venting HN_2-induced leukopenia in rabbits. On the contrary, cortisone induces a greater leukopenia as a result of increased lymphopenia. 2. Administration of cortisone to rabbits receiving L-cysteine prior to HN_2 administration results in a more severe leukopenia and neutropenia than administration of L-cysteine and HN_2 alone. 3. The combined use of cortisone and HN_2 therapeutically may induce a dangerous depression of hematopoietic tissue.

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Growth-Promotion by Lyxoflavin. II. Relationship to Riboflavin in Bacteria and Chicks.* (20186)

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Addition of L-lyxoflavin to appropriate experimental rations increases growth of rats (1-3), chicks(3,4), and pigs(5). To explain this action it has been suggested(2,4) that lyxoflavin may have vitamin action of its own, since: (a) it has no growth-promoting action for higher animals in the absence of riboflavin, (b) its addition to diets similar to those used for assay of unidentified factors stimulates growth, and (c) it has been reported to occur naturally(6), implicating a functional role for it in metabolism. The merit of these arguments is open to question. Growth stimulation by a given substance does not prove vitamin activity, as is abundantly evident from the growth-promoting action of a variety of antibiotics. Furthermore, although lyxoflavin stimulates growth of chicks on rations used for assay of unidentified stimulants, addition of crude supplements known to contain adequate amounts of these unidentified factors did not mask the response to lyxoflavin(4) as would be expected if their growth-promoting activity was due to this substance. Finally, the evidence for the natural occurrence of lyxoflavin is inconclusive(7); unless the compound actually occurs naturally, vitamin activity can scarcely be ascribed to it.

These findings with experimental animals are superficially similar to those in bacteria. We reported previously, without confirming data, that although lyxoflavin did not permit growth of *Lactobacillus casei* in the absence of riboflavin, low concentrations of lyxoflavin did increase the growth response of this organism to limiting amounts of riboflavin(4). Lyxoflavin resembled in this respect D- and L-araboflavins(8). At higher concentrations, lyxoflavin inhibits growth by acting as a competitive antagonist of riboflavin(4). These findings, which have been confirmed by others (3,9) are extended below. The report of Shorb(9) that *Lactobacillus lactis* grows in the absence of riboflavin when lyxoflavin is supplied has been confirmed and extended.

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Finally, it is shown that lyxoflavin, at high concentrations, acts as a riboflavin antagonist in chicks. Many toxic compounds stimulate growth in subinhibitory concentrations(10.11) and, although no generally applicable explanation for this behavior is known, it appears possible that stimulation of animal growth by lyxoflavin may represent an instance of this same phenomenon.

Procedures. a. *Bacterial experiments.* The assay medium used with *L. casei* 7469 was that described by Rabinowitz *et al.*(12) modified by omission of riboflavin and additon of 80 γ of pyridoxine hydrochloride and 0.1 mg each of adenine, guanine, and uracil for each 10 ml. For *L. lactis* 8000 this basal medium was supplemented with 5 mg of sodium ethyl oxaloacetate, 10 mg of polyoxyethylene sorbitan monopalmitate (Tween 40), 0.1 mg of sodium oleate, 10 γ of calcium pyridoxal phosphate, 2.5 γ of KCN, 0.04 γ of vit. B_{12}, and 0.025 γ of pantethine per 10 ml. Inoculum cells were grown for 18-24 hours in the appropriate basal medium supplemented with 1 γ of riboflavin per 10 ml, centrifuged, resuspended in sterile 0.9% NaCl solution, and diluted to a light transmission of 83% (Evelyn colorimeter, 660 mμ filter). One drop of this suspension was used for each experimental culture of 10 ml. Assays were incubated at 37°C; growth was determined turbidimetrically at appropriate times indicated with individual experiments. b. *Chick experiments.* Procedures were similar to those described previously(4). Day-old chicks (NH x SCWL) were separated into groups of 10 to 15 birds. They were maintained in electrically heated batteries and fed the rations and water *ad lib.* The basal ration was as follows, in g/kg: sucrose 630, alpha protein (Glidden) 250, salts V 60, soybean oil 45, feeding oil (300D-1500A) 5, DL-methionine 6, and glycine 3. Vitamin supplements in mg/kg were: vit. B_{12} 0.02, biotin 0.2, menadione 0.5, folacin 4, pyridoxine hydrochloride 4, calcium pantothenate 20, α-tocopherol acetate 3, niacin 50, p-aminobenzoic acid 100, inositol 1000, choline chloride 2000, and aureomycin 20. Thiamine was supplied by injection thrice weekly at levels of 0.5 mg per chick per injection the first week, and 1.0 mg during the second week. The experimental period was 15 days. Riboflavin and its analogs were fed at levels noted in the individual experiments.

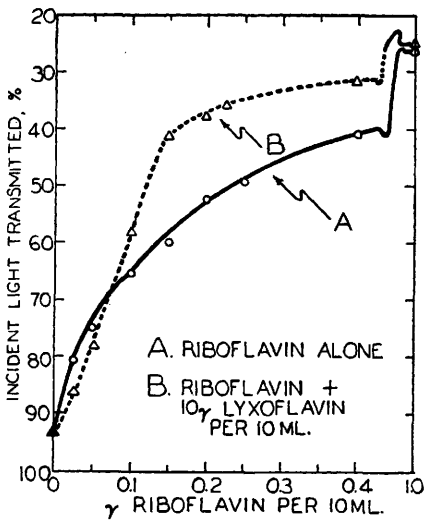

FIG. 1. Effect of a constant low level of L-lyxoflavin on response of *L. casei* to riboflavin. Incubated 17 hr.

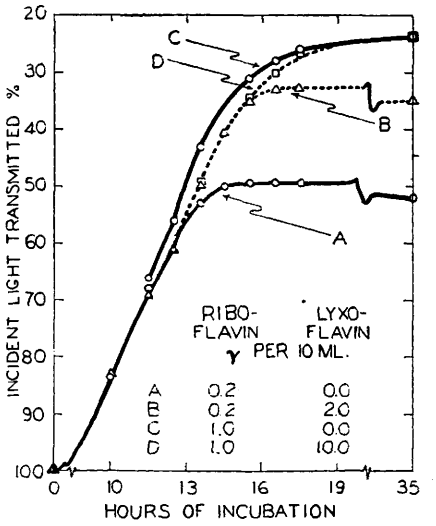

FIG. 2. Effect of riboflavin or riboflavin plus L-lyxoflavin on rate and extent of growth of *L. casei*.

Results. a. *Bacterial experiments.* The effect of a constant low level of L-lyxoflavin on the response of *L. casei* to riboflavin is shown in Fig. 1. At ratios of lyxoflavin to riboflavin above 100 to 1, inhibition of the response to riboflavin occurs; at ratios lower than this, stimulation of the response is ob-

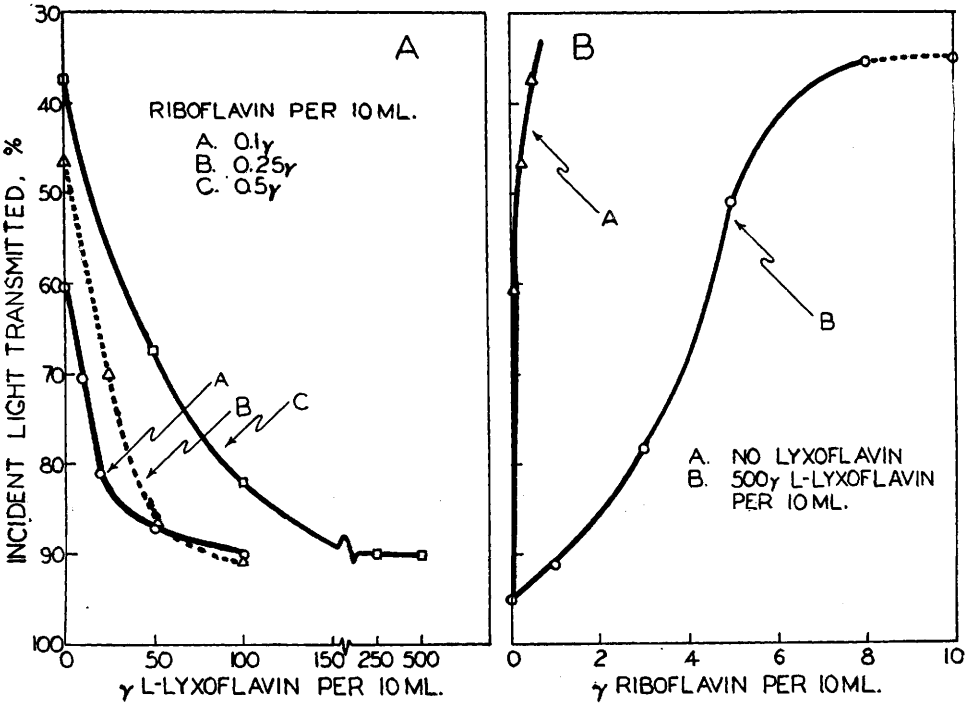

FIG. 3. Inhibition of response of *L. casei* to riboflavin by high levels of L-lyxoflavin. Incubated 17 hr.

served. With sufficient riboflavin, growth reaches the same maximum levels achieved with riboflavin plus lyxoflavin. In the absence of riboflavin, lyxoflavin permits no growth of *L. casei* (Fig. 1).

Data of Fig. 2 show that these levels of lyxoflavin act solely to increase the *amount* of growth when riboflavin is limiting; there is no stimulation of the rate of growth.

That inhibition by high levels of lyxoflavin is competitively related to the level of riboflavin present is evident from Fig. 3. Growth with 0.05 γ of riboflavin gives a light transmission of 70. When the ratio of lyxoflavin to riboflavin required to reduce growth to this level is calculated from the 4 curves of Fig. 3, values of 190, 125, 123, and 133 are obtained.‡ Very high levels of lyxoflavin are non-toxic if sufficient riboflavin is present (Fig. 3B).

‡ The ratios, R, were calculated according to the expression $R = \frac{C_L}{C_R - 0.05}$, where C_L is the amount of lyxoflavin (in γ) and C_R that of riboflavin present at growth equivalent to 70% light transmission.

The results shown in Fig. 4 emphasize the pronounced growth stimulation produced by subinhibitory amounts of L-lyxoflavin when riboflavin is limiting. D-galactoflavin behaves in the same way, but is a less potent inhibitor. Separate tests showed that inhibition by galactoflavin, like that by lyxoflavin, is competitively related to the concentration of riboflavin. The inhibitory action of this compound for *L. casei* correlates with its action in rats(13); growth stimulation in animals by subinhibitory levels of the compound has not been noted. Isoriboflavin showed the growth stimulation characteristic of the other 2 compounds, but in confirmation of a previous report(14) did not inhibit growth at levels which could be tested. The compound is an antimetabolite of riboflavin in rats(15). Dichlororiboflavin, in contrast to its action in several other bacteria(16), was largely inert toward *L. casei*.

In contrast to a report of Shorb(9), no essential change in these results was observed when the riboflavin-free medium of Snell and Strong was substituted for the more nearly

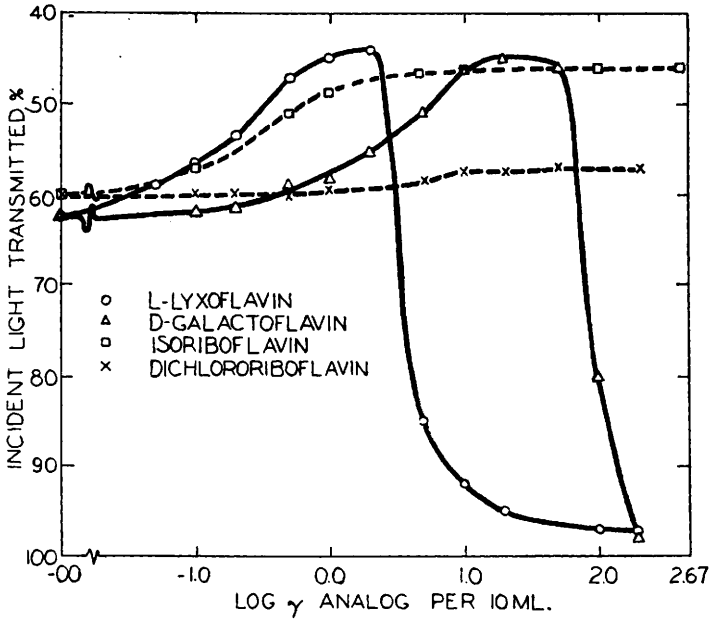

FIG. 4. Comparative effects of several analogs of riboflavin on growth of *L. casei*. Each culture tube was supplemented with 0.1 γ of riboflavin per 10 ml. None of the analogs permits growth in the absence of riboflavin.

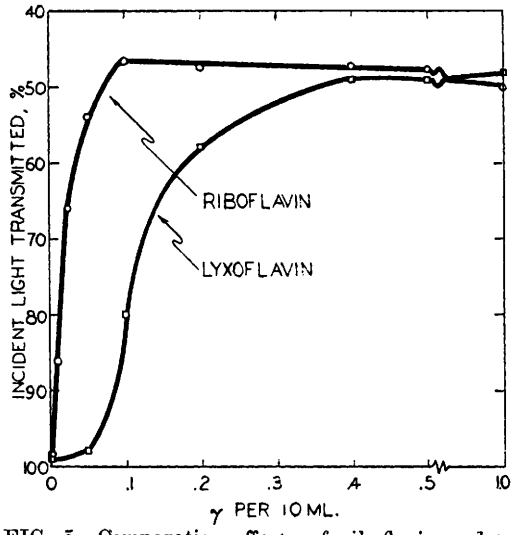

FIG. 5. Comparative effects of riboflavin and L-lyxoflavin on growth of *L. lactis*. Incubated 38 hr.

synthetic medium used in most of the experiments.

L. lactis behaves differently than *L. casei*. As first reported by Shorb(9), L-lyxoflavin supports as heavy growth of the former organism as does riboflavin, but is only one-tenth to one-third as active on a molar basis (Fig. 5). Growth of *L. lactis* with L-lyxoflavin as the sole supplement to the riboflavin-free medium continued without diminution through 7 consecutive transfers; there is thus no doubt of its ability to support growth of this organism in the absence of added riboflavin. Galactoflavin and isoriboflavin did not permit growth of *L. lactis* in the absence of riboflavin.

To determine whether lyxoflavin was used *per se* by *L. lactis*, or was converted to riboflavin, cells of this organism grown with riboflavin or lyxoflavin were harvested, washed once with water, then autoclaved for 20 minutes at 15 lb with 0.1 N HCl to release bound flavins. The extracts were then assayed in parallel with *L. casei* and *L. lactis*, against a riboflavin standard, and in the basal medium for *L. lactis*. Results (Table I) show that cells grown with riboflavin contained the same amount of flavin, within experimental error, by both assay methods. Cells grown with lyxoflavin contained no riboflavin (*L. casei* assay), but did contain lyxoflavin. These data and those of Fig. 5 indicate conclusively that lyxoflavin replaces riboflavin in the metabolism of *L. lactis*, presumably by incorporation into functional lyxoflavin coenzymes

TABLE I. Flavin Content of *L. lactis* Cells Grown with Riboflavin or Lyxoflavin.

Cells grown with	Flavin content,* γ/mg of cells, determined with *L. casei*†	*L. lactis*‡
Riboflavin (.01 γ/ml)	.073	.085
L-lyxoflavin (.03 γ/ml)	.00	.076§

* Riboflavin as standard.
† Specific for riboflavin.
‡ Responds to riboflavin and to lyxoflavin, see text.
§ Since lyxoflavin is only about one-third as active as riboflavin in promoting growth, this would correspond to a lyxoflavin content of approximately 0.23 γ/mg of cells.

TABLE II. Comparative Flavin Content of Cells of *L. casei* Grown with Riboflavin Alone vs. Riboflavin plus Stimulatory Levels of L-Lyxoflavin.

Cells grown with: Riboflavin, γ/250 ml	L-lyxoflavin, γ/250 ml	Yield of dry cells, mg	Total flavin content by *L. lactis* assay*: γ/mg of cells	Total γ
2.5	0	27.8	.033	.92
2.5	25	48	.022	1.03
2.5	250	37	.017	.64
25.0	0	84	.17	14.2

* Assayed against a riboflavin standard and expressed as riboflavin. Similar results, not shown, were obtained with *L. casei*, which does not respond to lyxoflavin.

analogous to those normally formed from riboflavin.

A similar experiment should reveal whether the sparing effect of lyxoflavin on the riboflavin requirement of *L. casei* results from utilization of lyxoflavin for some but not all of the purposes normally served by riboflavin. Accordingly cells of *L. casei* were grown with a suboptimal level of riboflavin, or with this level plus a stimulatory amount of lyxoflavin, and assayed for lyxoflavin plus riboflavin with *L. lactis*. Results (Table II) showed that the 48 mg of cells obtained from a medium containing suboptimal riboflavin plus lyxoflavin contained no more total flavin than the 28 mg of cells obtained with riboflavin alone. There is thus no evidence for the incorporaiton of lyxoflavin into these cells, a conclusion borne out by parallel assays conducted with *L. casei*. The concentration of riboflavin per mg of cells is decreased by culture in the presence of lyxoflavin; *i.e.*, the incorporation of riboflavin appears to be decreased by concentrations of lyxoflavin that are stimulatory. Increasing levels of lyxoflavin progressively decrease the amount of riboflavin incorporated in the cells; presumably growth inhibition results when this incorporation is decreased below a necessary minimum value. Thus lyxoflavin appears to "spare" riboflavin in *L. casei* not by functioning in its stead, but rather by causing (by means still unknown) a more efficient utilization of the limited amounts of riboflavin available. That riboflavin may be deposited in cells at concentrations far higher than are required for growth is evident from the high level present in those cells grown with an excess of this vitamin (Table II).

When this same differential assay procedure was applied to heart, brain, liver, kidney and leg muscle of adult rats grown on a stock diet based on crude natural foodstuffs (Purina Laboratory Chow), the flavin content was the same within experimental error whether *L. casei* or *L. lactis* was the assay organism. This is strong evidence against the natural occurrence of lyxoflavin.

b. *Chick experiments.* Results of feeding L-lyxoflavin and riboflavin at several different levels are shown in Fig. 6. Growth stimulation is consistently observed when ratios of lyxoflavin to riboflavin are 10:1 or below. At considerably higher levels lyxoflavin inhibits growth; the amount necessary for inhibition increases as the amount of riboflavin in the ration is increased. The relationship thus appears to be of the competitive type demonstrated earlier in *L. casei*. Cooperman *et al.* (3) suggested that further additions of riboflavin, above 6 mg/kg, stimulated an increase in growth similar to that elicited by lyxoflavin. Under our conditions, 3 mg of riboflavin per kg promotes the maximum growth obtainable with riboflavin alone. Although, on a percentage basis, lyxoflavin increases growth somewhat more at suboptimal levels of riboflavin, growth at superoptimal levels of riboflavin is also increased by additions of lyxoflavin (Table III). It is unlikely, therefore, that lyxoflavin acts *solely* by "sparing" riboflavin in the chick. These data suggest that the amount of lyxoflavin required to stimulate growth, like that required to inhibit

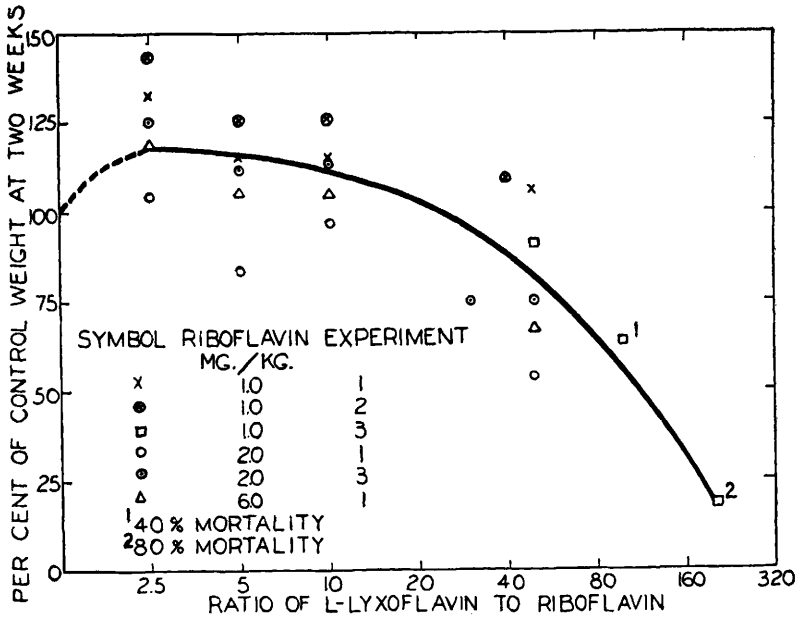

FIG. 6. Effect of lyxoflavin on growth of chicks fed various levels of riboflavin. The curve was fitted by the method of least squares, $Y = 119 - 0.824X + 0.00164X^2$.

growth, increases as the riboflavin content of the ration is increased, *i.e.*, maximum growth increases are obtained in all cases at a ratio of 2.5:1. However, since lower levels of lyxoflavin have not been adequately tested, data on this point are inconclusive.

With the possible exception of D-lyxoflavin, the other analogs of riboflavin were neither stimulatory nor inhibitory for chicks when fed with the maintenance level (1 mg/kg) of riboflavin (Table IV). The small amounts of these chemicals available made trials at higher levels impossible. From these results, galactoflavin would appear less potent as an antagonist for riboflavin in chicks than in rats.

TABLE III. Effect of Lyxoflavin on Chick Growth at Superoptimal Levels of Riboflavin. Gain in 14 days, g.

	mg riboflavin/kg ration				
	0	3	6	12	24
No lyxoflavin	5	72	71	73	71
L-lyxoflavin: riboflavin (2.5:1)	—	—	72	80	80

TABLE IV. Comparative Effects of Several Riboflavin Analogs on Chick Growth.* Avg wt of chicks at 15 days.

Analog fed	mg analog/kg ration					
	0	2.5	5.0	10	20	40
L-lyxoflavin	60	70	66	66		62
D-lyxoflavin	60	66	64	62	57	55
D-araboflavin	60	61	63	55	57	57
D-galactoflavin	60	60	60	60	58	61

* 1 mg riboflavin/kg of ration.

Discussion. The demonstration that lyxoflavin replaces riboflavin completely in the nutrition of *L. lactis*, and functions in this organism without prior conversion to riboflavin adds another instance to several of this type already known. Oxybiotin, although frequently less active quantitatively, effectively replaces biotin in the nutrition of many microorganisms and animals, and is not converted to biotin(11,17,18). No cases of inhibition of growth by oxybiotin are known. A closer analogy is provided by Rb^+, which replaces K^+ completely for *Streptococcus faecalis*, but not for *Leuconostoc mesenteroides*. At low levels it greatly reduces the requirement of the latter organism for K^+, apparently serving in its stead in some but not all metabolic reactions. At high levels, Rb^+ inhibits growth by competition with K^+ at sites where the latter is essential for growth(19,20).

Another probable instance of this same phenomenon is provided by analogs of riboflavin appropriately substituted in the 6- and

7- positions of the alloxazine nucleus, some of which have activity for microorganisms(8,11) and partial activity in animals(11). The most thoroughly examined of these is "diethylriboflavin"(21), which replaces riboflavin efficiently and completely for *L. casei* and *Bacillus lactis acidi,* and which stimulates growth of rats on riboflavin-deficient diets, but does not prevent their death when deprivation of riboflavin is continued. Although the growth effect of the analog in the latter instance was ascribed(2) to a mobilizing effect on riboflavin rather than to utilization *per se,* the possibility that it replaced riboflavin for certain functions but not for others was not eliminated. It appears unlikely from chemical considerations that these analogs are transformed to riboflavin by organisms that utilize them, although the experiments necessary to prove this have not been carried out.

An attractive postulate to explain the effects of lyxoflavin on growth of *L. casei* would be to assume that this compound could fill some but not all of the necessary functions of riboflavin. At subinhibitory levels it would then stimulate growth when riboflavin was limiting; at higher concentrations, by interfering with essential functions of riboflavin, it would inhibit growth. Evidence of Table II indicates, however, that lyxoflavin does not partially substitute for riboflavin, but rather "spares" riboflavin by interfering with its deposition at non-essential sites within the cell. At higher, growth-inhibitory concentrations, deposition of riboflavin at essential sites also must be prevented. The important point is that growth-stimulation at low concentrations of lyxoflavin thus results from operation of the same mechanism that causes growth inhibition at high concentrations. In the same way, other riboflavin antagonists might show a similar stimulating effect for this organism at sub-inhibitory concentrations; this expectation is fulfilled by D-galactoflavin. Growth stimulation without subsequent growth inhibition by isoriboflavin is not contradictory to this view, since an essential feature of the hypothesis is that deposition of riboflavin at non-essential sites is more readily inhibited than that at essential sites, *i.e.,* the cell receptors at non-essential sites have a lower "affinity" for riboflavin or its coenzyme forms than do cell-receptors at the essential sites.

In theory, riboflavin antagonists might also exist with specificity such that riboflavin would be prevented from combination with essential cell-receptors at concentrations as low or lower than that required to prevent combination with non-essential receptors. In such case, stimulation of growth by subinhibitory concentrations of the antagonist would not be observed. None of the riboflavin antagonists tested acted in this manner.

From Fig. 1 and 4 it is apparent that under conditions where it is maximally effective, lyxoflavin possesses only a small fraction of the activity of riboflavin in promoting growth of *L. casei.* Chemically, however, lyxoflavin and riboflavin are very similar. Since results of microbiological assays for riboflavin employing *L. casei* have been checked extensively against fluorometric assay with excellent agreement(22,23), and since such agreement would be impossible if lyxoflavin were widely distributed in nature in amounts comparable to riboflavin, it must be concluded that lyxoflavin, if it occurs naturally at all, must be present in very small amounts relative to riboflavin. This conclusion is supported by the agreement between results of assay of natural materials with *L. casei* and *L. lactis.* If lyxoflavin occurred naturally, the latter organism should give higher assay values; in trials on rat tissues it did not.

The growth responses to lyxoflavin observed in chicks on diets marginal in riboflavin may be due in part to operation of factors similar to those discussed for bacteria. In contrast to the results in *L. casei,* however, such growth responses, although less pronounced, are observed in the presence of an excess of riboflavin, provided sufficient lyxoflavin is fed. Such growth responses cannot be explained on the basis of present knowledge. They may result from an antibiotic-like action of lyxoflavin, or be akin to the unexplained growth-stimulating effects observed in microorganisms when a variety of toxic compounds are present in subinhibitory concentrations. The large amounts of lyxoflavin required to obtain such responses, coupled with the evidence against the natural

occurrence of this compound, make it highly improbable that the compound functions as a true vitamin.

Summary. 1. L-lyxoflavin, D-galactoflavin and isoriboflavin markedly increase the growth response of *Lactobacillus casei* to suboptimal amounts of riboflavin, but do not promote growth in the absence of this vitamin. Appropriate differential assays indicated that lyxoflavin was not deposited in cells of *L. casei* under conditions where it stimulated growth, and actually decreased the concentration of riboflavin deposited in such cells. Thus it does not serve as a partial substitute for riboflavin in *L. casei*, but appears to enhance the efficiency with which limited supplies of riboflavin are utilized. When riboflavin is present in excess, lyxoflavin does not increase the cell yield or the growth-rate. At higher concentrations, lyxoflavin and galactoflavin inhibit growth by acting as competitive antagonists of riboflavin. Dichlororiboflavin and isoriboflavin are ineffective as inhibitors of this organism. 2. L-lyxoflavin promotes maximum growth of *Lactobacillus lactis* in the absence of riboflavin, and such growth continues indefinitely upon subculture. Differential assay with *L. casei* and *L. lactis* showed that cells of the latter organism grown with lyxoflavin contain no riboflavin, but do contain lyxoflavin; *i.e.*, in this organism lyxoflavin can fill the essential metabolic roles normally played by riboflavin. Similar assays of tissues from rats grown on natural rations indicate that lyxoflavin does not occur naturally in significant quantities. 3. In chicks, lyxoflavin stimulated growth when fed at levels 2.5 to 10 times the amount of riboflavin in the diet. At ratios of lyxoflavin to riboflavin higher than 40 to 1, lyxoflavin inhibited growth. Amounts of lyxoflavin that inhibited growth on diets of low riboflavin content were non-toxic when the amount of riboflavin was increased. 4. Although the growth-responses observed in chicks when L-lyxoflavin is added to diets low in riboflavin may result in part from a sparing action similar to that found with *L. casei*, such a sparing action does not explain the growth effects of the analog observed in rations of high riboflavin content. Relatively large amounts of lyxoflavin are required for such growth stimulation, and since available evidence indicates that the compound does not occur naturally, it cannot be considered a vitamin.

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