9. A. M. Lands, A. Arnold, J. P. McAuliff, F. P. Luduena and T. G. Brown, *Nature, Lond.* **214**, 597 (1967).
10. J. B. Farmer, I. Kennedy, G. P. Levy and R. J. Marshall, *J. Pharm. Pharmac.* **22**, 61 (1970).
11. R. T. Brittain, D. Jack and A. C. Ritchie, *Prog. Drug Res.* **5**, 197 (1970).
12. P. Holtz, K. Stock and E. Westermann, *Nature, Lond.* **203**, 656 (1964).
13. R. Santi, A. Bruni, S. Luciani, C. E. Toth, G. Fassina and A. R. Contessa, *J. Pharm. Pharmac.* **16**, 287 (1964).
14. Y. Iwasawa and A. Kiyomoto, *Jap. J. Pharmac.* **17**, 143 (1967).
15. M. Sandler, S. B. Carter, K. R. Hunter and G. M. Stern, *Nature, Lond.* **241**, 439 (1973).

Biochemical Pharmacology, Vol. 24, pp. 1359–1361. Pergamon Press, 1975. Printed in Great Britain.

Vitamin B_{12} levels in drug-treated bacterial cells

(*Received* 12 *March* 1974; *accepted* 13 *January* 1975)

Up till now little attention has been paid to the action of bacteriostatics on the biosynthesis of cobalamins. In the presence of sulfathiazole and aureomycin, the content of a biologically active form of vitamin B_{12} synthesized by some species of Propionibacteria was found to be increased. However, this phenomenon has so far not been theoretically explained [1, 2]. On the other hand, Bukin and Proniakova found that sulfathiazole in the concentration of 1 mg% caused a marked inhibition of vitamin B_{12} formation by *Propionibacterium shermanii*, without influencing the yield of the bacterial mass [3]. According to Cohen the synthesis of vitamins was generally inhibited by sulfathiazole [4]. Kanopkajtie-Rozgiene found that an increase in vitamin B_{12} content in the biomass of *Azotobacter chroococcum* occurred only at the lag phase of growth [5]. In the previous studies an increase in the level of vitamin B_{12} was found in cells treated with 1·2% sulfathiazole, as compared with the B_{12} level in control cells of the mesophilic strain of *Bacillus megatherium* in their logarithmic phase of growth [6]. This fact has led the author to examine whether this phenomenon was connected with a specific action of this drug on vitamin B_{12} synthesis, or if it was due to changes in the metabolic pattern of the drug-treated cells. In these studies, for comparison, amethopterin, 5-fluorouracil, chloramphenicol, actinomycin D and puromycin were used. The experiments were performed on the mesophilic strain of *B. mehatherium*.

Culture conditions. Bacillus megatherium was grown in the following fermentation medium: broth 20 g, molasses 10 g, L-methionine 40 mg, Co (as chloride) 5 mg and water 1 litre. Prior to inoculation, the drugs were introduced into the culture media and afterwards the cells were seeded. The optical density of the cultures was then adjusted to about 0·3. The media in 60-ml amounts were distributed into 100-ml flasks. The cultures were kept at 30° in a shaker. At the experimental time periods the cells of control cultures and those of drug-treated cultures were centrifuged at 9000 rev/min. In parallel test samples the content of vitamin B_{12} per 1 mg of protein and the optical density of the cultures were estimated. Extraction of vitamin B_{12} from bacterial cells was performed by the method of Pawełkiewicz and Zodrow [7]. Vitamin B_{12} was determined microbiologically with *Lactobacillus leichmanii* ATCC 7830. Nephelometric readings were taken on a Coleman spectrophotometer, Model 14 at 600 nm. To determine the protein content in the test samples the method of Folin-Ciocalteau was employed [8].

Drugs. Sulphathiazolum solubile FP IV, sal. natric., Starogardzkie Zakłady Farmaceutyczne-Polfa was used at 0·05%, 5-fluorouracil sub forma sal. nitric., La Roche at

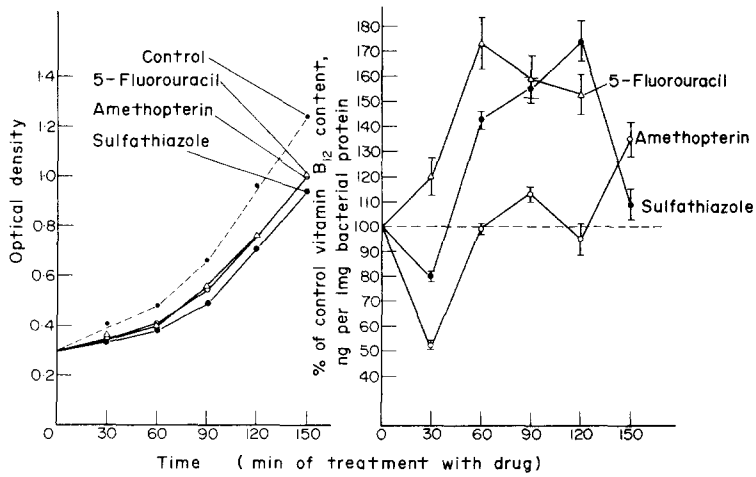

Fig. 1. Effects of sulfathiazole, amethopterin and 5-fluorouracil on the growth of cultures and on vitamin B_{12} content in cells of *B. megatherium*. Cell growth with sulfathiazole at the concentration of 0·05%, with 10^{-4} M amethopterin and with 0·15 mg/ml 5-fluorouracil. Points at 30 min on the B_{12} curve for sulfathiazole and amethopterin-treated cells differ significantly from corresponding point of control value ($P = 0·001$). Points at 30, 60, 90 and 120 min on the B_{12} curve for 5-fluorouracil-treated cells differ significantly from corresponding points of control values ($P = 0·05$ or $P = 0·01$ or $P = 0·001$).

Table 1. Effects of actinomycin D, chloramphenicol and puromycin on growth of cultures and vitamin B_{12} content in the cells of *B. megatherium*

Concn of drug added	Growth of culture (% of control value) 30 min	Growth of culture (% of control value) 60 min	Vitamin B_{12} content (% of control value) 30 min	Vitamin B_{12} content (% of control value) 60 min
Actinomycin D				
0·005 μg	98·0 ± 0·72	93·4 ± 1·81	82·2 ± 2·22‡	83·8 ± 2·10§
0·25 μg	80·4 ± 1·03§	58·7 ± 2·31§	216·3 ± 3·23§	314·2 ± 5·01§
Chloramphenicol				
1·0 μg	97·2 ± 2·29	84·9 ± 3·00†	77·8 ± 3·54*	107·1 ± 5·67
5·0 μg	82·0 ± 1·76‡	62·9 ± 2·82§	220·8 ± 8·31§	257·3 ± 12·04§
Puromycin				
2·0 μg	101·8 ± 1·45	94·4 ± 1·82	77·8 ± 4·89*	108·3 ± 8·02
4·0 μg	101·7 ± 1·23	88·2 ± 2·56†	81·2 ± 4·35*	110·6 ± 7·97

Values significantly different from control *(P = 0·05). †(P = 0·02). ‡(P = 0·01). §(P = 0·001).

0·15 mg/ml, Methotrexate Natrium® Lederle at 10^{-4} M, Chlorocid®, Egypt-Budapest at 1 μg/ml and 5 μg/ml, Lyovac Cosmogen® (Dactinomycin), Merck Sharp & Dohme at 0·005 μg/ml and 0·25 μg/ml and puromycin dihydrochloride, Sigma at 2 μg/ml and 4 μg/ml were employed. All drugs, with the exception of sulfathiazole, were introduced into the media sterilized by autoclaving.

In the studies performed a progressive decrease of the content of vitamin B_{12} in the cells of control cultures of *B. megatherium* was observed. This decrease of the content of the coenzyme in normal conditions was connected with a quick rate of cell division, as a result of which the mean value of vitamin concentration in the cells undergoing the lag phase of growth was 4·8 ng per 1 mg of bacterial protein, whereas the mean value of vitamin B_{12} content in the logarithmic phase was 0·95 ng per 1 mg of bacterial protein.

Figure 1 presents changes in vitamin B_{12} concentrations in the media with sulfathiazole, amethopterin (Methotrexate Natrium) or 5-fluorouracil. As can be seen, the growth in the antimetabolites-treated cultures was partially inhibited. The concentration of vitamin in sulfathiazole and amethopterin-treated cultures in the first experimental time period decreased to a greater extent than in the control. Determinations of vitamin B_{12} content in cells grown with 5-fluorouracil revealed higher levels of the coenzyme than the control cells. The most pronounced difference between these concentrations was parallel to the lengthening of the division rate of the drug-treated cells.

Vitamin B_{12} seems to be necessary for the conversion of N^5-methyltetrahydrofolate into other active folate coenzymes, presumably via methionine biosynthesis by the enzyme N^5-methyltetrahydrofolate homocysteine transmethylase. The enzyme catalyzing methionine bisoynthesis requires a cobalamin coenzyme. On the other hand, both the antifolates used in these studies, sulfathiazole and amethopterin each in its way make the formation of tetrahydrofolate impossible [9–11]. Since the level of vitamin B_{12} was in the first experimental time period lower than that in the control, it might be possible that vitamin B_{12} played a role in the change of the metabolic pattern induced by antifolates, most probably by the supply of active forms of folates. This supposition seems to be supported by the fact that in the second experimental time period the cells entered the logarithmic phase of growth. The results obtained with 5-fluorouracil might indicate lesser utilization of the vitamin than in the quickly dividing control cells, and might be connected with another mode of action of 5-fluorouracil than that of sulfathiazole and amethopterin on DNA synthesis [12].

In another set of experiments the effects of actinomycin D (Lyovac Cosmogen–Dactinomycin), chloramphenicol (Chlorocid®) and puromycin were investigated. As known, the action of these drugs, even at low concentrations, consists in primary or secondary inhibition of RNA and protein synthesis [13–14]. The results of these experiments are presented in Table 1. They show that the level of vitamin B_{12} in the cells of *B. megatherium* depends on the time of drug action as well on the drug concentration. The level of vitamin B_{12} in the cells grown in media with chloramphenicol in the concentration of 1 μg or with puromycin in the concentration of 2 μg and 4 μg per 1 ml 30 min after the beginning of the experiment decreased and afterwards increased to reach a level similar to that in control cultures. The decrease in the content of vitamin B_{12} in 0·005 μg actinomycin-treated cultures was in the experimental time period consistently more pronounced than in control cultures. The growth of the cells was either less (with chloramphenicol at concentration of 1 μg and with puromycin at concentration of 2 and 4 μg) or, as in the case of actinomycin-treated cells at concentration of 0·005 μg, similar to that in controls.

In conditions induced by the action of chloramphenicol or actinomycin D in high concentrations well pronounced inhibition of culture growth was observed. In actinomycin-treated cells the content of vitamin B_{12} was increased two-fold or three-fold and in the case of chloramphenicol-treated cells was two-fold and 2·5-fold higher as compared with that in controls. This very elevated level of vitamin B_{12} in the cells of cultures characterized by a pronounced inhibition of growth is probably due to the lack of its utilization in conditions in which most of the processes connected with active cell growth are inhibited. In contrast, the lower level of the vitamin in cells treated with the inhibitors of RNA and protein synthesis in low concentrations as compared with those in controls, at the same or at nearly the same rate of cell division, might indicate a more extensive utilization of the coenzyme. It seems, however, much more probable, that this decrease indicates a transient inhibition of B_{12} biosynthesis. This supposition is supported by the fact that, as in the case of cells grown with actinomycin D, the level of vitamin was lower than in the controls in conditions in which cell growth was parallel with that in controls.

Acknowledgement—The author wishes to express her thanks to Associate Professor Dr. J. Koziorowska for helpful discussions.

Department of Vitamin Drugs,
Institute for Drug Research and Control,
00–725 *Warsaw, Chelmska* 30, *Poland*
HANNA GAJCY

REFERENCES

1. J. Pawełkiewicz, *Acta Biochim. Polon.* **2**, 321 (1955).
2. J. Janicki and F. Pędziwilk, *Acta Biochim. Polon.* **5**, 299 (1958).

3. V. N. Bukin and G. V. Proniakova, in *International Symposium on B Vitamins*, Poznań 21–24. IX. p. 5 (1959).
4. S. S. Cohen, *Ann. N.Y. Acad. Sci.* **186,** 292 (1971).
5. S. I. Kanopkajtie-Rozgiene, in *Vitaminnyje Resursy i ich Ispolzovanije*, Vol. 5, p. 50. Academia Nauk USSR, Moskwa (1961).
6. H. Gajcy, *Acta Microbiol. Polon.* **5,** 119 (1973).
7. J. Pawełkiewicz and K. Zodrow, *Acta Microbiol. Polon.* **6,** 219 (1957).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
9. T. H. Jukes and H. P. Broquist, in *Metabolic Inhibitors* (Eds. R. M. Hochster and J. H. Quastel), Vol. 1, p. 481. Academic Press, New York (1963).
10. W. C. Werkheiser, *Cancer Res.* **23,** 1277 (1963).
11. J. S. O'Brien, *Cancer Res.* **22,** 267 (1962).
12. M. Reich, H. G. Mandel, *J. Bact.* **91,** 517 (1966).
13. T. Korzybski, Z. Kowszyk-Gindifer and W. Kuryłowicz, in *Antibiotics*, Vol. 1, p. 941. Pergamon Press, Oxford (1967).
14. I. H. Goldberg, in *Antibiotics and Chemotherapy* (Eds. H. Schönfeld and A. De Weck), Vol. XVII, p. 67. S. Karger, Basel (1971).

Biochemical Pharmacology, Vol. 24, pp. 1361–1362. Pergamon Press, 1975. Printed in Great Britain.

The displacing effect of a fatty acid on the binding of diazepam to human serum albumin

(*Received* 15 *October* 1974; *accepted* 20 *December* 1974)

Diazepam is a drug used very widely in the treatment of anxiety [1]. Studies on protein binding of diazepam in dog plasma and bovine serum showed extensive binding of the drug [2]. However, studies with human plasma or serum have been neglected. Drugs which are bound extensively to proteins present enhanced possibilities of interaction between drugs and endogenous substances [3]. This study was conducted to investigate the binding characteristics of diazepam to human serum albumin (HSA) and the effect of a fatty acid on the drug-protein binding.

Crystalline human serum albumin, essentially free of fatty acids, and sodium laurate were obtained from Sigma Chemical Co. 5-[^{14}C]Diazepam (sp. act., 197 μCi/mg) was supplied by Hoffman-La Roche Inc. The radiochemical purity of 5-[^{14}C]diazepam verified by thin-layer chromatography was found to be greater than 98 per cent [4]. Radioactive drugs were dissolved in absolute ethanol and mixed with unlabeled drug to achieve suitable concentrations. Unless otherwise indicated, 0·5% (w/v), $7{\cdot}46 \times 10^{-5}$ M, HSA was used and dissolved in 0·1 M phosphate buffer of pH 7·4. The drug solution (0·1 ml) was added to 4·9 ml HSA–buffer solution and stirred in a vortex mixer for 1 min. In some experiments, sodium laurate was added to the HSA–buffer solution and stirred for 1 min before adding diazepam. Diazepam concentrations ranged from $3{\cdot}6 \times 10^{-6}$ M to $1{\cdot}01 \times 10^{-4}$ M and laurate was $3{\cdot}5 \times 10^{-4}$ M. The molar ratios of laurate/diazepam ranged from 970 to 3·5. The molar ratio of laurate/albumin was 4·7 in 0·5% HSA. All experiments were carried out at the controlled room temperature of 22°.

Membrane cones (Centrifro CF-50 membrane ultrafilter, Amicon Corp.) were soaked in distilled water for at least 1 hr before use. After removing the water on the membrane by centrifugation, 4 ml of drug–HSA solution was pipetted into the cone, and centrifuged twice at 1000 rev/min for 2·5 min. After each centrifugation, approximately 0·1 ml of the ultrafiltrate was removed for radioactivity counting; the exact volume of ultrafiltrate was measured by weighing. Drug containing albumin solution (0·1 ml) from inside the cone was also taken for radioactivity counting. The radioactivity was determined using Aquasol (New England Nuclear) and Nuclear Chicago Mark II liquid scintillation counter. The amount of radioactivity in the ultrafiltrate was 1,500–10,000 dis./min/ml, and the standard deviation among repetitive determinations of radioactivity ranged from 1 to 3 per cent. Potentially major sources of error were protein leakage through the membrane and adsorptive binding of the drug to the membrane cone. These factors were carefully examined under these conditions. A small amount of albumin leaked through the membrane if the filtrate volume exceeded 0·2 ml. The quantity of drug adsorbed to the cone was determined by subtracting the arithmetic sum of the filtrate and supernatant drug from the original quantity of drug–albumin solution in the cone. Albumin concentrations in the ultrafiltrate were measured by the biuret method [5]. The percentage of drug bound to albumin was calculated by deducting the free drug concentration in the filtrate from the original concentration (free plus bound) in the supernatant. The calculated free drug concentration included the quantity of drug adsorbed to the membrane cone.

The binding capacity of diazepam was studied at a concentration observed to occur in therapy, that is, a concentration of 248 ng/ml with 3·5% HSA in buffer. Diazepam binding to HSA was 93·7 ± 0·8 (S.E.) per cent. Van der Kleijn [2] reported that the extents of binding of diazepam to dog plasma protein and bovine serum albumin (BSA) were 93–94 per cent and 84–87 per cent, respectively, as determined by an ultracentrifuge method. Per cent binding of diazepam to dog plasma and to BSA appeared to be independent of the drug concentration over a range

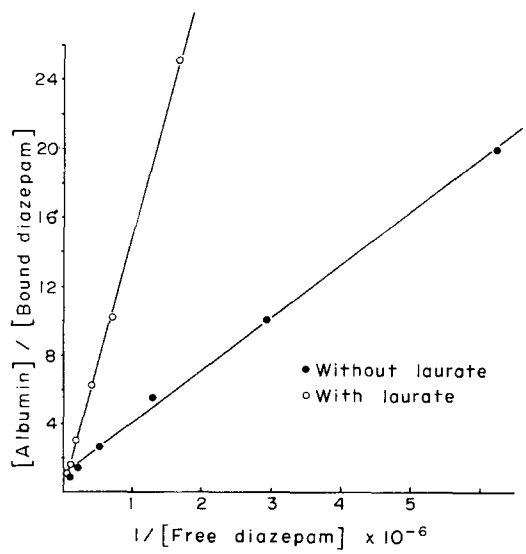

Fig. 1. Binding of diazepam to human serum albumin and the inhibiting effect of laurate. Albumin 0·5% (w/v), $7{\cdot}46 \times 10^{-5}$ M; laurate, $3{\cdot}5 \times 10^{-4}$ M. Both ordinates are calculated on the basis of molarities.