TABLE 3

RESULTS OF BLOOD AND SPLEEN CULTURES ON MICE SUBJECTED TO 450 R TOTAL BODY X-RADIATION*

Days after irradiation	Daily death rate† %	Blood and spleen positive No. of mice	Spleen only positive No. of mice	Blood *or* spleen positive %
2	0.2	1	2	8
3	0	0	0	0
4	0	1	5	17
5	2.0	0	3	8
6	1.0	4	1	14
7	4.2	3	1	11
8	4.3	8	1	25
9	4.6	4	2	17
10	6.0	15	4	54
11	3.6	15	2	48
12	4.0	15	2	48
13	3.0	13	3	45
14	1.0	12	1	37
15	0.8	8	3	31
16	0.2	10	3	36‡
17	0.1	3	0	8
18	0	5	1	17

* Thirty-five mice killed and cultured each day after irradiation. Cultures on mice that died spontaneously are excluded from these data.

† Percent of total number irradiated.

‡ One anaerobe.

In the 450-r series, a total of 595 mice were killed and cultured. Table 3 shows that a high incidence of positive cultures occurred during the period of greatest mortality, although the maxima were not as great as in the preceding series. The daily mortality rate was based on the total of 1,042 mice irradiated in this series. The bacteremia was not as severe; approximately 70% of the positive cultures developed more than 50 colonies but only 20% contained too many colonies to count.

TABLE 4

CLASSIFICATION OF MICROORGANISMS RECOVERED FROM HEART BLOOD OR SPLEEN OR BOTH

	%
Paracolobactrum	42
Coliform	22
Proteus	13
Pseudomonas	9
a Streptococcus	6
Unidentified Gram-negative rod	3
Alcaligenes	2
Anaerobes	0.3

Identity of the organisms. The cultures were identified by the customary methods. The relative frequence of the various species is given in Table 4. In a systematic survey of the flora at different levels of the intestinal tract of normal mice it was found that all of the species listed in Table 4 occurred regularly in the large bowel with the exception of *Pseudomonas*. This organism was found only occasionally. It is clear from these findings that the lower intestinal tract was the reservoir from which invasion of the blood stream occurred. In both the 600-r and 450-r series, 91% of the cases of bacteremia were caused by a single organism; in the remaining 9% no more than two species were present. This fact strongly suggests that multiplication of the organisms was occurring in the blood stream.

It was during the second week after irradiation, the period of greatest mortality, that bacteria from the lower intestinal tract invaded the blood stream and produced bacteremia, often of great severity. The maximum incidence of such infections in mice exposed to 600-r or 450-r x-radiation was 85% and 54%, respectively.

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The Effect of Cobalt on the Microbial Synthesis of LLD-active Substances

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Shorb (*7*) reported an unidentified growth factor in liver extract, LLD, which is required by *Lactobacillus lactis* Dorner. Crystalline vitamin B_{12}, a cobalt coordination complex isolated from liver (*5*), was found capable of satisfying the LLD requirement of this organism (*8*). It has also been shown that *Lactobacillus leichmannii* responds to vitamin B_{12} (*2*). Recently Rickes *et al.* (*6*) have reported the isolation of crystalline vitamin B_{12} from culture broths of a grisein-producing strain of *Streptomyces griseus.* In addition, they reported the presence of LLD-active substances in culture broths of other microorganisms. Other investigators have also observed the presence of materials having growth-promoting activity for *L. leichmannii* in various microbial broth cultures (*1, 4*). During an extensive screening program we have observed that large numbers of microorganisms are capable of synthesizing LLD-active substances. Chemical extraction data and paper strip chromatography carried out on some of these broths have shown them to contain vitamin B_{12}.

Various medium modifications were investigated in an attempt to increase the microbial synthesis of vitamin B_{12}. This paper presents the data obtained from a study on cobalt supplementation.

Medium. The medium employed in these studies was composed of 1% N-Z-Amine (Type A),[1] 0.3% Difco beef extract, and distilled water to volume. The medium was adjusted to pH 6.8–7.0 with NaOH, dispensed in 40-ml aliquots per 250-ml Erlenmeyer flask, and sterilized at 15 lb pressure for 20 min.

Inoculum. The various microorganisms were carried on nutrient agar slants. A 48-hr shake flask culture was used as inoculum in experiments with the grisein-produc-

[1] An enzymatic digest of casein manufactured by Sheffield Farms, Inc.

ing strain of *S. griseus*. In the case of the other microorganisms, a water suspension derived from a 24-to-48-hr agar slant was used as inoculum.

Incubation. The flasks were incubated for 5 days at 28° C on a shaking machine imparting a rotary motion of 220 rpm.

Estimation of LLD activity. Five-day-old fermentation broths were adjusted to pH 7.0 and diluted in distilled water to contain approximately 0.5 unit of vitamin B_{12} equivalent per ml. These samples were then assayed for LLD activity by the procedure previously outlined, using vitamin B_{12} as a standard (*3*).

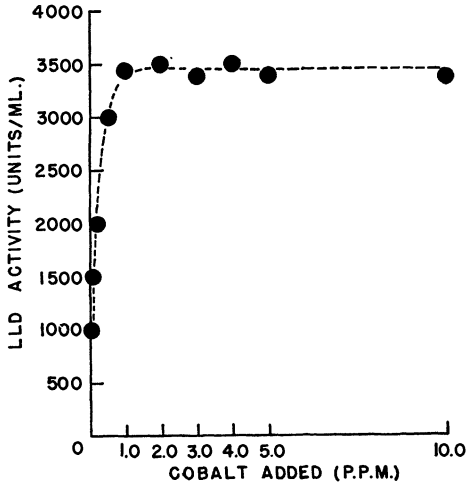

FIG. 1.

Experiments with S. griseus. In these experiments varying concentrations of cobalt in the form of $CO(NO_3)_2 \cdot 6H_2O$ were added to the N-Z-Amine medium prior to sterilization. LLD activity was then determined after 2, 3, 4, and 5 days' incubation. Maximum activity was observed between the 3rd and 5th days, with no significant loss in titer after peak production. For this reason, in these experiments, samples were assayed on the 5th day. Fig. 1 shows the response curve obtained with increased dosage of Co^{++}. An approximate threefold increase in LLD titer was obtained by the addition of Co^{++} to the basal medium. Although LLD activity is not identical with vitamin B_{12}, an increase in B_{12} production was also obtained which more or less paralleled the increase in LLD activity.[2] Maximal activities were obtained with as little as 1 to 2 ppm Co^{++}. Toxic manifestations became apparent at levels of 20 to 50 ppm Co^{++}. At these levels a marked decrease in growth and LLD activity was observed. It is apparent from these experiments that Co^{++}, a structural constituent of the B_{12} molecule, becomes the limiting factor in N-Z-Amine medium for the microbial synthesis of LLD-active substances. Supplementation of the medium with Co^{++} therefore gives rise to an increase in LLD activity and vitamin B_{12}.

Experiments with other microorganisms. It has previously been observed that microorganisms other than *S. griseus* produce significant yields of LLD-active substances (*6*). We have observed that a large number of microorganisms were capable of synthesizing LLD activity and that supplementation of the N-Z-Amine medium with Co^{++} (2 ppm) gave rise to increased yields of the growth factor. The results shown in Table 1 indicate the Co^{++} effect with several isolates obtained from cow rumen contents and manure, as well as with several cultures from the Merck Culture Collection. In every case, the addition of Co^{++} (2 ppm) resulted in a significant increase in the LLD titer of the broth.

TABLE 1

EFFECT OF Co^{++} ON LLD SYNTHESIS

Organism	LLD-active substances (units/ml)	
	No added Co^{++}	Co^{++} added (2 ppm)
R2*	1100	3500
R5	700	4900
R6	160	3000
R12	400	800
R36	1300	4800
R41	500	1140
C5†	260	480
C6	280	750
C7	390	950
C10	160	380
Mycobacterium smegmatis	540	3800
Pseudomonas sp.‡	640	3000
Streptomyces griseus G25	880	3500

* R series—isolates from cow rumen contents.
† C series—isolates from cow manure.
‡ Isolated from soil by J. W. Foster and tentatively named by him, *Pseudomonas lumichroma* n. sp., since it is capable of decomposing lumichrome.

These experiments throw some light on the biological significance of cobalt in the nutrition of the microbial cell. One role of cobalt, in the biosynthesis of vitamin B_{12}, has been experimentally substantiated with microorganisms.

In summary, data have been presented to show that, in N-Z-Amine medium, Co^{++} becomes the limiting factor in the biosynthesis of LLD-active substances by *S. griseus*. The addition of as little as 1 to 2 ppm Co^{++} gives rise to approximately a threefold increase in LLD activity.

In addition, an extensive survey has shown that large numbers of microorganisms synthesize LLD-active substances and that, in these cases as well, supplementation with Co^{++} (2 ppm) gives rise to a significant increase in LLD activity.

[2] We are indebted to E. L. Rickes and T. R. Wood for the isolation data.

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