., penicillin and streptomycin, do not kill if the organism exposed to these drugs fails to grow for lack of essential metabolites (Hobby *et al.*, 1942; Rosenblum and Bryson, 1953). With several of the antibiotics, the cells become filamentous, even as under conditions of thymineless death, and it appears possible that the antibiotics act by inducing unbalanced growth; i.e., they inhibit reactions essential to the biosynthesis of specific critical cell structures. With both penicillin and streptomycin, available evidence suggests the possibility of effects on nucleic acid biosynthesis and structure (Park, 1951; Cohen, 1947), and does not yet exclude these modes of action. The differentiation of the various inhibitors of bacterial growth as "bacteriostatic" or "bactericidal" tends to break down under these circumstances.

In recent years a number of chemicals have been found to be anti-tumor agents. The action of the anti-tumor nitrogen mustards when applied to bacteria has been shown to have an effect similar to that of a thymine deficiency, as discussed in an earlier paper (Barner and Cohen, 1955, Loveless *et al.*, 1954). The anti-neoplastic antibiotic, azaserine, induces filament formation in *E. coli*, i.e., cell growth without division, even as do the nitrogen mustards, or various types of radiation (Maxwell and Nickel, 1954).

Marked antileukemia activity has been obtained with various antifolic acid derivatives, such as aminopterin and A-methopterin. Toxic amounts of the former have been observed to inhibit RNA synthesis somewhat less than DNA synthesis (Goldthwait and Bendich, 1951). However A-methopterin has been observed to inhibit thymine synthesis in the spleens of the leukemic mice far more than even the synthesis of DNA purine, with very little effect on RNA synthesis (Balis and Dancis, 1955). This result has led to the suggestion that different folic acid-enzyme complexes are involved in thymine and purine synthesis. Thus the use of this compound may lead to a far more specific deficiency for thymine than does sulfanilamide, which prevents the synthesis of all folic acid coenzymes.

Since the lethal action of sulfanilamide requires a complete supply of metabolites other than thymine, it may be asked if the anti-tumor effect of the antifolic acid agents can not be improved by a supply of essential metabolites other than thymine. If these agents do have effects on systems other than that necessary for thymine synthesis, it may be anticipated that the most rapid rate of thymineless death or minimal survival will not be attained until such metabolites are provided. These considerations may bear on the survival of apparently resistant cells, a result which conceivably is attributable to slow growth rather than an insensitivity to the antifolic acid agent.

Antifolic acid compounds produce marked effects on mitosis (Himes and Leuchtenberger, 1950; Hughes, 1950). In a recent study of the antifolic acid compounds, the 1,2-dihydro-*s*-triazines, in plant material (Rudenberg *et al.*, 1955) it was observed that these compounds, like aminopterin, produced an accumulation of prophase cells. On incubation in the absence of the agent the cells were often found to be irreversibly damaged with aberrant mitoses characterized by "sticky" anaphase bridges. Of particular interest

in this connection is the ability of thymidine to reverse the inhibition produced by the triazine. Of comparable interest in this connection is the report that thymine prevents chromosomal breaks in urethane-treated animals and thereby reduces the toxic effects of this anti-tumor agent (Boyland and Koller, 1954).

Mitotic abnormalities as consequences of a pathological thymine metabolism have been particularly observed in plant material. The thymine analogue, 5-aminouracil, has been used to induce such effects in onion root tips, in a manner comparable to an antifolic acid agent (Duncan and Woods, 1953). The cells of treated roots accumulate in interphase. Cells which had started mitosis prior to the start of treatment continue through division and then stop in interphase. Thus thymine utilization would seem to be blocked by the analogue at interphase, when it is apparently required for the doubling of DNA, which has been shown to occur at this stage of development in plant and animal cells (Vendrely, 1955) and in bacteria (Barner and Cohen, 1955). The effect of 5-aminouracil on plant cell mitosis is completely reversed by thymine. Although the antithymine effect of uracil described in this paper does not appear to have been previously reported for bacteria, the incubation of onion root tip in uracil solutions has been observed to result in chromosome breaks in mitosis (Deysson, 1952). More recently the incorporation of highly radioactive thymidine or thymine in the nuclei of plant material has also been observed to produce chromosome breaks (McQuade *et al.*, 1955).

In the case of *E. coli*, strain 15_{T^-} in the absence of exogenous thymine, a new type of DNA is made in small amounts. The new DNA lacks thymine but contains a new base, 6-methylaminopurine (Dunn and Smith, 1955). The methyl group which is normally combined to uracil or to a derivative then appears to be transferred to adenine. The thymine analogues, 5-amino uracil or 2-thiothymine, are not themselves incorporated into DNA but are stated to stimulate the synthesis of the DNA containing methylamino purine. It is not known whether a similar effect is observed in plant material.

It is not known if a new type of DNA is made in thymine deficiency induced by sulfanilamide or an antifolic acid agent. The results with A-methopterin (Balis and Dancis, 1955) suggest the synthesis of a DNA lacking thymine. However, it does not appear likely that a methyl purine would be contained in such a DNA under conditions of a folic acid deficiency, since the generation and transfer of methyl groups appear to involve folic acid enzymes. Whether a deficient DNA is made at all in sulfanilamide-treated strain B which dies by unbalanced growth is of considerable interest with respect to the precise mechanism of thymineless death.

In contrast to 5-aminouracil, bromouracil has been found to be incorporated into DNA in strain 15_{T^-}. Since 5-bromouracil is found in such DNA as the deoxyriboside, it appears reasonable to suppose that exogenous bromouracil deoxyriboside is merely incorporated more readily than is free 5-bromouracil. However, unlike the methylaminopurine-containing DNA which is only synthesized to the extent of about 20 per cent of the DNA originally present, 5-bromouracil-containing DNA may be made to the extent of 500 per cent of the DNA originally present.

Thus four types of effects on DNA synthesis appear possible as a consequence of a specific thymine deficiency or antagonism. There may be, (1) a complete inhibition of DNA synthesis, (2) a synthesis of thymine-deficient DNA, in which the missing thymine deoxynucleotide is not replaced by another nucleotide, (3) a synthesis of a thymine-deficient DNA, in which a methylaminopurine replaces thymine, or (4) a synthesis of a thymine-deficient DNA, in which a thymine analogue replaces thymine. One may wonder which category is produced by high concentrations of uracil or whether spongothymidine produces yet another type of DNA, in which a thymine pentoside replaces thymidine.

Finally we must comment on the different sensitivities of various organisms to thymine or thymidine analogues. It is evident that the differential sensitivities of various organisms to these compounds will determine the chemotherapeutic significance of our observations. It is recorded in most texts of biochemistry that most cells will not utilize significant amounts of free pyrimidines but will incorporate pyrimidine nucleosides. Unfortunately studies of this type have been performed almost entirely on animal cells. The data mentioned in this discussion indicate that free pyrimidines, including thymine, can be used by some plant cells as well as by 15_{T^-}, and that this

apparently is a distinguishing metabolic feature of these plant cells in contrast to animals.[3] Thus a thymine analogue is conceivably of chemotherapeutic interest in plant and fungal systems, as well as in the few types of bacteria which possess requirements for free pyrimidine. It will be of considerable interest to explore the extent to which thymidine analogues will kill other types of cells and, as noted above, the incorporation of thymidine in animal cells may prove to supply but a minor part of the thymine necessary for DNA synthesis. Thus a route is known for the synthesis of pyrimidine nucleosides, which involves the condensation of orotic acid and 1-pyrophosphoryl ribose-5-phosphate to form orotidylic acid. It is conceivable that in *E. coli* strain B or strain W_{C-}, the synthesis of thymidylic acid for DNA by-passes thymidine by a similar type of mechanism. The existence of multiple alternative metabolic pathways in the pyrimidine series is barely beginning to be explored. The differences in these pathways which are beginning to be seen from organism to organism may be expected to determine the range of applicability of any chemotherapeutic agent designed to operate in this area of metabolism.

SUMMARY

Escherichia coli strain B grown in the presence of sulfanilamide has a thymine deficiency, among other requirements. If the requirements other than thymine are met by supplementing the medium, the bacteria die at the rate of approximately 90 per cent in the time necessary for one division. If an amino acid or purine requirement is not met, then the bacteria do not grow and are not killed. Prevention of growth by imposing an amino acid requirement on the thymine requirement markedly reduces death in the absence of thymine. The significance of these results is discussed in relation to the use of antifolic acid agents in tumor chemotherapy, in the inhibition of plant growth, and in the induction of chromosome breaks in mitosis.

A number of pyrimidines and their analogues were tested for their ability to compete with thymine in the multiplication of 15_{T-}. It was observed that uracil at high concentrations acts as a thymine analogue. 5-Bromouracil appears to compete with and to replace thymine in supporting growth and nucleic acid synthesis in 15_{T-}. However, cells incorporating this pyrimidine in their DNA were capable of only one division and then were killed irreversibly as incorporation of the analogue continued. The compound was relatively ineffective in the presence of thymidine.

In tests on 15_{T-} thymidine analogues, thymidine ribofuranoside, spongothymidine, and 5-bromouracil deoxyriboside, were inhibitors of division in the presence or absence of thymidine. The compounds were inactive with *E. coli* strain B and strain W_{C-}. Under conditions of normal growth, the riboside was slowly degraded by 15_{T-}. Spongothymidine was much less active than the bromouracil deoxyriboside. The significance of these results has been discussed.

[3] One may speculate that the ability to incorporate thymine may be associated with a route through thymidine by reaction with deoxyribose-1-phosphate. This sugar phosphate is formed by the condensation of triose phosphate and acetaldehyde, and the latter compound is most readily generated by carboxylase, an enzyme found in plants and not in animals. *E. coli* strain B which does not incorporate thymine into DNA does not make deoxyribose during growth by a condensation of triose phosphate and acetaldehyde (Lanning and Cohen, 1954) and lacks carboxylase.

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