CXIII. LACTOFLAVIN IN MICRO-ORGANISMS.

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IN three years very rapid development has taken place in the study of the water-soluble, nitrogen-containing pigments, commonly called flavins or lyochromes, which occur in widely varying plant and animal tissues. In this time several members have been identified and their derivatives synthesised, especially by Karrer [1934, 1] and Kuhn [1934], lactoflavin has been suggested as identical with vitamin B₂ by György *et al.* [1934], and it has been shown in yeast to be bound to protein, forming an oxido-reduction enzyme, the "gelbes Ferment" of Warburg and Christian [1933]. As yet, however, almost nothing is known as to the method of production of lactoflavin in living material and the changes it undergoes. Although widely distributed in animals, it seems probable that flavin comes originally from plants. Lactoflavin has been found in yeast and in a few bacteria. The present work shows that yeast forms the flavin itself, that under various conditions it forms amounts of flavin different from normal, that some bacteria do not contain flavin, and that under various conditions blue-fluorescing substances may arise which are possibly related to flavin.

A review of early work on this subject by Wagner-Jauregg [1934] and preliminary reports by Pett [1935, 1, 2] should be consulted. The chemistry of lactoflavin may be briefly reviewed. Kuhn *et al.* [1933] obtained from milk a yellow-coloured, green-fluorescing substance which received the name lactoflavin, and around which the chief interest now centres. Lactoflavin has the formula C₁₇H₂₀O₆N₄. It easily accepts hydrogen thereby losing its colour and fluorescence. Oxygen from the air readily oxidises the reduced form. Under the influence of alkali and strong light it loses a carbohydrate group and the resulting yellow pigment, called lumilactoflavin, has the formula of 6:7:9-trimethylisoalloxazine, which has been synthesised. The carbohydrate is probably in position 9. If lumilactoflavin is heated with alkali, urea is split off. A blue-fluorescing photo-derivative, 6:7-dimethylalloxazine, has been described by Karrer [1934, 2].

Flavin has been found in brewer's yeast in amounts varying between 18 and 30γ per g., and in slightly higher amounts in baker's yeast. In bacteria the amounts vary from the low value of 2γ per g. for *Thermobacterium helveticum* [Adler and Euler, 1934] and 10γ for *Bacterium pasteurianum* to the high values of 77–115γ for *Bact. delbrückii* and 91–136 for *Clostridium butyricum* [Warburg and Christian, 1933]. The present paper reports some further analyses for different organisms. Euler and Adler [1934, 1] showed that flavin in yeast is chiefly present in undialysable, or bound, form.

EXPERIMENTAL.

The yeast was grown in a medium containing per litre: 30 g. glucose, 5 g. (NH₄)₂SO₄, 3 g. Na₂HPO₄, H₂O, 3 g. KH₂PO₄, 2 g. NaCl, 0.1 g. MgSO₄, 7H₂O, and about 0.1 g. marmite; initial *p*_H about 6.5, final 4.5 to 5.0 unless controlled. To this control solution additions were made in the following amounts: urea,

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10 g. per litre; FeSO_4 , FeCl_3 , 0.01 g. per litre; CuSO_4 , $5\text{H}_2\text{O}$, 0.001 g. per litre; nucleic acid, 2 g. per litre; S, 3 g. per litre; Na_2S , 5 g. per litre. Cyanide and pyridine solutions, sterilised by filtration, were added after autoclaving the medium.

The yeast used for inoculation was a specially isolated strain of bottom yeast from a Stockholm brewery. Bacteriological technique was used throughout. An inoculum of 5 ml. per flask was used and contained about 1 γ of flavin.

After maximum fermentation had been reached (about 5 days) the yeast was centrifuged and washed twice with distilled water. Part was then taken for moisture determinations and the remainder weighed in a small Erlenmeyer flask and treated with 6–10 ml. of 95 % ethyl alcohol. After some hours the mixture was heated at 70° for 2 minutes, under a condenser, and filtered. The yeast and filter were returned to the flask and re-extracted with a few ml. of 60 % alcohol. After 2–3 hours the mixture was again heated, filtered and re-extracted. If allowed to stand 12 hours or more the extract may be filtered without heating. Usually two treatments with 60 % alcohol were sufficient. The volume of the combined extracts was taken and a portion filtered through a hard paper. After one or more filtrations the solution was clear enough to be examined using a carbon arc lamp, the beams of which were concentrated by a lens. Comparison of the intensity of the green fluorescence is made with standard tubes of lacto-flavin. Often a cloudiness prevents measurement, but is sometimes removed by shaking with light petroleum. This method is essentially that described by Euler and Adler [1934, 2].

More exact measurements, necessitated by the small amount of material available, were also made. The extracts were made alkaline, until equal to 0.5N NaOH, and exposed under a 500 watt lamp for 3 hours, using a special system of cooling and aeration. This solution was acidified to Congo red paper and extracted with chloroform. Several extractions remove the lumilactoflavin; the chloroform was then dried with sodium sulphate and filtered, and the fluorescence intensity was determined in a Zeiss "Stufenphotometer", previously calibrated by standard solutions.

All experiments were done two or more times. Results are expressed in γ of flavin per g. dry weight of substance. With any given solution the results usually agreed within ± 3.0 , but sometimes the error was about 6.0 %. Thus considerable differences are necessary to be significant.

RESULTS.

Flavin of yeast grown in different solutions.

The results shown in Table I indicate that the only method of influencing the amount of flavin was to affect the metabolism of the yeast, especially in relation to the physiological functioning of oxidation-reduction systems.

Table I. *The amounts of lactoflavin in yeast grown in different solutions.*

Flavin is expressed as γ per g. dry weight.			
Control	...	...	32
Aerobic	...	...	17
Anaerobic (vacuum)	...	...	40
plus malt (50 % of vol.)	...	...	20
plus urea, Fe''	...	...	18
plus Fe'' (p_{H} 4)	...	...	20
(p_{H} 7)	...	...	24
plus KCN (see Fig. 1)	...	...	65
plus cysteine	...	...	45
plus active iron	...	...	19
plus pyridine (see Fig. 2)	...	...	50
plus Fe'''	...	...	32
plus Cu (p_{H} 4)	...	...	30
(p_{H} 7)	...	...	33
plus urea, Cu	...	...	26
plus urea	...	...	28
plus nucleic acid	...	...	29
plus sulphur	...	...	28
plus sodium sulphide	...	...	32
plus Cu, Fe''	...	...	40
Phosphate (see Fig. 3)	...	...	

The results with cyanide, pyridine and phosphate are best seen from curves, such as those given in Figs. 1, 2 and 3.

Fig. 1.

Fig. 2.

Fig. 1. The relation between the flavin content of yeast and the concentration of cyanide in which it was grown.

Fig. 2. The relation between the flavin content of yeast and the concentration of pyridine in which it was grown.

Fig. 3. The relation between the flavin content of yeast and the concentration of phosphate in which it was grown.

Discussion of flavin changes in yeast. Since only 2–3 γ of flavin per flask were present at the start, and after some days' growth the yeast present contained 15 γ or more, yeast must form flavin. The lower content of flavin resulting when yeast is grown with much malt extract suggests that favourable conditions for growth in the medium depress the development of flavin.

Under aerobic conditions, produced by a flow of air through the medium, the amount of flavin was lower than normal. Such conditions have long been known to depress fermentation and to promote reproduction and spore-formation. Since flavin was also depressed, some connection with fermentation is suggested.

Fig. 3 shows that a certain concentration of phosphate was necessary for normal flavin production. This seems clear in spite of some undetermined factor which caused considerable variation in the flavin contents of yeasts grown with little phosphate. This observation has great interest in view of the recent suggestion by Theorell [1934] that phosphate forms part of the active group of lactoflavin, when attached to protein to form the yellow enzyme. Since free phosphate was still present, the effect cannot be due to lack of phosphate. The explanation possibly lies in a lowering of the phosphatase activity of the yeast [Pett, 1934].

Of great interest is the increase of flavin in the presence of potassium cyanide, cysteine and pyridine (Table I, Figs. 1, 2). That flavin-enzyme has some kind of respiratory function was suggested as its properties became known in the original work of Warburg and Christian. It is, however, usually considered that iron-containing haemin compounds are chiefly concerned in cellular respiration, and the precise function of the yellow enzyme is still not clear, being possibly more related to fermentation. Nevertheless, when the normal, iron-containing oxidation mechanism is poisoned by the yeast being grown in cyanide, more flavin than normal is produced. Thus flavin can be considered an alternative system, developed to aid the cell's respiration, and it may be the only system left in cyanide-yeast.

Yeast grown in cyanide shows the characteristic spectrum bands of haemochromogen compounds, showing that these are present to some extent, even though not active. No evidence of utilisation of the cyanide by yeast was found: quantitative determinations at the beginning and end of several fermentations yielded about the same values.

Fermentation and respiration of cyanide-yeast.

For these experiments 5-litre flasks containing 4 litres of medium were used. Of the centrifuged yeast, washed free from KCN, part was used directly for flavin and moisture determinations, part was dried for dialysis in collodion, to determine the bound flavin, and part was suspended in water. This last portion was aerated according to the method of Ambrus *et al.* [1931], to remove easily oxidisable substances, and was then used for fermentation and respiration estimations and for Thunberg methylene blue reduction tests.

Fermentation was carried out, according to the well-known methods of Euler and Myrbäck, in small tubes using 0.5 ml. of 2 % KH_2PO_4 solution, 1 ml. of 10 % glucose and 0.5 ml. of yeast suspension. Results are expressed as ml. of gas evolved in one hour by 1.0 g. (dry) of yeast. The Thunberg test was performed with a final methylene blue concentration of $M/20,000$, and results are expressed in minutes required for decoloration, using fresh yeast equivalent to 0.01 g. The same amount of yeast was used for the Warburg respiration and results give ml. of oxygen per hour. Table II shows the results obtained with yeasts grown normally, with added iron and with added cyanide.

Table II. *Some properties of yeasts grown with added iron and cyanide.*

	Flavin			Fermentation	Warburg respiration	Thunberg MB reduction		
	Total	Bound	Bound %			Without subst.	Glucose	Alcohol
First experiment:								
Control	32	27	84	252	128	24	25	19
plus Fe	28	18	72	160	162	38	44	31
plus KCN	55	51	93	248	110	20	27	13
Second experiment:								
Control	30	25	81	170	190	—	—	—
plus Fe	26	21	81	118	246	—	—	—
plus KCN	46	44	95	178	140	—	—	—

The respiration of yeast grown in cyanide is somewhat less than normal but normal fermentation is maintained. The respiration was reduced from 140 only to 128 in the presence of $M/500$ KCN in the apparatus, indicating that the increased flavin content is chiefly responsible for the respiration observed, since

under these conditions normal yeast respiration is inhibited 70 to 90 % [see Dixon and Elliott, 1929].

The alternative action between iron compounds and flavin is supported by the observation of Malkov [1933] that phosphates inhibit respiration and stimulate fermentation by forming insoluble iron compounds. The work of Elvehjem [1931] had already shown that yeast grown with iron in the solution has an increased cytochrome content and respiration, the latter being clearly indicated also in Table II. Elvehjem also indicated that the longer yeast is grown the less its fermentation is inhibited by cyanide in the Warburg apparatus, and that the cyanide-stable system is concerned in glucose metabolism. Kuhn and Meyer [1929] have, on the contrary, suggested that this system is concerned in fat, not carbohydrate, metabolism. Zuckerkandl and Messiner-Klebermann [1932] have suggested that the cyanide-insensitive system concerned in fermentation also contains iron. From our work it might appear that this cyanide-stable part is flavin.

Effect of continuous culture of yeast in KCN.

When yeast was grown in a medium containing $M/1000$ KCN apparently normal gas production took place. Also, as shown above, the lactoflavin content was higher than normal. If yeast was taken from such a flask and added to another, also containing KCN, the same effect was observed. But when these transferences were continued a yeast was obtained, usually after the third to fifth culture, giving an extract with a blue fluorescence. In some cases all the fluorescence was blue, but more commonly some green (lactoflavin) fluorescence was also present. This blue persisted in subsequent transferences. It was not reducible by hydrosulphite. Inoculation into medium free from cyanide brought the green fluorescence back to normal, though sometimes some blue was present in the first transference.

It seems possible that during long-continued growth in cyanide yeast develops a special mechanism involving a blue-fluorescing substance which may or may not be related to lactoflavin.

Flavin contents of various organisms.

An examination of the quantity of lactoflavin found in various yeasts and bacteria, as shown in Table III, shows that flavin is not as generally distributed in bacteria as in higher organisms. All determinations were made as previously described.

Table III. *The amounts of flavin in various organisms.*

γ per g.			
Bottom yeast ...	...	20-25	<i>Aspergillus</i> ... Nil (blue)
Top yeast:			<i>Penicillium</i> ... Nil (blue)
Baker's (Stockholm)	...	25	<i>Proteus vulgaris</i> ... Nil
Race "M" ...	...	33	<i>Staphylococcus albus</i> ... Nil (blue)
Race XII ...	...	38	<i>Bacterium coli</i> A ... Nil (blue)
Wild yeast:			<i>Bacterium coli</i> B ... Trace <1
<i>Torula utilis</i> ...	...	27	(pathogenic)
<i>Mycoderma cerevisiae</i> ...	...	12	<i>Bacterium aerogenes</i> ... 9.8
<i>Pichia membranifaciens</i> ...	...	30	<i>Bacillus subtilis</i> ... 7.5

The idea that flavin is an alternative to actively aerobic, iron-containing systems and that it has a relation to fermentation finds some support in Table III. Thus a weakly fermenting, strongly aerobic yeast like *Mycoderma* has a very low content of flavin. *Pichia* and top yeasts are aerobic, but strongly

fermenting, and have higher amounts. *Torula* is not so strongly fermenting, but has a fairly high flavin content, possibly because it is not strongly aerobic and is said to have incomplete haemin systems.

Moulds, like *Aspergillus* and *Penicillium*, of which the species were unfortunately not known, although containing green-fluorescing substances, showed only blue fluorescence after the treatment for the preparation of lumilactoflavin. They are, of course, strongly aerobic, but according to Frei *et al.* [1934] they contain neither cytochrome nor haematin compounds in general.

Flavin and bacteria.

No systematic study of flavin distribution in bacteria, such as that made for haemin compounds by Frei *et al.* [1934], has been made, but the results in Table III, together with previously mentioned determinations, give some idea of the situation. Undoubtedly also many older observations of colours in bacteria, especially of yellow colours, are significant in this connection. While some of these are undoubtedly carotenoids, the work of Ingraham and Baumann [1934] shows that this is true only in aerobes. Anaerobes, such as *B. butyricus*, do not contain carotenoids, but contain much lactoflavin.

That many bacteria do not contain flavin is also evident. While flavin is undoubtedly present in many kinds of tissue, it is thus not universal. Some bacteria contain other respiration pigments, like the pyocyanin of Wrede and Strack [1929], or the chloraphin of Kögl *et al.* [1932].

The possibility that some bacteria not only do not contain flavin but can actually transform it was indicated by Pett [1935, 1]. In that communication it was pointed out that an organism had been isolated from a lactoflavin solution which had become blue-fluorescing; and that this organism if placed in very small amount in a flavin solution caused the disappearance of the green fluorescence, and the development of a blue fluorescence. A few more facts can be presented. The use of fresh centrifuged, washed bacteria has been found more convenient. Dried bacteria in extremely minute quantity also effect the change described above. Their activity is about the same from p_H 6.5 to 8 but falls off in the more acid and alkaline ranges. The blue-fluorescent substance, either extracted from bacteria or developed as above, and then purified a little by extractions, fluoresces between 330 and 370 $m\mu$; some preparations showed a faint absorption band about 264 $m\mu$.

Of the organisms mentioned in Table III only *Bact. coli* A and *Bact. aerogenes* can act like the organism described by Pett [1935, 1], but *Bact. coli* acts much more slowly, and *Bact. aerogenes* produces a blue fluorescence reducible by hydrosulphite.

DISCUSSION.

This work contributes to two interesting points in the study of flavins: (1) the function of lactoflavin and (2) the importance of blue-fluorescing substances possibly related to lactoflavin.

The function of flavin in yeast might seem to be similar to that of the many other intermediate carriers for the oxidation-reduction activities, such as cytochrome or glutathione, assisted by the fact that it is autoxidisable. The increase of flavin in yeast grown in cyanide, shown in this paper, suggests that it is an alternative mechanism to iron-containing catalysts, but it is known that most of the normal respiration of yeast is not effected through flavin. The evidence advanced for a function in fermentation rather than respiration is not very strong for yeast but receives considerable support by analogy with bacteria.

In considering the function of lactoflavin in bacteria, as well as yeast, it must be remembered that the transference of oxygen is slower by way of flavin than by way of haemin compounds. Thus any cell containing haemin would not use flavin-enzyme for the same purpose. Using a lactic acid organism, which contained no haemochromogen, Warburg and Christian observed simultaneously the respiration and the amount of reduced flavin. They considered their results to show that all the respiration occurred by means of flavin. However, the spectrographic method of estimating the oxygen-transporting enzyme has been criticised as unreliable by Keilin [1933]. Adler and Euler [1934] showed an increase in respiration of bacteria in the presence of flavin, and especially if cozymase was also present.

In considering these results there is involved the old problem of what distinction must be made between fermentation and respiration. The respiration of lactic acid bacteria in the presence of fermentable substrate is quite different from the respiration of the bacterial cells themselves [Davis, 1933; Bertho and Glück, 1932]. Stimulation of the respiration of lactic acid bacteria by methylene blue is observed only if fermentation is taking place, and this may be true of flavin. An association with cozymase is of interest in this connection, though this appears to be related simply to the dehydrogenase system [Euler and Adler, 1934, 3]. The fact that the highest content of lactoflavin is found in anaerobic bacteria may be related to the strong fermenting ability of these bacteria rather than to their anaerobicity.

The present paper has shown that some organisms do not contain flavin, that other organisms contain it in only very small amounts, and that these results can be related to the fermentative power of the organisms. It has also been shown that if haemochromogen compounds are poisoned, the flavin system can develop and carry on suitable respiration. The function of flavin is, therefore, still obscure, except in the latter specific case.

Blue-fluorescing substances, possibly related to flavin, have been indicated from time to time by most of the workers in this field. These observations are complicated by the fact that many normal cell constituents, such as proteins or nucleotides, give a blue fluorescence in ultra-violet radiation, though not so many with a carbon arc lamp as used in this work. Karrer [1934, 1, 2] has described certain chemically prepared flavin derivatives with blue fluorescence. The chief example, lumichrome, loses its colour in the presence of hydrosulphite. Other apparent derivatives [see Euler and Adler, 1934, 4] are stable to hydrosulphite. Reduction by hydrosulphite is probably not a good criterion of lactoflavin but is of interest.

Pett [1935, 2] showed that a blue fluorescence appears during autolysis of yeast and during starvation of yeast. The present paper reports a blue fluorescence developed in yeast after continuous culture in cyanide, as well as from bacterial sources. The explanation of these observations cannot at present be given, but blue-fluorescing substances related to lactoflavin seem to be a promising field for future work.

SUMMARY.

Using a standard nutrient solution with the addition of various substances an attempt was made to vary the lactoflavin content of bottom yeast. The chief effect was observed by influencing the metabolism of the yeast, especially the functioning of redox systems. The following observations were made:

1. The presence of cyanide, cysteine or pyridine, which can poison the iron-containing catalysts, produced a flavin content much higher than normal.

2. Yeast grown in cyanide has a normal fermentation but a respiration below normal.

3. A certain amount of phosphate must be present in the medium for normal flavin development.

4. Under strongly aerobic conditions, which depress fermentation, the amount of flavin is also lowered. Some other evidence of a relation to fermentation is adduced.

5. Continued subculture of yeast in media containing cyanide resulted in the development of a blue-fluorescing substance, which partially or wholly replaces flavin.

6. The flavin contents of various organisms are reported.

The results are discussed in relation to flavin function and to blue-fluorescing substances.

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