TABLE III. Hematological Data on Rats Receiving High Levels of Glycine and Choline With and Without Added PGA.

Diet	No. of animals	R.B.C. 20th day $10^6/mm^3$	W.B.C. 20th day $10^3/mm^3$
Experiment I			
Basal	4	7.63	17.8
Basal + 500 γ PGA/100 g	7	7.30	18.7
Basal + 10% glycine	5	7.43	18.0
Basal + 10% glycine + 500 γ PGA/100 g	5	7.10	16.6
Basal + 10% glycine + 2% choline	5	9.23	17.1
Basal + 10% glycine + 2% choline + 500 γ PGA/100 g	5	9.78	17.9
Experiment II		32nd day	
Basal	8	8.31	18.8
Basal + 6% choline	8	8.49	18.9
Basal + 6% choline + 500 γ PGA/100 g	8	8.84	17.8

the basis of an altered rate of conversion of choline to betaine.

The red cell counts, hemoglobin and hematocrits of the various groups showed no significant differences.

Summary. Weanling albino rats were fed a standard purified diet, a similar diet with 10% glycine replacing an equal amount of sucrose, and a diet with 10% glycine and 2% choline replacing an equal amount of sucrose. Other groups received these diets supplemented with 100 γ of riboflavin per day or 100 γ of riboflavin and 100 γ of PGA per day. The growth of the rats receiving the high glycine and high glycine plus high choline diets was greatly reduced as compared to the basal group. Supplementation of these diets with riboflavin brought about no effect on the growth; the combination of riboflavin and PGA overcame the growth depression to a considerable extent and also brought about a marked increase in gain per unit of food consumed. Supplementing the various diets with both riboflavin and PGA brought about a leucocytosis which was very marked when the diet contained a high level of both glycine and choline. The data would suggest that there is an interrelationship between riboflavin and PGA in white cell production.

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Further Observations on the Microbiological Assay for Vitamin B_{12} Using *Lactobacillus leichmannii.* (18639)

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Conflicting reports have appeared regarding the comparative potencies of vit. B_{12} and B_{12b} in microbiological assays with *Lactobacillus leichmannii* and *L. lactis* Dorner. The two compounds were found to have approximately equal activity for *L. leichmannii* by Pierce *et al.*(1,2). However, in subsequent studies in our laboratories, the potency of vit. B_{12b} often appeared to be only 0.7 to 0.8 that of vit. B_{12} in the *L. leichmannii* assay. In contrast, Jackson and co-workers(3) found that vit. B_{12b} was 1.4 to 1.5 times as potent as vit. B_{12} when the vitamins were added

1. Pierce, J. V., Page, A. C., Jr., Stokstad, E. L. R., and Jukes, T. H., *J. Am. Chem. Soc.*, 1950, v72, 2615.

2. Fricke, H. H., Lanius, B., DeRose, A. F., Lapidus, M., and Frost, D. V., *Fed. Proc.*, 1950, v9, 173.

3. Jackson, W. G., Whitfield, G. B., DeVries, W. H., Nelson, H. A., and Evans, J. S., *J. Am. Chem. Soc.*, 1951, v73, 337.

aseptically to the culture media. Vit. B_{12b} had about 60% of the activity of vit. B_{12} for *L. lactis* Dorner when autoclaved with the medium but the two substances showed equal activity when added aseptically. Kaczka *et al.*(4) reported the following relative values for vit. B_{12b} with different assay organisms assigning vit. B_{12} a potency of 1.0: with *L. lactis* 0.62 (titrimetric), 1.0 (cup), with *L. leichmannii* in the absence of reducing agent 0.91 (aseptic addition), and 0.17 (autoclaved with the medium), while vit. B_{12a}, which appears to be formed from vit. B_{12b} by heating at 100° for 2 hours and perhaps may be an "anhydro-form" of vit. B_{12b} which reverts on standing in solution, behaved similarly to vit. B_{12b}.

In the present investigation it was found that the potency of vit. B_{12} for *L. leichmannii* 4797 was less than that of vit. B_{12b} when both substances were assayed by aseptic addition to the previously autoclaved culture medium. However, when vit. B_{12} was autoclaved with the culture medium, its apparent potency was found to be greater than that of either vit. B_{12b} or of aseptically-added vit. B_{12}. The experimental basis of these observations was examined.

Experimental. Vit. B_{12b} was prepared by Mr. J. V. Pierce from *Streptomyces aureofaciens* as described previously(1). Solutions in water were standardized by measurement of the absorption spectrum, $E^{1\%}_{1cm}$ at 351 $m\mu$ = 167. Vit. B_{12} was purchased from Merck and Co. and aqueous solutions were standardized by measurement of the absorption spectrum, $E^{1\%}_{1cm}$ at 361 $m\mu$ = 207. Vit. B_{12c} (nitrosocobalamin) was kindly supplied by Dr. E. Lester Smith of Glaxo Laboratories. The concentration of the solution was not known, but was estimated to be approximately 50 γ/ml by spectrophotometric analysis using vit. B_{12b} as standard. A sample of "ammino-cobalamin" (Compound AC2) was kindly furnished by Dr. V. Petrow of British Drug Houses. Thiomalic acid, obtained from Allied Chemical and Drug Co., was recrystallized twice from water before use in the culture medium.

Microbiological assay technic. The microbiological assay technic employed in the present investigation was that of Skeggs *et al.*(5) using *Lactobacillus leichmannii* 4797. The only modification of the method was that a final volume of 2 ml of culture medium was employed rather than 10 ml; separate experiments showed this modification did not alter the experimental results. Assay results were obtained by estimating growth turbidimetrically after a 24-hour incubation at 37°C.

Results. In an attempt to compare vit. B_{12b} with vit. B_{12}, the technic of assaying the two vitamins by aseptic addition to the medium was reinvestigated. This was accomplished by diluting concentrated solutions of the vitamin by aseptic technic into sterile water and finally adding the appropriate dilution aseptically to the assay tubes containing autoclaved, cooled medium. No trouble with contamination was experienced with this technic. It was repeatedly found that the growth response of *L. leichmannii* was always less when vit. B_{12} was added aseptically to the medium as compared to when vit. B_{12} was autoclaved in the medium. Typical results are shown in Table I. In contrast liver extract, vit. B_{12b}, B_{12c} and ammino-cobalamin gave essentially the same growth response when added to the culture medium aseptically as when autoclaved. As a consequence when these samples were assayed against vit. B_{12} using aseptic technic, erroneously high results were obtained. If the samples and vit. B_{12} standard were autoclaved separately from the medium, or if sterilization of the assay was accomplished with ethylene oxide at reduced temperatures(6), results were obtained similar to those described with the aseptically-added samples in Table I. Also, if tomato juice or asparagus juice were added to the assay medium in amounts commonly employed in other vit. B_{12} microbiological assays, these additions did not alter the experimental

4. Kaczka, E. A., Denkewalter, R. G., Holland, A., and Folkers, K., *J. Am. Chem. Soc.*, 1951, v73, 335.

5. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *J. Biol. Chem.*, 1950, v184, 211.

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TABLE I. Microbiological Assay of Vitamin B_{12} with *Lactobacillus leichmannii* Comparing Aseptic and Non-Aseptic Assay Technic.

Additions to 2 ml medium deficient in vit. B_{12}	Sample autoclaved with medium		Sample added aseptically to sterile, cooled medium	
	Optical density	γ B_{12}/ml found	Optical density	γ B_{12}/ml found
0	.0		.0	
.01 mγ vit. B_{12}	.08		.08	
.03 " " "	.24		.12	
.10 " " "	.51		.20	
.3 " " "	.62		.33	
1.0 " " "	.62		.46	
3.0 " " "	.62		.56	
Vit. B_{12b}, 10 γ/ml				
6 × 10-6 ml	.21	4.3	.24	27.0
20 × 10-6 ml	.45		.40	
Ammino cobalamin, 30.5 γ ml				
1.5 × 10-6 ml	.23	19.4	.25	150
5 × 10-6 ml	.49		.45	
Vit. B_{12c} sol. (ca 50 γ ml)				
0.6 × 10-6 ml	.19	40	.21	195
2 × 10-6 ml	.45		.37	
15 unit liver extr.				
1.5 × 10-6 ml	.30	26	.33	220
5 × 10-6 ml	.58		.47	
Vit. B_{12}, thiomalic-acid-reaction-product* equivalent to 2 γ vit. B_{12}/ml				
15 × 10-6 ml	.20	1.9	.26	13.2
50 × 10-6 ml	.47		.42	

* See text for details of preparation.

results shown in Table I.

The poor microbiological response to vit. B_{12} obtained when the vitamin is added aseptically to the medium suggested that when vit. B_{12} is autoclaved in the medium it may be converted to another and more active form of the vitamin. Because of the prominent role of reducing agents in microbiological assays for vit. B_{12}, the effect of heating vit. B_{12} with thiomalic acid was investigated. Fifty milligrams of thiomalic acid was dissolved in 4 ml water and the pH adjusted to 6.5; 10 γ of a solution of crystalline vit. B_{12} was added to the thiomalic acid solution, the volume adjusted to 5.0 ml, and the solution autoclaved for 10 minutes at 15 lb pressure. When this preparation was compared microbiologically with untreated vit. B_{12} it was found (Table I) to give essentially the same growth response when assayed aseptically as when autoclaved with the medium. Thus microbiologically the vit. B_{12} compound formed by heating vit. B_{12} and thiomalic acid differs from untreated vit. B_{12}. Further evidence that vit. B_{12} is transformed into a different compound when heated with thiomalic acid was obtained by paper chromatography.

The technic for chromatography followed that described by Woodruff and Foster(7). Filter paper strips buffered with 0.66M KH_2PO_4 were employed and n-butanol saturated with water used as a developer. The descending method of paper strip chromatography was used, and the chromatograms were run at 37°C for 48 hours. The position of the vitamins on the paper strips was determined bioautographically. Results of a typical experiment are given in Table II. As reported by others(7) solutions of vit. B_{12} following paper strip chromatography exhibited two zones of activity, one in the position occupied by vit. B_{12b} (35 mm) and another more rapidly-moving component characteristic of pure vit. B_{12} (180 mm). Of great interest was the finding that the preparation made by auto-

7. Woodruff, H. B., and Foster, J. C., *J. Biol. Chem.*, 1950, v183, 569.

TABLE II. Chromatographic Evidence for the Transformation of Vit. B_{12} to an Unidentified Compound Following Treatment with Thiomalic Acid.

Substance chromatographed	Zone	Distance from origin,† mm
Vit. B_{12}	1	35
	2	180
Vit. B_{12b}	1	35
Reaction product obtained by autoclaving vit. B_{12} and thiomalic acid*	1	60

* See text for details of preparation.

† Results are recorded as distance the vitamin moved on the paper strip from the origin rather than R_f values, as the developing solvent was allowed to drip off the end of the paper during the extended period of time employed to develop the chromatogram.

claving vit. B_{12} and thiomalic acid was chromatographically different from pure vit. B_{12}, as the reaction product moved only one-third as rapidly as vit. B_{12} but somewhat faster than vit. B_{12b}. Similar results were obtained by autoclaving vit. B_{12} in the complete assay medium containing either thiomalic acid or thioglycolic acid. From the results of Tables I and II it may be concluded that when vit. B_{12} is autoclaved in the medium in the presence of thiomalic acid it is transformed to a new compound having greater microbiological activity than vit. B_{12}, when compared by aseptic assay technic.

Earlier work from this laboratory(8) had suggested that thioglycolic acid and various reducing agents protected vit. B_{12} in crude materials from heat destruction during autoclaving. The present results indicate that the reducing agent might exert its favorable effect on growth by participating in a chemical reaction with vit. B_{12} to form a compound more readily utilized by *L. leichmannii*. To test these hypotheses further, the experiment outlined in Table III was carried out. In the absence of thiomalic acid a very poor growth response to the test samples was obtained. However, if after autoclaving the samples in the absence of reducing agent, thiomalic acid was added to the tubes and the samples reautoclaved, vit. B_{12} now exhibited essentially full activity demonstrating conclusively that the vitamin was not destroyed during autoclaving but had to be reheated with thiomalic acid to produce a good growth response. It was of particular interest that vit. B_{12b} (and liver extract) when autoclaved in the medium in the absence of thiomalic acid, gave only a slight growth response, as the data of Table I indicate that these preparations gave a good growth response when added aseptically to the medium. It is possible that heating vit. B_{12b} in the medium without thiomalic acid converts the vitamin to an inactive form which can be partially reactivated by reheating the samples with thiomalic acid. Welch and Wilson(9) have noted that thermal inactivation of vit. B_{12} may occur yielding products of which a portion can be converted by reducing agents to microbiologically more active forms.

Discussion. The results indicate that under the experimental conditions described vit. B_{12} was transformed into a new compound when it was autoclaved with the culture medium. The new compound appeared to be about five times as potent as vit. B_{12} when compared by aseptic addition. The implications of this finding in the evaluation of the vit. B_{12} content of natural materials are important. If vit. B_{12}, autoclaved with the medium, is used for the construction of the "standard curve," then a somewhat low value may be obtained for the potency of samples which contain predominantly vit. B_{12b}. An erroneously high value for such samples may be obtained if the standard curve is constructed from values obtained with vit. B_{12} added aseptically. If solutions of vit. B_{12} are inadvertently exposed to light, the resultant decomposition to form vit. B_{12b} may complicate the microbiological findings.

Two possibilities suggest themselves for resolving these discrepancies: (I) treatment of the samples with cyanide to convert vit. B_{12b} into vit. B_{12}(10-12) and use of vit. B_{12} as a standard, (II) photolysis of the samples if vit. B_{12} is present to convert it into vit. B_{12b}

8. Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffmann, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, v8, 257.

9. Welch, A. D., and Wilson, M. F., *Arch. Biochem.*, 1949, v22, 486.

TABLE III. Effects of Thiomalic Acid (TMA) on the Microbiological Assay of Vitamin B_{12}.

Addition to 2 ml medium deficient in vit. B_{12}	Samples autoclaved in medium containing		
	1 mg TMA per tube	No TMA	Following autoclaving 1 mg TMA added/ tube and assay reautoclaved
	Optical density		
0	.02	.02	.02
.03 mγ vit. B_{12}	.30	.04	.22
.08 " " "	.57	.10	.52
.27 " " "	.78	.27	.80
.1 mγ vit. B_{12b}	.38	.03	.14
.3 " " "	.63	.06	.29
1.0 " " "	.84	.16	.66
.67 × 10^{-6} ml liver extract	.19	.01	.09
2.0 " " " " "	.39	.03	.17
6.66 " " " " "	.69	.06	.35

(10) or perhaps standing in dilute HCl(13) to convert to a vit. B_{12b}-like compound, and use of vit. B_{12b} as a standard. These possibilities are being investigated.

Vit. B_{12c} and "ammino-cobalamin" both appeared, like vit. B_{12b}, to give the same growth response when the substance autoclaved with the medium was compared with the aseptically-added substance. The results obtained in patients with pernicious anemia (2,14,15) did not indicate a quantitative difference in clinical potency between vit. B_{12} and B_{12b}. The two compounds appear to have the same potency when injected into chicks (1). The potency of both vit. B_{12} and B_{12a} for *L. lactis* was enhanced by autoclaving with the culture medium(16). The addition of thioglycolic acid to the culture medium further increased the potency of both substances and their activity became equal. The results in their Fig. 1 indicate that the potency of vit. B_{12} for *L. leichmannii* was increased somewhat by autoclaving with the culture medium as compared with the potency of the vitamin when added aseptically. This increase did not appear to have been observed for vit. B_{12a} and B_{12b}.

Summary. 1. A comparison was made of vit. B_{12}, B_{12b}, B_{12c} and "ammino-cobalamin" in the microbiological assay with *Lactobacillus leichmannii* using aseptic or non-aseptic assay technic. The growth response of *L. leichmannii* was significantly less when vit. B_{12} was added aseptically to the medium as compared to when vit. B_{12} was autoclaved in the medium. This effect was not noted for vit. B_{12b}, B_{12c} and "ammino-cobalamin." 2. By means of paper strip chromatography it was demonstrated that when vit. B_{12} is autoclaved with thiomalic acid, vit. B_{12} is transformed into a new compound which is distinct from vit. B_{12b}. The vit. B_{12}-thiomalic acid reaction product is more potent microbiologically than vit. B_{12} when the compounds are compared by aseptic assay technic. 3. The significance of these results in the interpretation of the microbiological assay of the vit. B_{12} content of natural materials when vit. B_{12}

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is used as a standard is briefly discussed.

Addendum. During the progress of this work we received a copy of a paper by H. W. Long and O. L. Kline(17) in which, from studies similar to ours, it was postulated that both vit. B_{12} and vit. B_{12b} following heating in the basal medium are converted to a form of vit. B_{12} more readily utilizable by *L. leichmannii.*

The assistance of Mrs. Lucy Smith is gratefully acknowledged.

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Estrogen-Androgen Antagonism: Histology of Mammary Glands and Vaginal Grafts of Male Mice Receiving Estrogens.* (18640)

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The concept of an essential antagonism between estrogens and androgens has been the subject of much endocrinological investigation and considerable debate. In the early endocrine literature, Steinach and Kun(1), Lipschutz and others(2,3) refer to what they considered to be antagonistic actions between the gonads of the opposite sexes and later applied this concept to the sex hormones as well. However, extensive work culminating in the classic publication of Moore and Price (4), refuted this hypothesis, contending that gonad hormones stimulate homologous reproductive accessories but are without direct effect upon heterologous accessories and have no direct effect on the gonads of either the same or opposite sex, gonadal activity being governed by the function of the pituitary gland. Although most instances of "hormonal antagonism" observed prior to 1932 probably were effected through the suppression of pituitary function, several more recent reports seem clearly to demonstrate the existence of both cooperative and antagonistic interactions between estrogenic, androgenic and progestational hormones(5-16). Although there are certain differences in the usage of the terms hormone "antagonism" and "cooperation", it would seem best to restrict the term "antagonism" to those situations in which one hormone reduces the magnitude of the response of a target tissue or end organ to another hormone. To demonstrate such an-

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