J Clin Pathol: first published as 10.1136/jcp.22.5.554 on 1 September 1969. Downloaded from <http://jcp.bmj.com/> on April 26, 2023 by guest. Protected by copyright.

Effect of antimicrobial agents on the Euglena method of serum vitamin B₁₂ assay¹

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SYNOPSIS Antimicrobial agents in the serum may affect the results of the Euglena method of serum vitamin B₁₂ assay. Sulphonamides suppress the growth of Euglena in concentrations attainable in the serum during treatment; streptomycin, chlortetracycline, erythromycin, kanamycin, and nitrofurantoin bleach Euglena but only when present in concentrations far exceeding the normal peak therapeutic blood levels. False low results of serum vitamin B₁₂ assay due to inhibitory and/or bleaching substances in the serum can be readily detected by microscopy of the assay cultures and Euglena cell counts.

Microbiological assay with *Euglena gracilis* is a specific and sensitive method of determining serum vitamin B₁₂ levels, provided constant and optimal assay conditions are observed (Anderson, 1964). On the basis that sulphonamides, when present in a patient's serum in concentrations attainable during treatment, suppress the growth of Euglena (Lear, Harris, Castle, and Fleming, 1954), Hutner, Bach, and Ross (1956) suggested that the addition of para-aminobenzoic acid to the serum should be included in the assay procedure to

overcome the inhibitory effect of sulphonamides. Other workers have demonstrated that certain antibiotics also affect the growth of Euglena (Robbins, Hervey, and Stebbins, 1953; Zahalsky, Hutner, Keane, and Burger, 1962; McCalla, 1962). We report here our investigation of the effect of a number of currently used antimicrobial agents on the Euglena method of serum vitamin B₁₂ assay.

MATERIALS AND METHODS

The concentration of vitamin B₁₂ in the standard serum was assayed by the method described by Anderson (1964) using *Euglena gracilis* strain z. Two dilutions of the serum, 1 in 40 and 1 in 80, were used for each deter-

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Received for publication 31 December 1968.

TABLE I
EFFECT OF ANTIMICROBIAL AGENTS IN NORMAL PEAK THERAPEUTIC BLOOD LEVELS ON THE EUGLENA METHOD OF SERUM VITAMIN B₁₂ ASSAY¹

Drug Added to Serum	Concentration (μg/ml)	Mean Cell Count on Day 7 (10 ⁶ /ml) ²	Colour of Euglena	Mean Vitamin B ₁₂ (pg/ml)
None	0	30	Green	550
Sulphonamides	100	2.5	Green	<20
Benzylpenicillin	10 (unit/ml)	31	Green	564
Streptomycin	50	30	Green	470
Chlortetracycline	25	30	Green	498
Erythromycin	50	31	Green	502
Chloramphenicol	50	31	Green	546
Methicillin	25	32	Green	638
Ampicillin	2	31	Green	588
Cephaloridine	25	30	Green	516
Colistimethate	5	31	Green	602
Kanamycin	25	29	Green	484
Gentamicin	2	30	Green	574
Nitrofurantoin	2	29	Green	472

¹Data from Goodman and Gilman (1965) and Carroll, Brennan, and Jacques (1955); the assay serum was diluted 1 in 80.
²Size of Euglena inoculum (day 0) : 0.2 × 10⁶ cells/ml.

mination. The size of the *Euglena* inoculum was 0.2×10^6 cells per millilitre. The antimicrobial agents were added to the standard serum in various concentrations, including those likely to be found in the human body during treatment with these drugs. Para-aminobenzoic acid was added to the serum in a concentration of 1.25 $\mu\text{g}/\text{ml}$. In addition to the usual colorimetric measurement of the assay cultures to determine the serum vitamin B₁₂ levels, the growth of *Euglena* was also assessed at regular intervals by light microscopy, with particular attention to the size, colour, and motility of the flagellates. *Euglena* cell counts were made in a haemocytometer.

RESULTS

The mean value of vitamin B₁₂ in the standard serum from 30 determinations was 550 pg/ml , with a range of 468 to 634 pg/ml . The average *Euglena* cell count at the time of harvest (day 7) was 30×10^5 cells/ml. All the flagellates grown in the drug-free standard serum and in sera containing low concentrations of vitamin B₁₂ (less than 20 pg/ml) were plump and green; over 90% of the population were motile.

THE EFFECT OF ANTIMICROBIAL AGENTS IN CONCENTRATIONS ATTAINABLE IN THE SERUM DURING TREATMENT With the exception of sulphonamides, all drugs tested had no significant effect on the growth of *Euglena*, as judged by the *Euglena* cell counts, the appearance of the flagellates, and the serum vitamin B₁₂ measurements (Table I).

Eight short- and long-acting sulphonamides were tested (Table II); all exhibited a profound inhibitory

FIG. 1. Growth chart of *Euglena gracilis* in assay serum diluted 1 in 40, with sulphadiazine (SDZ) added in different concentrations.

effect on the growth of *Euglena* in direct proportion to the concentration of the drug added to the standard serum. The *Euglena* cell counts were markedly reduced, the flagellates remained green but appeared smaller and moved sluggishly; less than one-third of the population were motile. The effect of sulphadiazine was representative of that of other sulphonamides. In concentration as low as 20 $\mu\text{g}/\text{ml}$, sulphadiazine reduced the growth of

TABLE II
SULPHONAMIDES TESTED

Sulphadiazine
Sulphacetamide
Sulphadimidine
Sulphafurazole (Gantrisin)
Sulphamethoxypyridazine (Midicel, Lederkyn)
Sulphamethoxydiazine (Bayrena, Kirocid)
Sulphadimethoxine (Madrison)
Sulphamethizole (Urolucosil, Thiosulfil)

TABLE III

EFFECT OF SULPHADIAZINE ON THE GROWTH OF EUGLENA¹

Sulphadiazine Concentration in Serum ($\mu\text{g}/\text{ml}$)	Euglena Cell Counts ($10^5/\text{ml}$) ²				Mean Vitamin B ₁₂ (pg/ml)
	Day 1	Day 3	Day 5	Day 7	
0	2.1	6.8	13.5	30	550
20	1.6	4.8	9.2	16	180
40	1.2	3.6	6.8	11	32
60	1	2.8	5.2	8.5	16
80	0.8	2.5	4.5	7	8
100	0.7	1.6	2.2	2.5	4

¹Dilution of assay serum: 1 in 40.

²Size of *Euglena* inoculum (day 0): 0.2×10^6 cells/ml.

FIG. 2. The effect of sulphadiazine (SDZ) added to the assay serum in different concentrations on the results of serum vitamin B₁₂ assay. A = assay serum diluted 1 in 40; B = assay serum diluted 1 in 80.

Euglena as measured by the cell counts to approximately one-half of that in the drug-free serum; with 100 µg/ml, the growth was negligible (Table III and Fig. 1). The inhibitory effect of sulphadiazine was more marked in serum diluted 1 in 40 than in serum diluted 1 in 80 (Fig. 2), but could be abolished by simultaneously adding 1.25 µg/ml of para-aminobenzoic acid to the serum before assay. Para-aminobenzoic acid alone did not enhance or suppress the growth of *Euglena*.

THE EFFECT OF ANTIMICROBIAL AGENTS IN CONCENTRATIONS EXCEEDING THE NORMAL PEAK THERAPEUTIC BLOOD LEVELS The results are summarized in Table IV. In concentrations exceeding 10 times the normal peak therapeutic blood levels, benzylpenicillin, chloramphenicol, ampicillin, cephaloridine, methicillin, colistimethate, and gentamicin had no significant effect on the growth of *Euglena*. Streptomycin, erythromycin, chlortetracycline, and kanamycin affected the growth of *Euglena* resulting in significantly lower colorimetric measurements of serum vitamin B₁₂ levels. Microscopy of assay cultures and *Euglena* cell counts showed that the low results were due to the bleaching effect of the drugs and not to inhibition of cell multiplication. Nitrofurantoin was unique in that it not only bleached *Euglena* but also suppressed the multiplication of the cells.

DISCUSSION

The presence of certain antimicrobial agents in the serum affects the growth of *Euglena gracilis* by suppression of cell multiplication, inhibition of chlorophyll formation, or both, resulting in false low results of serum vitamin B₁₂ assay.

Both short- and long-acting sulphonamides suppress the multiplication of *Euglena* without bleaching the flagellates even when the concentration of the drug in serum is only a fraction of the normal peak therapeutic blood levels (Tables I and III). The selection of serum diluted 1 in 80 for assay as suggested by Anderson (1964) reduced the inhibitory effect of sulphonamides but only slightly (Fig. 2). The inhibitory effect of sulphonamides can be overcome by the addition of para-aminobenzoic acid to the serum before assay in a concentration of 1.25 µg/ml. Since para-aminobenzoic acid alone does not

TABLE IV

EFFECT OF ANTIMICROBIAL AGENTS IN CONCENTRATIONS 10 TIMES THE NORMAL PEAK THERAPEUTIC BLOOD LEVELS ON THE EUGLENA METHOD OF SERUM VITAMIN B₁₂ ASSAY

Drug Added to Serum	Concentration (µg/ml)	Mean Cell Count on Day 7 (10 ⁶ /ml) ^a	Colour of <i>Euglena</i>	Mean Vitamin B ₁₂ (pg/ml)
None	0	30	Green	550
Benzylpenicillin	100 (unit/ml)	29	Green	496
Streptomycin	500	27	White	116
Chlortetracycline	250	28	White	194
Erythromycin	500	28	White	208
Chloramphenicol	500	29	Green	496
Methicillin	250	31	Green	504
Ampicillin	20	30	Green	562
Cephaloridine	250	31	Green	530
Colistimethate	50	29	Green	518
Kanamycin	250	27	White	188
Gentamicin	20	29	Green	482
Nitrofurantoin	20	22	60% Green 40% White	176 176

^aDilution of assay serum : 1 in 80

^aSize of *Euglena* inoculum (day 0) : 0.2 × 10⁶ cells/ml

enhance or suppress the growth of *Euglena*, and sulphonamides are among the most frequently used antimicrobial agents, the addition of para-aminobenzoic acid should be a routine step in the *Euglena* method of serum vitamin B₁₂ assay. This was recommended by Hutner *et al* (1956) and accepted by Anderson (1964) but has not been universally adopted. Because neither pteroylglutamic acid nor citrovorum factor corrected the inhibitory effect of sulphonamides on the growth of *Euglena*, Lear *et al* (1954) suggested that para-aminobenzoic acid was concerned with nucleic acid and/or aminoacid synthesis rather than folic acid synthesis.

We have also confirmed that streptomycin, chlortetracycline, erythromycin, and kanamycin inhibit the formation of chlorophyll in *Euglena* without suppressing cell multiplication—'apochlorosis' (Robbins *et al*, 1953; Zahalsky *et al*, 1962)—and that nitrofurantoin not only bleaches *Euglena* but also suppresses their multiplication (McCalla, 1962). The effect of these drugs is apparent only in concentrations exceeding 10 times the normal peak therapeutic blood levels (Tables I and IV). The serum vitamin B₁₂ assay is likely to be affected only when these drugs are administered in exceptionally high doses or when severe renal and/or hepatic failure delays their clearance from the blood. The inability of *Euglena* to form chloroplasts may be due to interference with the replication of DNA-con-

taining cytoplasmic entities which are responsible for plastid formation (Smillie, 1963).

From a clinical point of view, it is essential to be sure that a low result of serum vitamin B₁₂ assay is actually due to low serum vitamin B₁₂ level. Microscopy of assay cultures and *Euglena* cell counts are simple methods of detecting false low results of serum vitamin B₁₂ assay due to the presence of inhibitory and/or bleaching substance in the serum.

We are grateful to Mr Neil Naismith, Chief Pharmacist of the Royal Melbourne Hospital, and his staff, for their help in preparing the antimicrobial agents used in this investigation.

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