# MICROBIOLOGICAL ASPECTS OF RIBOFLAVIN

## III. Oxidation Studies with Pseudomonas Riboflavina[1]

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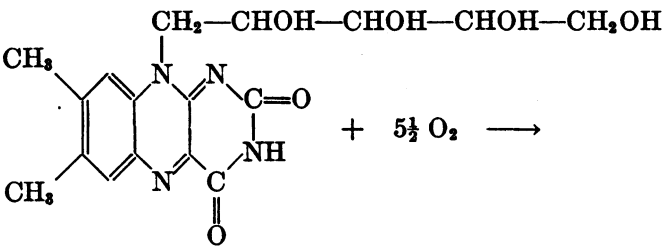
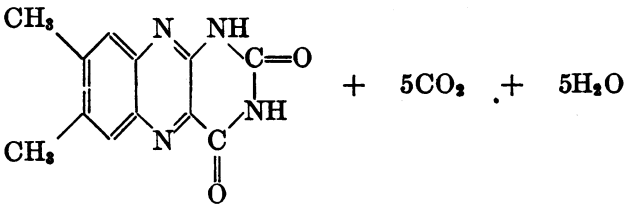
In the previous paper of this series (Foster, 1944) was reported the isolation of a riboflavin-decomposing bacterium, *Pseudomonas riboflavina*, its description and the establishment of the following equation as representing the degradation of riboflavin by this organism.




d-Riboflavin




Lumichrome

Riboflavin is converted stoichiometrically to lumichrome (6,7-dimethyl alloxazine). The reaction is essentially an oxidation of the ribityl side chain of the riboflavin molecule, leaving the ring structure intact. It may be here mentioned that a second organism has been discovered whose distinctive character it is to attack and degrade this ring structure (lumichrome) without, however, being able to attack the parent riboflavin molecule. Description of the organism and details of the lumichrome degradation will be the subject of a later publication. The present paper is a continuation of the biochemical studies on *Pseudomonas riboflavina* begun in Part II of this series.

### EXPERIMENTAL

Except for special details, the methods and apparatus used were essentially those employed earlier; their repetition here is unnecessary.

[1] This was previously *P. riboflavinus*. The feminine form, as above, is correct.

*Oxidation of d-ribose*

Consideration of the structures of the ribityl side chain of riboflavin and of d-ribose leads to the supposition that since *P. riboflavina* attacks the former, it would, consequently, attack the latter; and, moreover, because of the close similarity between the two structures, presumably one and the same enzyme system would be responsible in both cases. Preliminary experiments demonstrated the correctness of the first of these suppositions: d-ribose is oxidized by resting cell suspensions of *P. riboflavina*. All attempts to provide experimental substantiation for the second hypothesis have, however, failed, and, seemingly,

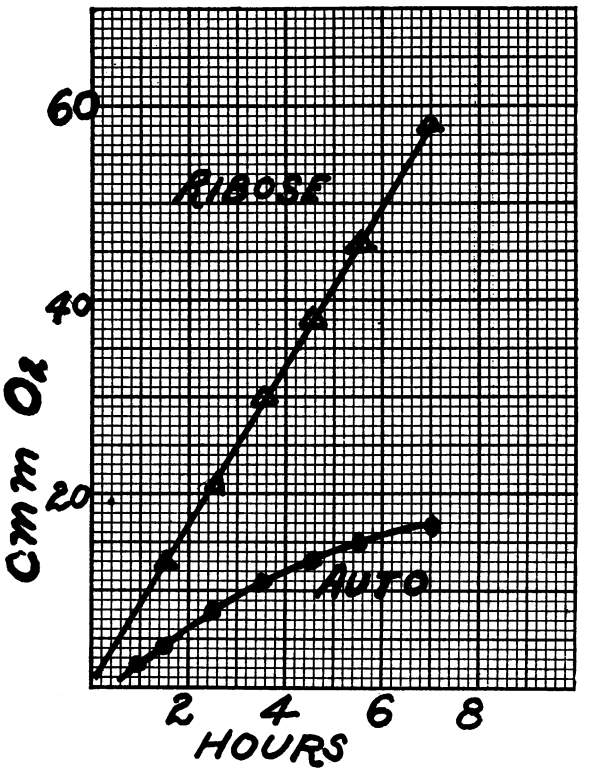



FIG. 1. OXIDATION OF RIBOSE BY PSEUDOMONAS RIBOFLAVINA

the only interpretation is that different enzyme systems attack the d-ribityl group and d-ribose. A summary of pertinent details and data follows: fig. 1 shows curves for ribose oxidation in one experiment (A). Another experiment, (B), provided quantitative data for a respiratory balance and also evaluation of the degree of oxidative assimilation (see below).

In order to make these calculations it was necessary to decide whether the value for endogenous respiration is to be deducted from that for the sugar, i.e., does endogenous respiration proceed simultaneously with oxidation of the substrate, or does it, in the presence of the readily utilizable energy source,

become suppressed? Unless this can be settled, the validity of quantitative interpretation is open to serious question. Examination of the curves in the experiment of fig. 1 permits the conclusion that the presence of ribose inhibits endogenous respiration of *P. riboflavina*, in this respect being similar to the instances reported by Barker (1936), Doudoroff (1940) and Van Niel and Cohen (1942), for other readily oxidizable substrates.

It is evident that over the duration of the experiment the rate of endogenous respiration clearly fell off with time, whereas the rates when (unconsumed) ribose was present were maintained constant throughout. If the curves for ribose actually represented total and simultaneous oxygen uptake for ribose and endogenous respiration, the shape of the curve would show a falling off in rate similar to the endogenous curve, since the sum of a constant rate plus a changing rate is a changing rate. The curves in fig. 1 clearly show this not to be true; hence, it may be assumed with considerable assurance that in these experiments the endogenous respiration was substantially inhibited during ribose oxidation.

There is, however, another important contention deducible from the foregoing line of reasoning and which must be eliminated before the argument advanced is wholly acceptable. If the ribose curve represented the maximum capacity for oxygen uptake by the bacteria, i.e., saturation of the oxidation mechanisms, it might be that a concomitant oxidation of ribose and endogenous material could keep the system fully saturated. As the endogenous substrate becomes exhausted, this deficiency is compensated by oxidation of proportionally more riboflavin, up to the limit of the capacity of the cells. Thus, the ribose curve would in reality be inclusive of endogenous respiration. There is, however, ample evidence that the total oxidative capacity of the cells of *P. riboflavina* far exceeds the oxidation rate of ribose, and that the rates obtained with ribose and with riboflavin are limited only by the ability of the cells to activate separately these specific substrates. Thus, it seems that endogenous respiration and ribose oxidation, were they occurring simultaneously, would have been additive, manifesting a changing rate curve as mentioned above, instead of the straight line actually observed. In support of the statement about the high oxidative capacity of *P. riboflavina* are the following experimentally observed facts: The rate of oxygen uptake for riboflavin definitely exceeded that for ribose (fig. 3); oxygen uptake in the simultaneous presence of ribose and riboflavin was additive (fig. 3); oxygen uptake rates with other substrates (notably, glucose and sodium acetate) have been observed to be substantially greater than for riboflavin (or ribose), the ratios varying rather widely from experiment to experiment and depending apparently on the condition of the cells.

In the experiment B, 0.00055 millimol of ribose was consumed, representing a theoretical oxygen uptake and $CO_2$ production of 130 $mm^3$ each, assuming oxidation according to the following reaction:

$$C_5H_{10}O_5 + 5O_2 \rightarrow 5CO_2 + 5H_2O$$

The actual values found experimentally were 59 $mm^3$ $O_2$ and 51 $mm^3$ $CO_2$, 45 and 40 per cent of the theoretical. In line with the foregoing discussion endog-

enous respiration is assumed to have been repressed. Analysis for initial and final bound $CO_2$ in another experiment, with air containing 5% $CO_2$ in equilib-

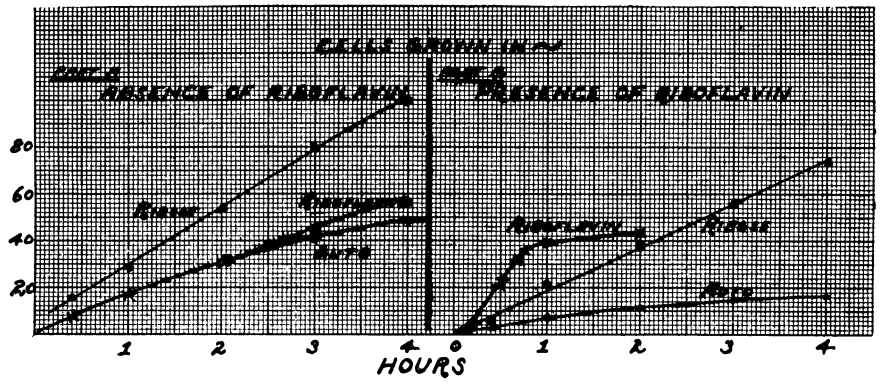



FIG. 2. OXIDATION OF RIBOFLAVIN AND OF RIBOSE BY CELL SUSPENSIONS OF PSEUDOMONAS RIBOFLAVINA GROWN IN THE ABSENCE AND IN THE PRESENCE OF RIBOFLAVIN

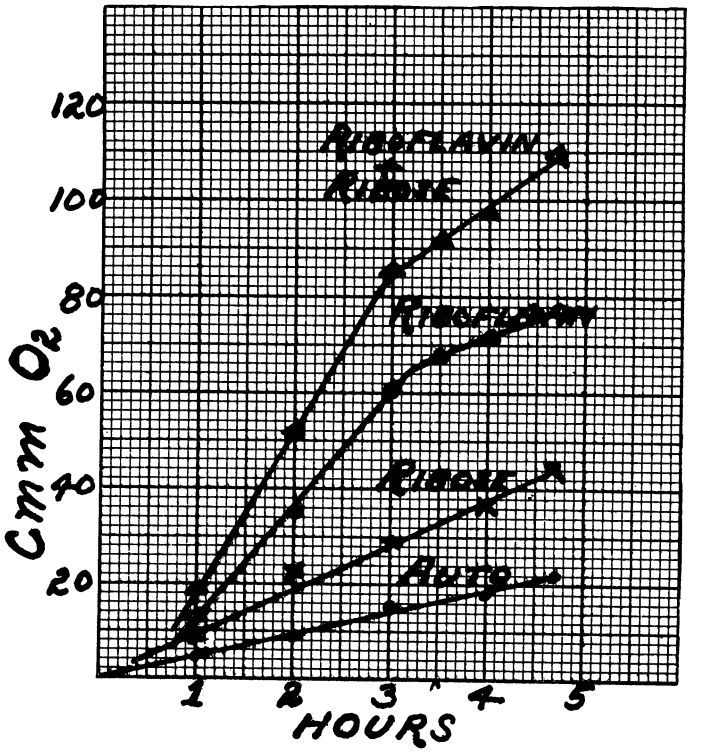



FIG. 3. SIMULTANEOUS OXIDATION OF RIBOFLAVIN AND RIBOSE

rium with 0.05% $NaHCO_3$ in the liquid phase, gave no indication of acid formation. Almost certainly the fate of the missing $O_2$ and $CO_2$ can be assigned to the

now well-known phenomenon of oxidative assimilation (see van Niel, 1940). The indications are that of each mole of ribose decomposed, two atoms of carbon are oxidized to $CO_2$ and the other three are converted into cell substance or reserve materials. The increased $O_2$ uptake observed during ribose oxidation in the presence of 2,4-dinitrophenol (see Clifton, 1937; Clifton and Logan, 1939; Pickett and Clifton, 1943) further supports this idea.

*Ribose and ribityl oxidation*

Two lines of evidence that the ribityl and ribose substrates are not attacked with identical enzyme systems are given in figs. 2 and 3. In part B of fig. 2 are depicted oxygen consumption rates by cells of *P. riboflavina* harvested from 1% yeast-extract broth containing 0.05% added riboflavin. It is plain that only when the cells were grown in the presence of riboflavin (ribityl-) was it oxidized immediately and at a significant rate. The adaptive nature of the enzyme(s) concerned with this process has been established in the previous paper of this series (Foster, 1944). Ribose, however, was oxidized immediately and at a significant rate, quite independent of the presence or absence of added riboflavin during the growth of the cells. Thus, the enzyme(s) oxidizing ribose is constitutive (Karström, 1930, 1937); it must, therefore, be distinct from the adaptive ribityl-oxidizing enzyme(s). Traces of ribose admittedly present in yeast extract conceivably could have been sufficient to allow for complete adaptation of the cells to ribose in the absence of riboflavin prior to the harvesting. But this should also apply to traces of riboflavin, which, however, as known from above, were insufficient to cause adaptation. Furthermore, the fact does remain that cells not capable of oxidizing ribityl could oxidize ribose.

The second line of evidence is given in fig. 3. Ribose and riboflavin were furnished as substrates separately and together to fully adapted cells. When present together both were oxidized simultaneously and the rates were almost quantitatively additive. At the 3 hour point:

| | |
|---|---|
| Riboflavin | = 61 $mm^3$ $O_2$ |
| Ribose | = 29 |
| Combined, theory | 90 |
| Combined, found | 86 |

If one and the same enzyme system were limiting in the oxidation of the two compounds, there would be a competition by the 2 molecular species for that one enzyme, but total rate could not exceed the larger of the two separately; this is because the enzyme was acting at its maximum rate under the conditions where concentration of substrate was not the limiting factor (straight line portion of the curves; also other experiments). Moreover, if riboflavin and ribose were being oxidized indiscriminately by one system, the break in the curve indicating complete disappearance of the riboflavin should have occurred sometime after it broke in the absence of ribose, for a good portion of the oxygen value at the break in the former case came from ribose oxidation, and the time of disappearance of

the riboflavin would therefore have been greatly prolonged. Actually, the breaks indicative of complete disappearance developed almost at the same time, independent of the presence of ribose. After disappearance of the riboflavin in the combined treatments, the rate of oxygen uptake continued to be that characteristic for ribose, since plenty of this remained.

d-Arabinose was attacked even more rapidly than ribose, and combination experiments with riboflavin also gave additive results indicative of simultaneous oxidation similar to the case of ribose. The degree of oxidative assimilation was about the same as that for ribose, approximately 40% of the theoretical oxygen being consumed, and indicating assimilation of about 3 carbon atoms per molecule of arabinose oxidized.

TABLE 1

*Oxidation of riboflavin homologues by P. riboflavina*

| COMPOUND | OBSERVED $O_2$ UPTAKE AFTER 90 MINUTES | ACTUAL $O_2$* UPTAKE DUE TO COMPOUND | PER CENT OF RIBOFLAVIN |
|---|---|---|---|
| | *mm*³ | | |
| None (autorespiration) | 8 | — | — |
| I 7-methyl-9-(1-1′-arabityl)-isoalloxazine | 40 | 32 | 50.9 |
| II 7-methyl-9-(d-1′-arabityl)-isoalloxazine | 18 | 10 | 15.9 |
| III 6,7-dimethyl-9-(d-1′-arabityl)-isoalloxazine | 20 | 12 | 19.1 |
| IV 7-methyl-9-(d-1′-ribityl)-isoalloxazine | 31 | 23 | 36.5 |
| V 5,6-dimethyl-9-(d-1′-ribityl)-isoalloxazine (iso-d-riboflavin) | 8 | — | 0 |
| 6,7-dimethyl-9-(d-1′-ribityl)-isoalloxazine (d-riboflavin) | 71 | 63 | 100 |

* Autorespiration deducted from observed uptake. It is likely that endogenous respiration was not entirely suppressed in the case of the more slowly attacked substances. Either way does not alter significantly the interpretation of the results.

It is worthy of record that riboflavin was not attacked by *P. riboflavina* under anaerobic conditions, or if it was the rate was extremely low compared to that under aerobic conditions.

*Oxidation of riboflavin homologues.* The compounds[2] listed in table 1 were compared for their rates of attack by *P. riboflavina* as judged by oxygen consumption in Warburg respirometers.

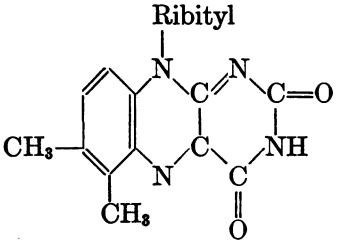
The results show that removal of the methyl group in the 6 position caused a two-thirds reduction in rate of oxidation of riboflavin. All the arabityl compounds are attacked, the 7-methyl-1-arabityl compound even more rapidly than the 7-methyl-d-ribityl compound. Whereas the 6,7-dimethyl ribityl-compound (d-riboflavin) was oxidized at a rate 3 times its 7-methyl ribityl-homologue, there was little difference between the 6,7-dimethyl and the 7-methyl-d-arabityl-compounds. However, the 7-methyl-1-arabityl-compound was attacked almost three times faster than its d-arabityl-isomer. Snell and Strong (1939) have tested a number of synthetic flavins for their vitamin activity with

[2] Supplied through the courtesy of Dr. Max Tishler.

*Lactobacillus casei* and *Streptococcus lactis*. Two from the present list were included, III and IV. IV could support growth of these lactic acid bacteria, but III possessed detectable activity only in the presence of suboptimal amounts of riboflavin. Kuhn and Rudy (1936) found III to be inactive in their enzymatic dehydrogenation test and it had no vitamin action in rats. IV has been found active in rats (Karrer and Quibell, 1936; Kuhn, Vetter and Rzeppa, 1937; Karrer, von Euler, Malmberg and Schöpp, 1935).

*Iso-riboflavin (V)*[3]

This compound has the following structure:

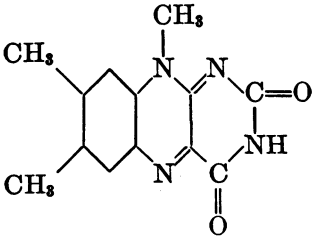



5,6-dimethyl-9-(d-1′-ribityl)-isoalloxazine.

Iso-riboflavin was not attacked by *P. riboflavina*. Iso-riboflavin tetraacetate did, however, lead to a substantial rate of oxygen uptake which undoubtedly can be ascribed to hydrolysis of the compound and subsequent oxidation of the liberated acetate. *P. riboflavina* rapidly oxidizes acetic acid (Na salt). Iso-riboflavin and tetraacetyl iso-riboflavin were tested in growth experiments with *L. casei* for riboflavin activity, and both were found to have negligible activity (less than 0.5 per cent). The free compound was also tested for its ability to compete with riboflavin in the metabolism of *L. casei* since its inactivity and close chemical similarity might enable it to block riboflavin from the enzyme carrier protein. At 10, 100, 10,000 and 100,000 times the concentration of riboflavin necessary for maximum growth (0.15 $\gamma$ per 10 ml) there was no sign of competitive inhibition detectable by reduction in titre of the lactic acid formed during growth.

*Lumiflavin (6,7,9-trimethyl-isoalloxazine)*

This degradation product of riboflavin, first isolated by Warburg and Christian (1933), has the following structure (Kuhn and Rudy, 1934):




[3] I am especially grateful to Drs. Karl Pfister and Max Tishler for making liberal amounts of this compound available to me. The synthesis of this compound will be described elsewhere.

An aqueous solution of lumiflavin was prepared by alkaline photolysis of pure riboflavin as described by Warburg and Christian (1933) except that the benzene purification step prior to the chloroform extraction was omitted. Two ml of the final aqueous solution (containing 150 $\gamma$ by fluorimetric analysis) was used as substrate for *P. riboflavina* in respirometer experiments. This solution was shown to be free of riboflavin by *L. casei* growth tests. Three separate oxidation experiments were run, each using an equivalent amount of riboflavin as a comparison for the lumiflavin. At the conclusion of the experiment, fluorimetric analyses were made for residual flavin. Table 2 contains the results of one experiment, typical of all three. The oxygen uptake in the lumiflavin treatment exceeded the autorespiration, but there is good likelihood that this extra $O_2$ consumption can be ascribed to the presence of oxidizable impurities in the lumiflavin preparation, these doubtless being derived during photolysis from the carbohydrate chain of riboflavin. This is based on the fact that very little lumiflavin, if any significant amount at all, disappeared (see flavin figures in table 2) even after 5½ hours, which was almost four times as long as the time for which the oxygen levels are reported. The comparison is made at the 90-

TABLE 2

*Oxidation of lumiflavin by P. riboflavina*

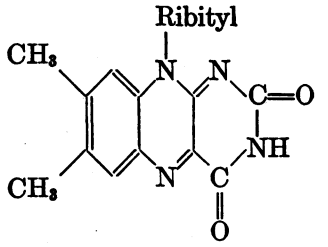
| | $O_2$ UPTAKE AT 90 MIN. | FLAVIN LEFT AFTER 5½ HRS |
|---|---|---|
| | | $\gamma$ |
| Riboflavin (147 $\gamma$) | 34 | 9.6 |
| Lumiflavin (150$\gamma$) | 19 | 145.0 |
| Autorespiration | 10 | — |

minute mark because breaks in the oxygen curves indicated that riboflavin was entirely consumed at that time. There was no break in the lumiflavin curve.

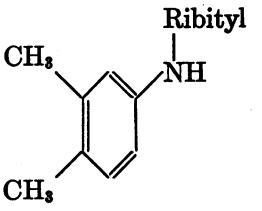
At the time residual lumiflavin was measured, this treatment showed 52 $mm^3$ $O_2$ consumed as compared to 27 for autorespiration. Even assuming that endogenous respiration occurred simultaneously and is therefore deductible (not a remote possibility in view of the low rate of $O_2$ uptake with lumiflavin), this amount of oxygen (25 $mm^3$) is far in excess of that theoretically possible from the few micrograms of lumiflavin reported as disappearing. The reported difference in the lumiflavin figures before and after the action of the bacterial suspension may well be within the limits of accuracy of the measurement under these conditions.

It may be concluded, therefore, that (1) the oxygen uptake observed with lumiflavin was due to oxidation of associating impurities (note that the benzene purification step was not applied to the lumiflavin preparation used); (2) lumiflavin is not attacked by *P. riboflavina*, or if it is, the rate compared to that of riboflavin is insignificant; and (3) lumiflavin is not an intermediate in the decomposition of riboflavin by *P. riboflavina*.

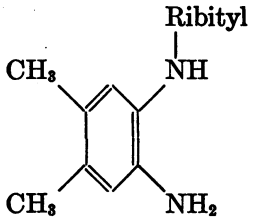
*Other ribityl-compounds.* Those available and tested are listed with structural formulas to indicate their relation to riboflavin.



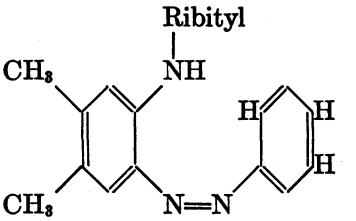
Riboflavin
6,7-dimethyl-9-(d-1′-ribityl)-isoalloxazine



(VI) 3,4-dimethylphenyl-d-1′-ribamine



(VII) 2-amino-4,5-dimethyl-phenyl-d-1′-ribamine



(VIII) 2-phenylazo-4,5-dimethyl-phenyl-d-1′-ribamine

They were chosen particularly to compare the oxidizability of their ribityl groups with that of riboflavin.

Although it was not definitely proved that oxygen uptake with these compounds in the respirometer represented destruction of the ribityl side chain, it is extremely likely that this group would be attacked first. The relative rates

TABLE 3

*Oxidation of ribityl-compounds by P. riboflavina**

| -d-1′-RIBAMINE DERIVATIVE | OBSERVED $O_2$ UPTAKE | $O_2$ UPTAKE DUE TO† COMPOUND | PER CENT OF RIBOFLAVIN RATE |
|---|---|---|---|
| | *mm³* | *mm³* | |
| No substrate (auto) | 6 | — | — |
| Compd. (VI) 3,4-dimethylphenyl | 9 | 3 | 11 |
| Compd. (VII) 2-amino-4,5-dimethylphenyl | 23 | 17 | 63 |
| Compd. (VIII) 2-phenylazo-4,5-dimethylphenyl | 6 | 0 | 0 |
| d-riboflavin | 33 | 27 | — |

* Readings made at 90 minutes at which time all rates were maximum.

† Autorespiration deducted from observed $O_2$ uptake.

at which these compounds were attacked are given in table 3. Compounds I and II were definitely oxidized by *P. riboflavina* but at rates less than that for riboflavin, and the presence of an amino group in the 2-position led to a 5½-fold oxidation rate over compound I. Substitution of the phenylazo group (compound VIII) prevented entirely the oxidation of the ribityl group.

*Competitive inhibition experiments*

The susceptibility to attack of the ribityl groups of intermediates VI and VII and of the homologues I–IV, and especially the fact that their oxidation was slower than that of d-riboflavin, invited experiments to test whether such compounds could compete with the latter for the bacterial enzymes and thereby retard the oxidation rate of riboflavin.

For a preliminary experiment in Warburg respirometers three different possible competitors, IV, VI and VII, were added separately from side cups to cell suspensions oxidizing riboflavin (0.00064 M) at a constant rate, and their effect on rate of $O_2$ uptake thereafter noted. In the remaining three vessels the additions were made in the reverse order. The data follow:

| *Compound and order of addition* | *$O_2$ uptake per 45 min.* |
|---|---|
| Riboflavin alone | 51 mm³* |
| Riboflavin then VI | 50 |
| VI alone | 15 |
| VI then riboflavin | 33 |
| Riboflavin then VII | 43 |
| VII alone | 15 |
| VII then riboflavin | 20 |
| Riboflavin then IV | 47 |
| IV alone | 18 |
| IV then riboflavin | 38 |

* 17 mm³ per 15 min. × 3.

(VI) Addition of this compound (0.0016 M) to the riboflavin-oxidizing suspension had no measurable effect on the rate of $O_2$ uptake one way or the other. However, addition of the riboflavin after the cells had been acting on VI at a low rate definitely increased the rate of $O_2$ uptake till it approached, but did not equal, that of riboflavin oxidation alone.

These data would indicate that riboflavin can displace the previously present VI from the bacterial enzymes, whereas in the reverse order this is not true.

(VII) Secondary addition of riboflavin caused very slight increase in $O_2$ uptake due presumably to effective blocking of the enzymes by the previously added VII. Indeed, addition of VII effectively reduced the oxidation of riboflavin after the latter was well under way.

(IV) Secondary addition of IV reduced slightly the rate of riboflavin oxidation but $O_2$ uptake with IV alone was markedly increased by addition of riboflavin. This again probably indicates a preferential affinity for riboflavin by the enzyme(s).

*Concentration of inhibitor*

2-Amino-4,5-dimethylphenyl-d-1′-ribamine (VII) was tested at the rate of $\frac{1}{10}$, 1, 10 and 100 times the molar concentration of riboflavin oxidized as substrate (121 $\gamma$ = 0.000089 M). Both substances were added simultaneously at the beginning. The ability of this compound to inhibit successfully the oxidation of riboflavin is evident from data in table 4. The values for $O_2$ uptake show

that the inhibitor itself was attacked slowly in the absence of riboflavin, confirming the previous findings. The $\frac{1}{10}$ level of VII was without significant effect on the rate of riboflavin oxidation but the one-molar equivalent was definitely inhibitory. The heightened value (over endogenous) for the ten-molar concentrations (and the one-molar also) doubtless was due to a direct attack of the inhibitor itself, since the (fluorimetric) analysis for riboflavin shows that its consumption was greatly retarded by the presence of the inhibitor. The accelerated $O_2$ uptake with certain intermediate concentrations of VI and VII has been observed in a number of different experiments. However, 100-molar equivalents became more inhibitory, but the clearly significant oxygen uptake occurring in this treatment must nevertheless have been due to oxidation of the poison because, as evidenced from the column (table 4) showing riboflavin consumption, all the riboflavin present initially was shown to be still present at the end of the experiment. This level of VII did, therefore, prevent completely the oxidation

TABLE 4

*Inhibition of riboflavin oxidation by compound VII*

| CONCENTRATION OF VII IN MOLAR EQUIVALENTS OF RIBOFLAVIN PRESENT | 0.000089 M RIBOFLAVIN PRESENT (121 γ) | $O_2$ UPTAKE AFTER FIVE HOURS | | RIBOFLAVIN AFTER 8 HOURS | |
|---|---|---|---|---|---|
| | | Observed | Due to oxidation of substrate† | Left | Total, consumed |
| | | | | γ *per ml* | γ |
| 1 | No | 22 | 7 | 8.6 | — |
| 0 | Yes | 38 | 23 | 1.3 | 116 |
| $\frac{1}{10}$ | Yes | 38 | 23 | 3.3 | 109 |
| 1 | Yes | 25 | 10 | 20.6 | 47 |
| 10 | Yes | 47 | 32 | 27.2 | 23 |
| 100 | Yes | 35 | 20 | 40.0 | 0 |
| 0 | No | 15* | — | | |

* Estimated from theoretical value (60%) oxidative assimilation of 121 γ riboflavin during complete conversion to lumichrome.

† Autorespiration deducted from observed $O_2$ uptake.

of available riboflavin and was itself selectively oxidized. It is also obvious from table 4 that lower concentrations of VII were proportionately inhibitory to riboflavin destruction.

Without presenting the data in detail, it may be stated that almost identical results were obtained in a similar concentration experiment employing as inhibitor of riboflavin oxidation 3,4-dimethylphenyl-d-1′-ribamine (VI). This substance appeared to be slightly less effective in blocking riboflavin consumption.

*Specific competitor or non-specific poison? Bacterial growth experiment*

The experiments just described seem to substantiate the hypothesis expressed earlier, namely, that these ribityl-compounds block riboflavin oxidation by successfully competing with the latter for the active centers on the riboflavin-oxidizing enzymes. The following experiments were designed to test further this idea.

Compound VII was first tried for its ability to displace riboflavin from the active enzymes in *Lactobacillus casei* and to inhibit, thereby, growth of this organism since the vitamin is indispensable for its development. Two lots of the basal medium of Snell and Strong (1939 b) received 0.075 $\gamma$ and 0.15 $\gamma$ riboflavin respectively per tube containing 10 ml. These amounts permit approximately $\frac{1}{2}$ and full growth of *L. casei* in this medium. Each lot of these media now was divided into six portions containing 0, 10, 100, 1000, 10,000 and 100,000 molar equivalents of Seitz-filtered VII based on the riboflavin present. All treatments were set up in six replicates, duplicates of which were titrated after 1, 2 and 3 days' growth of *L. casei*. There was no evidence of riboflavin competition (growth inhibition) as measured by titration of the lactic acid formed, either in rate or in final total, even with 100,000 molar equivalents of VII.

*Failure to reverse with higher concentrations of riboflavin the inhibition of riboflavin oxidation caused by VI*

One of the characteristics of competitive inhibitions by structurally related homologues is that the degree of inhibition caused by any concentration of the inhibitor, can, within solubility limits, be relieved by adjusting to higher levels the concentration of the normally required or active substance (in this case riboflavin). Without here considering the theoretical aspects, this reversibility action seems to be a generally accepted criterion of competitive inhibition. Fig. 4 is an example of the data of several similar experiments designed to overcome with high concentrations of riboflavin the inhibition caused by VI (or VII). The lower concentration of riboflavin (117 $\gamma$ = 0.000086 M) was in the range generally used for oxidation studies in this work and a definite reduction in its rate of oxidation by *P. riboflavina* by five molar equivalents of VI is apparent from the full line curves in fig. 4. This relatively small amount of riboflavin was so quickly oxidized that analysis of the inhibition from the slopes of the curves is open to question. But the rates can easily be computed from the times required for the completion of the oxidation of the available riboflavin in the presence and in the absence, respectively, of the inhibitor as denoted by the breaks in the curves at the points where the riboflavin was exhausted. These times were 35 and 60 minutes respectively, with an inhibition of 58.5 per cent. The inhibition caused by the identical concentration of inhibitor as above, 0.00043 M, only with 1932 $\gamma$ riboflavin as substrate, a 16.5 fold increase over that of the control, can be determined from the slopes of their curves in Fig. 4. At the 90-minute level the respective oxygen uptakes were 46 and 76 $mm^3$ (autorespiration deducted), an inhibition of 60.5 per cent.

Thus, no alleviation of the inhibition was observed by a 16.5 fold increase in riboflavin concentration. Were there an effect, it should have been apparent, for the 0.00043 M level of inhibitor was nearly a border line concentration for inhibiton and even a modest antidote action would be expected to manifest itself. The likelihood that still higher levels of riboflavin would be effective could not be experimentally verified because of solubility limitations, but the experiments reported below belie this possibility.

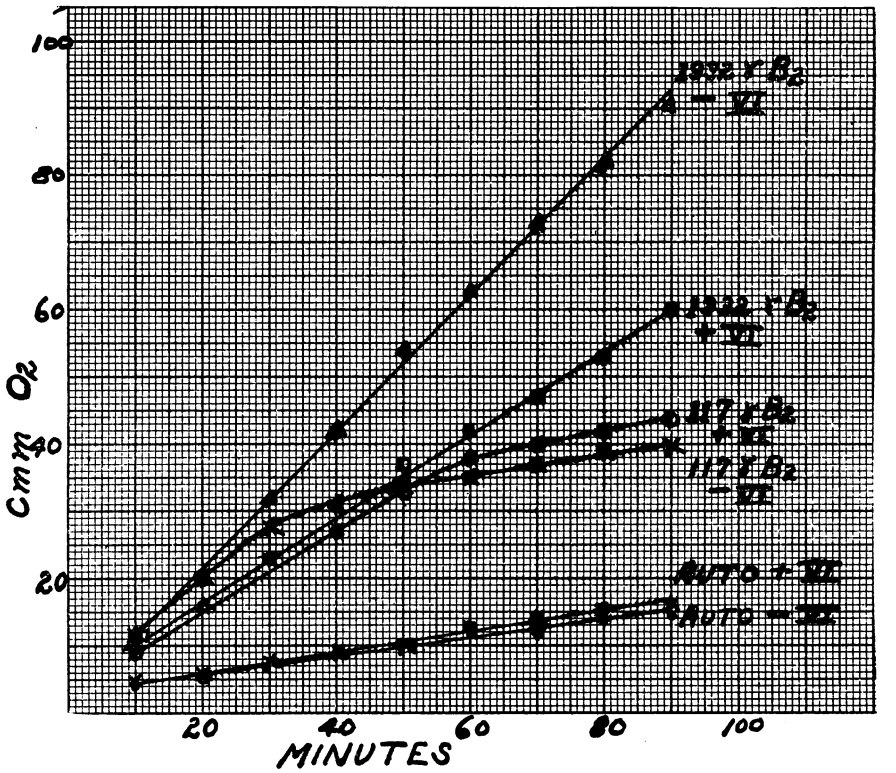



FIG. 4. INHIBITION BY VI OF OXIDATION OF TWO CONCENTRATION LEVELS OF RIBOFLAVIN

TABLE 5

*Inhibition of oxidation of riboflavin and of glucose by 3-4-dimethylphenyl-d-1'-ribamine. (Compound VI)**

| TREATMENT | $O_2$ UPTAKE | $O_2$ DUE TO SUBSTRATE OXIDATION | PER CENT INHIBITION BY VI |
|---|---|---|---|
| | *mm*³ | | |
| Autorespiration | 15 | | |
| Autorespiration plus VI | 12 | | 20 |
| Riboflavin | 44 | 29 | |
| Riboflavin plus VI | 12 | 0 | 100 |
| Dextrose | 46 | 31 | |
| Dextrose plus VI | 15 | 3 | 90 |

* 0.00043 M (=100 molar equivalent of the riboflavin used).

† At 100 minutes. Up to this the rate of $O_2$ uptake was constant.

*Inhibition of glucose oxidation by VI*

The indicated non-specificity for riboflavin made it seem likely that the inhibitors should affect the oxidation rate for *P. riboflavina* with other substrates, and this proved to be the case with glucose. Table 5 compares the inhibition

with riboflavin and glucose respectively by 100 molar equivalents of compound VI, based on the (117 $\gamma$ = 0.000086 M) riboflavin present. The glucose was present in excess throughout the duration of the experiment (0.01 M per 2.2 ml). The inhibition of glucose was only slightly less than that in the case of riboflavin. The fact that inhibition by VI was obtained independent of the presence of riboflavin (in the absence of added substrate (autorespiration) and with glucose) eliminates this compound from the rôle of a structurally related competitive inhibitor of riboflavin oxidation.

Compounds VI and VII have a rather general inhibitory action. This effect was noted in the oxidation by *P. riboflavina* of all other utilizable substrates tested. Further evidence for the non-specificity of these compounds is furnished by the similar repressive actions on the oxidation of glucose by *Escherichia coli*, *Bacillus subtilus* and *Pseudomonas aeruginosa*. Usually 0.0043 to 0.0086 M of the inhibitors sufficed to stop respiration of the bacteria completely, either in the presence or absence of oxidizable substrates.

### *Bacterial growth and VI*

In one experiment in which the bacterial growth inhibiting properties of VI were tested by streaking 18 different common bacterial species, gram-positive and gram-negative, on brain-heart agar containing different concentrations of the inhibitor, no inhibition was observed at the highest level tested, e.g., 0.1 per cent (0.0038 M).

### *Animal experiments with VI*

Through the courtesy of Dr. Gladys Emerson of the Merck Institute (to whom I wish to express my thanks), 3,4-dimethylphenyl-d-1′-ribamine (VI) was tested in rats for its ability to inhibit the utilization of riboflavin. Seventy male weanling rats were employed in the prophylactic study designed to show the effect of varying levels of the compound upon the growth response incurred by the feeding of riboflavin. No anti-vitamin activity was demonstrable in this experiment. The compound did show some toxicity. The 10 animals receiving 140 mg of VI daily became cold and exhibited a transient flaccid paralysis following dosing. Recovery was spontaneous for the majority of the rats in the group. However, 2 animals succumbed following the second feeding. The dosage was accordingly decreased to 70 mg daily, a quantity that was apparently well tolerated. The toxic manifestations are similar to those which have been observed following the administration of xylene. Since compound VI is a substituted (d-1′-ribamine-) xylene it is possible that the carbohydrate side chain fails to mask the toxicity characteristic of xylene or that the compound itself is metabolized by the animal, yielding xylene or a closely allied substance.

### SUMMARY

*Pseudomonas riboflavina* attacks ribose, but by different enzyme systems from those with which it attacks the ribityl group of riboflavin. Lumiflavin apparently is not attacked. Ribityl groups of a number of other riboflavin homo-

logues and related compounds are also attacked. Some, like 4,5-dimethyl-phenyl-isoalloxazine, inhibit riboflavin oxidation by this organism. This action is not competitive since it cannot be reversed by higher concentrations of riboflavin and it inhibits oxidation of glucose by the *P. riboflavina* and other bacteria also. It has no anti-vitamin activity in rats.

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