The Effect of 5-Bromouracil Deoxyriboside on the Synthesis of Antibody *in vitro*

By R. W. DUTTON,* ANNE H. DUTTON* AND J. H. VAUGHAN

Departments of Medicine and Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York, U.S.A.

(Received 17 August 1959)

The synthesis of antibody *in vitro*, in common with other protein synthesis, can be inhibited by the addition of amino acid analogues to the medium (Stavitsky & Wolf, 1958; Vaughan, Dutton, Dutton George & Marston, 1960). In an earlier paper it was shown that antibody synthesis could also be inhibited by the purine antagonist 8-azaguanine (Dutton, Dutton & George, 1958). It was established in a spleen-cell system, however, that 8-azaguanine, in addition to inhibiting the synthesis of ribonucleic acid, was affecting other pathways of metabolism and was thus of little use in elucidating biochemical pathways involved in the formation of antibody (Dutton *et al.* 1958).

The present paper describes the inhibition of antibody formation by the thymidine analogue 5-bromouracil deoxyriboside, which may be expected to affect a much more limited number of metabolic pathways. The incorporation of amino acids into the trichloroacetic acid-insoluble fraction of the spleen cells is not affected. The inhibitor appears to affect deoxyribonucleic acid but not ribonucleic acid metabolism. The implications of these findings are discussed.

A preliminary account of part of this work has already been presented (Dutton, Dutton & Vaughan, 1959*a, b*).

EXPERIMENTAL

Materials

[¹⁴C]Amino acids. These were obtained from Nuclear-Chicago Corp., Ill., U.S.A. In most experiments [*carboxy*-¹⁴C]DL-leucine was used. In some cases uniformly labelled L-[¹⁴C]leucine, L-[¹⁴C]lysine or L-[¹⁴C]phenylalanine was used. (Specific activities ranged from 0.5 to 5.0 mc/mm.)

Inhibitors. 5-Bromouracil deoxyriboside was obtained from the California Corporation for Biochemical Research, Los Angeles, U.S.A.

Methods

The techniques used for the preparation of the spleen-cell cultures, measurement of antibody synthesis *in vitro* and the uptake of radioactive phosphate are described in detail

elsewhere (Vaughan *et al.* 1959; Dutton, Dutton, George, Marston & Vaughan, 1960). The following is a brief description.

Immunization. Rabbits weighing 2–3 kg. were immunized with 16 intravenous injections of alum-precipitated ovalbumin over a period of 4 weeks. One or 2 months later the animals were stimulated with 2 mg. of alum-precipitated ovalbumin.

Spleen-cell preparation and incubation. Two days after the anamnestic stimulation, the animals were killed, the abdomen was opened aseptically and the spleen perfused and removed. A cell suspension was made by mincing the spleen and sieving it through a double 60-mesh screen. The isolated cells were washed three times in phosphate-buffered NaCl soln. containing 2–3% of bovine albumin and were finally resuspended in a modified Eagle's medium containing 15% of normal rabbit serum. Eagle's medium contained penicillin (100 i.u./ml.) and streptomycin (0.1 mg./ml.). Portions of this final suspension were pipetted into 10 ml. beakers. The incubation mixture was made up as follows: cell suspension, containing approx. 3×10^7 cells, 1 ml.; medium, with or without inhibitor, 0.5 ml.; [¹⁴C]amino acid, 0.5 μ C, dissolved in medium, 0.2 ml. The beakers and contents were incubated at 37° in an atmosphere of O₂ + CO₂ (95:5, v/v), saturated with water vapour.

Measurement of antibody synthesis. After incubation for prescribed periods the cells were separated from the supernatant by centrifuging at 150 g. The cells were washed once in aq. 0.85% NaCl and frozen until required. The supernatant was recentrifuged at 1000 g for 1 hr. at 5°. The radioactive antibody was co-precipitated from the supernatant by the addition of non-radioactive antibody, followed by the amount of antigen required to give maximal precipitation. The washed precipitate was dissolved in 0.25 N-acetic acid, a portion was plated and the radioactivity measured. Repeated determinations of [¹⁴C]antibody in the supernatants from duplicate cultures generally agreed well with a standard deviation of one-tenth of the mean. Most determinations have been made in triplicate, the rest in duplicate. The results presented are averages of all replicates, no selection being made against isolated aberrant results.

Measurement of the incorporation of [¹⁴C]amino acid into 'cell protein'. The washed cells (wet wt. 10–20 mg.) were thawed and were washed three times in 1 ml. of cold 5% trichloroacetic acid, once in hot 5% trichloroacetic acid, twice in acetone, once in ether and were finally dried in an oven at 60°. The fraction thus obtained is referred to below as 'cell protein'. The cell protein was then

* Present address: Postgraduate Medical School, Ducane Road, London, W. 12.

dissolved in 1 ml. of 6N-HCl (60°, overnight). A portion (0.1 ml.) of this solution was plated on to a 5 cm.² disk for counting.

Measurement of the incorporation of [³²P]phosphate into the cell-phosphate fractions. Approximately 50 µC of [³²P]-phosphate was added to cultures containing 2×10^8 cells, after the desired incubation period. The cells were incubated for an additional 1 hr. period and then harvested. The phosphate fractions of the cells were separated by a modification of the Schmidt and Thannhauser technique (see Dutton *et al.* 1960). The specific activity of the phosphate in these fractions was determined.

Measurement of radioactivity. Samples containing ¹⁴C were counted in a D 47 gas-flow counter (Nuclear-Chicago Corp.) used with a Micromil window. The samples were plated so thinly that no correction for absorption was necessary.

Samples containing ³²P were counted in a 20th Century Electronics liquid counter, type M 6.

RESULTS

It has been shown (Vaughan *et al.* 1960) that spleen cells, isolated as described, will synthesize antibody *in vitro* for at least 48 hr., although in occasional experiments (four out of seventeen) the rate of synthesis falls off after 24 or 36 hr. In the experiments to be described the spleen cells were isolated 2 days after the anamnestic stimulation *in vivo* and incubated *in vitro* for a period of 48 hr. Under these conditions (Fig. 1) the rate of antibody synthesis is slow initially but increases approximately threefold during incubation.

Inhibition of antibody production. When the incubation medium contained 5-bromouracil de-

Fig. 1. Incorporation of [¹⁴C]amino acid into antibody synthesized by spleen cells incubated *in vitro*. The spleen cells were removed from rabbit 138 two days after anamnestic stimulation *in vivo* and were incubated in medium containing [¹⁴C]amino acids. Individual cultures containing the spleen-cell suspension were removed after varying periods of incubation and the radioactivity of the antibody, recovered from the supernatant, was estimated.

oxyriboside the antibody produced in a 48 hr. period was reduced to about one-third of the control value. The effect of concentration of the inhibitor is shown in Fig. 2. It can be seen that as little as 0.02 mM caused marked inhibition. An increase up to 2.5 mM did not reduce antibody

Fig. 2. Effect of the concentration of 5-bromouracil deoxyriboside on the amount of antibody synthesized. Spleen cells isolated from rabbits 2 days after anamnestic stimulation *in vivo* were incubated for 48 hr. with [¹⁴C]-amino acids and varying concentrations of 5-bromouracil deoxyriboside. The amount of radioactive antibody in the supernatant at 48 hr. was measured. Results obtained with five different rabbits are illustrated: ○, R 302; ●, R 309; △, R 125; ▼, R 153; ■, R 140.

Fig. 3. Reversal of the 5-bromouracil deoxyriboside-inhibition of antibody synthesis by thymidine. The amount of antibody made by spleen cells isolated from rabbit 302 2 days after anamnestic stimulation and incubated for 48 hr. *in vitro* was measured for uninhibited cells (x), for cells inhibited with 5-bromouracil deoxyriboside (y), and for cells in which the inhibition was reversed by the further addition of varying concentrations of thymidine (z). The percentage reversal of inhibition, $(z - y)/(x - y) \times 100$, is plotted against the molar ratio of thymidine/5-bromouracil deoxyriboside. Measurements were made at two concentrations of 5-bromouracil deoxyriboside, 6 µg./ml. (○) and 50 µg./ml. (●).

Table 1. *Effect of uridine and thymidine on the inhibition of antibody synthesis by 5-bromouracil deoxyriboside*

Spleen cells were incubated with [14 C]amino acid for 48 hr. under the conditions indicated and the radioactivity incorporated into antibody in this period was determined.

Additions to the medium	Antibody (total counts)
Rabbit 131	
None	980
5-Bromouracil deoxyriboside (350 μ g./ml.)	340
5-Bromouracil deoxyriboside (350 μ g./ml.) plus uridine (350 μ g./ml.)	443
5-Bromouracil deoxyriboside (350 μ g./ml.) plus thymidine (350 μ g./ml.)	813
Rabbit 148	
None	7235
5-Bromouracil deoxyriboside (200 μ g./ml.)	3340
5-Bromouracil deoxyriboside (100 μ g./ml.)	2805
5-Bromouracil deoxyriboside (200 μ g./ml.) plus uridine (350 μ g./ml.)	3115
5-Bromouracil deoxyriboside (100 μ g./ml.) plus uridine (175 μ g./ml.)	3205
5-Bromouracil deoxyriboside (200 μ g./ml.) plus thymidine (350 μ g./ml.)	4380
5-Bromouracil deoxyriboside (100 μ g./ml.) plus thymidine (175 μ g./ml.)	6030

Fig. 4. Effect of time of addition of 5-bromouracil deoxyriboside on the synthesis of antibody. The antibody synthesized by '2-days spleen cells' incubated *in vitro* is plotted against the time of incubation. In ○, ●, [14 C]amino acids and inhibitor were added at 0 hr. and in □, ■, [14 C]amino acids and inhibitor were added at 17 hr. ○, □, Controls; ●, ■, with 5-bromouracil deoxyriboside (50 μ g./ml.). Rabbit 132.

synthesis appreciably further. The inhibition was partially reversed by the presence of thymidine (Fig. 3), but not by uridine (Table 1). The reversal with thymidine appeared to be competitive, although the system is not sufficiently defined to allow an inhibition analysis.

Effect of time of addition of 5-bromouracil deoxyriboside. In several experiments the time course of inhibition of antibody synthesis by 5-bromouracil deoxyriboside was studied. The result of one such experiment, which was typical, is illustrated in Fig. 4. When the inhibitor and [14 C]leucine were added at the beginning of incubation (Fig. 4) the

Fig. 5. Effect of 5-bromouracil deoxyriboside on the incorporation of [14 C]amino acid into 'cell protein' of spleen cells incubated *in vitro*. ○, Control; ●, incubated with 5-bromouracil deoxyriboside (50 μ g./ml.). In series A, [14 C]amino acids were added at 90 min.; in series B, at 24 hr. 5-Bromouracil deoxyriboside was added to the inhibited cultures of both series at 0 hr. Rabbit 144.

incorporation of amino acid into antibody lagged steadily behind that of the control cultures. In marked contrast, when the inhibitor and [14 C]leucine were added to a portion of this same spleen-cell suspension after incubation for 17 hr. (Fig. 4) there was little demonstrable inhibition of antibody synthesis.

It was thought possible that the cells might release thymidine, or an enzyme capable of destroying the inhibitor, into the medium during the course of incubation. However, if fresh cells were suspended in old medium, i.e. the supernatant obtained from cells after incubation for 24 hr., the initial inhibiting effect observed with 5-bromouracil deoxyriboside was not reduced. Furthermore, the antibody production of cells already incubated for

Table 2. Effect of 5-bromouracil deoxyriboside on the uptake of [³²P]phosphate into phosphate fractions of spleen cells

Cultures were incubated with or without inhibitor. At 2, 16 or 28 hr. [³²P]phosphate was added and the cultures were incubated for a further 60 min. The cells were then harvested, the phosphate fractions isolated and the specific activities determined. The antibody produced in parallel cultures, incubated for 48 hr., was also measured.

Rabbit no.	Incubation time (hr.) before addition of radioactive phosphate	Specific activity reached after incubation for 60 min. with [³² P]phosphate (counts/μg. of P)					Radioactive amino acid incorporated into antibody (total counts 48 hr.)
		Acid-soluble phosphate	Fat-soluble phosphate	Ribonucleic acid phosphate	Deoxyribonucleic acid phosphate		
R 140	2	780 } 745	65 } 63	72 } 68	3-6 } 3-8		4024
		710 }	61 }	64 }	3-9 }		
		700 } 655	73 } 68	68 } 64	3-9 } 3-9		
		610 }	62 }	60 }	3-9 }		
R 135	16	12	- 8	6	- 3		82
		480 } 510	52 } 68	38 } 42	2-7 } 2-9		
		540 }	84 }	45 }	3-0 }		
		500 } 535	65 } 71	36 } 37	2-0 } 2-1		
R 149	28	570 }	76 }	37 }	2-1 }		2008
		- 5	- 4	12	28		
		534 } 542	47 } 60	33 } 33	2-0 } 2-5		
		550 }	73 }	33 }	2-9 }		
		570 } 565	53 } 52	32 } 30	1-4 } 1-4		235
		560 }	51 }	28 }	1-3 }		
		- †	13	9	34

24 hr. and resuspended in fresh medium was still not inhibited by 5-bromouracil deoxyriboside added at this time.

Incorporation of amino acid into cell protein. The incorporation of [^{14}C]leucine into the trichloroacetic acid-insoluble residue of the cell is illustrated in Fig. 5. In this experiment the inhibitor was added at the beginning of incubation and [^{14}C]-amino acids were added at either 90 min. (Fig. 5, A) or at 24 hr. (Fig. 5, B). It can be seen that the presence of the inhibitor caused no significant inhibition of cell-protein synthesis even after prolonged incubation.

Incorporation of phosphate into the phosphate fractions of spleen cells. Owing to difficulties of technique, differences in specific activities of less than 15 % cannot be regarded as significant. This is especially true when the measurement is made several hours after incubation has begun. The reductions in the specific activity of deoxyribonucleic acid phosphate at 16 hr. and 28 hr., however, are 28 and 34 % (Table 2). There was no demonstrable effect by the inhibitor on the uptake of [^{32}P]phosphate into acid-soluble, fat-soluble or ribonucleic acid phosphate fractions even after incubation for 28 hr. with 5-bromouracil deoxyriboside at 200 $\mu\text{g./ml.}$ These findings are in contrast with the effect of 8-azaguanine (Dutton *et al.* 1958), which, at 300 $\mu\text{g./ml.}$, had brought about a 30 % reduction of incorporation into acid-soluble phosphate, 28 % reduction of incorporation into ribonucleic acid phosphate and a complete elimination of incorporation into deoxyribonucleic acid after only 4 hr.

DISCUSSION

In the experiments described, the spleen cells were isolated 2 days after anamnestic stimulation with antigen. Under these conditions the rate of antibody formation *in vitro* increased approximately threefold during the first 24 hr. of incubation. In contrast, a constant rate of antibody formation was described when cells were isolated at 3 days (Vaughan *et al.* 1960). The increasing rate of synthesis occurring during incubation of cells obtained at 2 days can be interpreted in two ways. Either the templates utilized in the synthesis of antibody protein are working faster, or more templates are coming into use.

The total amount of antibody produced in incubation for 48 hr. by the '2-day' spleen cells is greatly reduced by the presence of 5-bromouracil deoxyriboside. The degree of inhibition is little affected by the concentration of the inhibitor over the wide range tested. The production of antibody in this system thus appears to be divisible into two parts. The synthesis of approximately one-third is little affected by the presence of the inhibitor,

whereas the production of the other two-thirds is strongly inhibited by the inhibitor even in low concentrations (Fig. 2). It is tempting to surmise that the increasing rate of antibody synthesis in the '2-days system' is due to the development of increased number of templates, and that 5-bromouracil deoxyriboside selectively inhibits this development.

The time study of the inhibition is consistent with this possibility. When the inhibitor was added at the beginning of the incubation period the rate of antibody production failed to show the usual increase. Instead, a slight fall was seen. However, if the inhibitor is added after the rate of antibody synthesis has already increased, there is little or no effect on the subsequent synthesis (Fig. 4).

5-Bromouracil deoxyriboside is a structural analogue of thymidine and has been shown to inhibit the utilization of this compound for deoxyribonucleic acid synthesis in bacteria (Bardos, Levin, Herr & Gordon, 1955; Beltz & Visser, 1955, 1957) and in mammals (Kit, Beck, Graham & Gross, 1958; Eidinoff, Knoll & Marano, 1957). It has been shown both in bacteria (Dunn & Smith, 1957; Litman & Pardee, 1956; Price, Hudson, Hinds, Darmstadt & Zamenhof, 1956) and in mammalian tissues (Eidinoff, Cheong & Rich, 1959) that 5-bromouracil deoxyriboside, or 5-bromouracil, is, under certain conditions, incorporated into deoxyribonucleic acid, partially replacing thymidine. The effect of such incorporation on the genotype and phenotype of a cell needs clarification (for a discussion see Zamenhof, De Giovanni & Rich, 1956).

It seems reasonable to assume that in the present experiments 5-bromouracil deoxyriboside inhibits cell division by inhibiting spleen-cell synthesis of deoxyribonucleic acid although there has been, as yet, no direct demonstration of cell division in the spleen-cell system. It is possible that some few cells may synthesize deoxyribonucleic acid containing 5-bromouracil, or may undergo division in the absence of deoxyribonucleic acid synthesis (cf. the findings of Linder, 1959, with 5-fluorouracil). It is likely that either of these last-named metabolic complications, however, would result in abnormal or deficient cytoplasmic function and thereby inhibit antibody formation.

It has generally been considered that thymidine is incorporated solely into deoxyribonucleic acid (Reichard & Estborn, 1951; Friedkin, Tilson & Roberts, 1956). It has also been suggested that thymidine may have some other metabolic function (Prusoff, 1957; Beltz & Visser, 1957). However, Kit *et al.* (1958), working with mouse spleens, rat thymus and tumour cells, were unable to find evidence of such other function. 5-Bromouracil deoxyriboside had no effect on the methylation of

deoxyuridine to thymidine, the hydroxymethylation reaction leading to serine synthesis, the anabolic reactions involved in the incorporation of [^{14}C]formate into protein or the energy-yielding processes of glycolysis and respiration. In their experiments, the cells were incubated for only 3 hr. In the present study, during the 48 hr. no effect could be observed on the uptake of [^{32}P]phosphate into cell fractions other than deoxyribonucleic acid. Also the incorporation of [^{14}C]amino acid into cell protein was not affected by the inhibitor, making it seem unlikely that thymidine or some derivative is involved in activation and incorporation of amino acids into protein.

In summary, there is a stage in antibody formation which is independent of inhibition by 5-bromouracil deoxyriboside. This is indicated by the persistence of significant antibody formation even when the concentration of inhibitor is raised many times above the minimal inhibitory level (Fig. 2), and in the complete lack of inhibitory effect in preparations allowed to incubate through a critical time interval (Fig. 4). It would seem that the inhibitor does not inhibit antibody formation, but affects an earlier developmental stage associated with increasing capacity to form antibody.

The mechanism by which 5-bromouracil deoxyriboside brings about this inhibition is not yet established. The inhibitor may affect some as yet undemonstrated function of thymidine or a thymidine derivative necessary for the development of synthetic ability. The inhibitor does inhibit the synthesis of new deoxyribonucleic acid. This inhibition may prevent the division of cells which either are, or become, capable of antibody formation. Further experiments are needed to differentiate these alternatives.

SUMMARY

1. The rate of synthesis of antibody by rabbit-spleen cells, isolated 2 days after anamnestic antigenic stimulation *in vivo*, increases roughly three-fold during the first 24 hr. of incubation *in vitro*.

2. This increase is prevented by 5-bromouracil deoxyriboside and the inhibition appears to be reversed competitively by the addition of thymidine.

3. The incorporation of radioactive amino acid into cell protein, the synthesis of antibody and the incorporation of radioactive phosphate into cell-phosphate fractions other than deoxyribonucleic acid, do not appear to be affected by the inhibitor.

4. The implications of these findings are discussed.

This investigation was supported by a grant E-2349 from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

We wish to thank Mr D. Krupcale for technical assistance.

REFERENCES

- Bardos, T. J., Levin, G. M., Herr, R. R. & Gordon, H. L. (1955). *J. Amer. chem. Soc.* **77**, 4279.
 Beltz, R. E. & Visser, D. W. (1955). *J. Amer. chem. Soc.* **77**, 736.
 Beltz, R. E. & Visser, D. W. (1957). *J. biol. Chem.* **226**, 1035.
 Dunn, D. B. & Smith, J. D. (1957). *Biochem. J.* **67**, 494.
 Dutton, R. W., Dutton, A. H. & George, M. (1958). *Nature, Lond.*, **182**, 1377.
 Dutton, R. W., Dutton, A. H., George, M., Marston, R. Q. & Vaughan, J. H. (1960). *J. Immunol.* (in the Press).
 Dutton, A. H., Dutton, R. W. & Vaughan, J. H. (1959a). *Fed. Proc.* **18**, 565.
 Dutton, R. W., Dutton, A. H. & Vaughan, J. H. (1959b). *Fed. Proc.* **18**, 219.
 Eidinoff, M. L., Cheong, L. & Rich, M. A. (1959). *Fed. Proc.* **18**, 220.
 Eidinoff, M. L., Knoll, J. & Marano, B. J. (1957). *Proc. Amer. Ass. Cancer Res.* **2**, 198.
 Friedkin, M., Tilson, D. & Roberts, D. (1956). *J. biol. Chem.* **220**, 627.
 Kit, S., Beck, C., Graham, O. L. & Gross, A. (1958). *Cancer Res.* **18**, 598.
 Linder, A. (1959). *Cancer Res.* **19**, 189.
 Litman, R. M. & Pardee, A. B. (1956). *Nature, Lond.*, **178**, 529.
 Price, T. B., Hudson, P. B., Hinds, H. A., Darmstadt, R. A. & Zamenhof, S. (1956). *Nature, Lond.*, **178**, 684.
 Prusoff, W. H. (1957). *J. biol. Chem.* **226**, 901.
 Reichard, P. & Estborn, B. (1951). *J. biol. Chem.* **188**, 839.
 Stavitsky, A. B. & Wolf, B. (1958). *Biochim. biophys. Acta*, **27**, 4.
 Vaughan, J. H., Dutton, A. H., Dutton, R. W., George, M. & Marston, R. Q. (1960). *J. Immunol.* (in the Press).
 Zamenhof, S., De Giovanni, R. & Rich, K. (1956). *J. Bact.* **71**, 60.