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The Auxanographic Responses of certain Aneurin-requiring Yeasts to Aneurin, Aneurin Components and Derivatives

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SUMMARY: Three yeast species, *Rhodotorula mucilaginosa*, *Trichosporon cutaneum* and *Torulopsis albida* (*Cryptococcus albidus*) respond in a simple auxanographic spot test to the presence of aneurin, aneurin split products and cocarboxylase. Differences in the behaviour of these three species towards the different biologically active entities make it possible to differentiate between these substances and to identify them. Moreover, from the appearance of the auxanogram conclusions can be drawn regarding certain dynamic aspects of aneurin metabolism such as velocity of uptake, storage and splitting of aneurin, or of its derivatives. The test is not only qualitative but also, within certain limits, quantitative.

An auxanographic procedure for the determination of vitamin requirements of yeasts by means of drops of vitamin solutions applied with a bacteriological loop has been described earlier (Mager & Aschner, 1947). It was noted at that time that the diameter of the growth field was roughly proportional to the concentration of the vitamin in the drop applied. As the preparation of the auxanogram is very simple and the amount of test solution needed extremely small (a loopful being quite sufficient) it seemed desirable to examine how far this procedure could serve as a method for quantitative or semi-quantitative determination of a vitamin. We were especially interested in the determination of aneurin because, with suitable yeasts as test organisms, a much simpler test medium could be employed than the one proposed in the auxanographic assay method of Bacharach & Cuthbertson (1948) where a lactobacillus serves as the test organism.

Our conclusions are based on the comparative study of the auxanographic responses of three aneurin-requiring organisms towards aneurin, aneurin pyrophosphate (cocarboxylase) and two synthetic compounds representing respectively the pyrimidine and the thiazole moieties of the aneurin molecule. Different biologically active split products of aneurin served as controls.

MATERIALS AND TECHNIQUE

Experiments were carried out with three yeast species belonging to three different genera (nomenclature according to Lodder, 1934, and Diddens & Lodder, 1942): *Rhodotorula mucilaginosa*, *Trichosporon cutaneum* and *Torulopsis albida* (*Cryptococcus albidus*). Some experiments were also undertaken with *T. neoformans* (*Cryptococcus neoformans*) which behaved in every respect like *T. albida*. The strains were kept as stock cultures on a glucose peptone Marmite medium and were transferred 24 hr. before the test on to

a medium of the following composition: glucose, 1.0 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.2 g.; K_2HPO_4 , 0.1 g.; MgSO_4 , 0.04 g.; agar (Difco), 2.0 g.; tapwater to 100 ml. The medium used for the auxanographic plates was of the same composition.

The materials tested were: aneurin chloride hydrochloride (U.S.P. reference standard); the synthetic pyrimidine moiety (2-methyl-5-ethoxymethyl-6-aminopyrimidine, Merck); the synthetic thiazole moiety (4-methyl-5-(β -hydroxyethyl)-thiazole, Merck) and aneurin chloride pyrophosphate (cocarboxylase, Merck). The following preparations of split products were compared with the synthetic compounds: aneurin treated at 120° at acid and alkaline pH; aneurin treated with fish 'thiaminase'; and aneurin which was in contact with resting yeast cells. All these substances, and combinations of them, were usually tested in the range of 0.01–2 $\mu\text{g.}/\text{ml.}$

The auxanographic plates were prepared in the following way. To a test-tube containing 6 ml. of melted agar cooled to 50°, 0.5 ml. of a suspension of test organism was added as an inoculum and the plates poured after quick but thorough mixing. Care was taken to have an even thickness of the agar layer all over the plate. Therefore only plates with perfectly flat bottoms were used and these were kept on a water-level plane during solidification of the agar. For the inoculum a 24 hr. slope culture of test organism was washed into 3 ml. sterile distilled water. One half ml. of these washings contained about 50 million cells, an amount which in previous experiments was found to give satisfactory results. Before the application of the test solutions the plates were kept for 3 hr. at 30° in the incubator.

The test solutions were deposited as drops on the agar surface by means of a bacteriological platinum loop (diameter *c.* 3 mm.) which was flamed before each application. Six to ten solutions could be tested simultaneously on the same plate. The incubation temperature was 30°. The circular areas of growth responses became visible macroscopically after several hours' incubation and then remained practically unchanged in diameter. The microcolonies composing this growth, however, continued to grow for several days, so that the density within the growth area increased with time. Results were usually read after 20 hr. or more.

Quantitative estimations of aneurin were carried out by comparing on the same plate several dilutions of the material to be tested with a series of known solutions of aneurin in the range of 0.1–2 $\mu\text{g.}/\text{ml.}$

EXPERIMENTAL

Auxanograms of aneurin, its pyrimidine and thiazole moieties and cocarboxylase

Auxanogram plates were prepared in the manner described above with the three test organisms, and drops of aneurin solutions of different strengths applied to the agar surface. Well-defined growth areas were obtained with concentrations in the range 0.05–2 $\mu\text{g.}/\text{ml.}$ After 20 hr. the growth within the positive areas was already so dense that, although composed of numerous microcolonies, it gave the impression of one single large colony. The density of

growth appeared equal throughout the whole growth area, except for a small border zone, barely visible to the naked eye, where a gradual decrease in the size of the colonies could be observed (Pl. 1, fig. 1(1) and fig. 2(1)). Responses were also obtained with much lower concentrations such as 0.005 $\mu\text{g./ml.}$ These areas of growth, however, were too poorly defined to serve for quantitative determinations and can only be referred to as traces. With higher concentrations some interfering phenomena were observed; for instance, an outer zone of sparser growth in the case of *T. albida*, and a transparent central area in the growth response of *R. mucilaginosa*. An explanation for these phenomena will be attempted later. With all three strains the size of the growth areas increased with increase in aneurin concentration applied. However, at least a twofold to fivefold ratio between different concentrations was necessary in order to produce measurable differences in the diameters of the growth areas. Table 1 contains actual measurements of the diameters of the auxanographic responses produced by aneurin solutions in varying concentration. No discernible differences in the sensitivity of the three different strains were noted.

Table 1. *Extent of the areas of auxanographic response of R. mucilaginosa and Tr. cutaneum resulting from the application of varying aneurin concentrations*

Aneurin ($\mu\text{g./ml.}$)	Diameter of growth area (mm.)
5	8
1	6
0.2	5
0.04	4

Mixtures of the pyrimidine and thiazole moieties, equimolar in concentration to the aneurin solution tested previously, gave areas of response which were always considerably larger than the corresponding aneurin auxanograms (Pl. 1, fig. 1(4) and fig. 2(4)). The ratio between the diameters of growth areas was about 2:1 in the case of *R. mucilaginosa* and 4:1 in the case of *Tr. cutaneum*. In addition to the larger diameters, the responses to mixtures of the two moieties showed another characteristic difference; the microcolonies composing the growth response remained smaller, and the transition zone from the positive to the negative area was much broader than in the corresponding aneurin auxanogram. The whole growth area was therefore less opaque and its outlines less sharply defined than in the case of aneurin (Pl. 1 figs. 3 and 4).

All three yeast species required for a threshold response a higher concentration of the moieties than of aneurin. The corresponding values in the case of *R. mucilaginosa* and *T. albida* were a twofold concentration, and in the case of *Tr. cutaneum* a fivefold concentration of the moieties (Table 2).

Further differences in the behaviour of the three yeast species became evident when they were tested against each of the two moieties separately: *R. mucilaginosa* reacted to the pyrimidine as to a mixture of both moieties, while *Tr. cutaneum* and *T. albida* gave no response with the pyrimidine alone

even when the concentration was raised to 50 $\mu\text{g./ml.}$ (Pl. 1, fig. 1(2) and fig. 2(2)).

When the ratio of the pyrimidine and thiazole moieties in the mixture was changed, the dimensions of the growth areas of *R. mucilaginosa* were affected only by the concentration of pyrimidine. Responses of *Tr. cutaneum* and *T. albida* were not perceptibly influenced by a preponderance of either of the two moieties.

The thiazole moiety in the absence of the pyrimidine moiety produced very weak responses with all three yeast species. With *R. mucilaginosa* responses remained small and faint even when 1 $\mu\text{g.}$ thiazole/ml. was applied.

Table 2. *The lowest concentrations of growth-promoting substance ($\mu\text{g./ml.}$) necessary to produce a discernible response*

Test organism	$\mu\text{g./ml.}$				
	Aneurin	Pyrimidine moiety	thiazole moiety	Mixture of both moieties in the ratio of their molecular weights, pyrim. + thiaz.	Coccarboxylase
<i>Rhodotorula mucilaginosa</i>	0.005	0.005	0.04	0.005 + 0.004	2.7
<i>Trichosporon cutaneum</i>	0.005	— *	0.2†	0.025 + 0.021	0.14
<i>Torulopsis albida</i>	0.005	— *	0.2†	0.005 + 0.004	0.014

* No responses even at 50 $\mu\text{g./ml.}$

† These responses remain faint even following the application of 1 $\mu\text{g./ml.}$

With concentrations above 0.1 $\mu\text{g./ml.}$ *Tr. cutaneum* and *T. albida* yielded rather extensive areas of growth which were, however, so weak as to be barely visible. The microcolonies in the centre were somewhat larger and so gave the impression of a more dense growth, but even the central part was considerably less opaque than the growth in the auxanogram of the mixture of both moieties (Pl. 1, fig. 1(3) and fig. 2(3)). With concentrations below 0.1 $\mu\text{g./ml.}$ only the central part was visible.

By using an agar medium with 0.5 $\mu\text{g./ml.}$ pyrimidine moiety for the auxanogram plate and *Tr. cutaneum* or *T. albida* as test organism it was also possible to produce well-defined growth areas on adding the thiazole moiety. Plates prepared in this way gave responses to a mixture of the two moieties which were dependent only on the thiazole concentration in the test solution. *R. mucilaginosa* of course always grew homogeneously throughout the whole plate under these conditions.

Mixtures of aneurin with its pyrimidine and thiazole moieties (0.5 aneurin + 0.25 pyrimidine + 0.21 thiazole, $\mu\text{g./ml.}$) produced auxanograms in which it was obvious that aneurin and the moieties produced their responses separately. The growth area was composed of a dense central part comparable with the response produced by aneurin alone and a sharply separated outer zone which was less dense, like the auxanogram of the moieties (Pl. 1, fig. 1(5) and fig. 2(5)).

Coccarboxylase in this range of concentrations gave no response with *R. mucilaginosa*, a clearly perceptible response with *T. albida*, and a definite

but less sharply pronounced response with *Tr. cutaneum* (Pl. 1, fig. 1(6) and fig. 2(6)). Even concentrations as high as 50 $\mu\text{g./ml.}$ produced only traces of a response with *R. mucilaginosa*. Table 2 summarizes the lowest concentrations of all substances tested giving a discernible reaction.

Auxanograms of different split products of aneurin

Aneurin solutions which were autoclaved at 120° for 30 min. at pH 8.0 or 10.0 stimulated *R. mucilaginosa* as did solutions which contained the free pyrimidine moiety, i.e. they gave more extensive but less dense responses than the untreated control solution of aneurin. However, as the response areas with *Tr. cutaneum* and *T. albida* were not only weaker but also less extensive than with the aneurin control solution, we must conclude that the thiazole moiety was lacking entirely or was present only in traces. Indeed these two species gave no response at all to 1/5 dilutions of the treated solutions, whereas *R. mucilaginosa* responded well in this case. Beadle, Greenwood & Kraybill (1943) concluded from spectrophotometric data that the pyrimidine moiety was present in the split products of similarly treated aneurin. Our results concerning the pyrimidine moiety agree with this conclusion. Solutions of cocarboxylase treated in the same way likewise indicated the presence of the pyrimidine moiety alone. Heating a solution of aneurin in the autoclave at 120° at pH 5.0 also effected a cleavage of the aneurin molecule, as shown by the more extensive growth response of *R. mucilaginosa* in comparison with the response to the untreated aneurin solution. In this case, however, both *Tr. cutaneum* and *T. albida* yielded an increased area of growth, an effect which is brought about only when the thiazole moiety is present in addition to the pyrimidine moiety. We must assume therefore that, in contrast to the alkaline cleavage, at the acid pH the aneurin was split without simultaneous destruction of the thiazole moiety.

Aneurin, after 15 hr. contact with thiaminase prepared from carp intestine according to Krampitz & Woolley (1944), gave with *Tr. cutaneum* an extensive response indicative of the presence of a mixture of the two moieties, yet no response at all was given by *R. mucilaginosa*. According to the behaviour of *Tr. cutaneum* the two moieties of aneurin are present in the treated solution. As, however, *R. mucilaginosa* failed to indicate the presence of the pyrimidine moiety this substance was apparently present in a modified form which *Tr. cutaneum*, but not *R. mucilaginosa*, was able to utilize. Krampitz & Woolley (1944) had a similar experience in their assay with *Endomyces vernalis*. This organism, which is an indicator for the pyrimidine moiety gave only a very weak reaction in this case. They concluded that cleavage of aneurin by their thiaminase preparation produced the thiazole moiety and a derivative of the pyrimidine moiety.

Action of yeast cells on aneurin solutions

Uptake of aneurin by resting yeast cells was demonstrated in the following experiment. About 50 million cells of *Saccharomyces carlsbergensis* were suspended in 10 ml. of an aqueous solution (2 $\mu\text{g./ml.}$). At intervals samples

were taken and the suspended yeast cells were removed by centrifugation. The supernatant fluids were then analysed by the auxanographic method. The response areas became progressively smaller until after 1 hr. auxanograms of the supernatant fluids were negative with all three yeasts species. The missing aneurin was detected, by the thiochrome method, in the sedimented cells. The yeast cells obviously had taken up aneurin from the supernatant fluid. When a mixture of moieties was tested in the same way no perceptible decrease of concentration could be demonstrated in the supernatant. The uptake of split products by yeast cells is obviously much smaller than the uptake of intact aneurin.

An additional action of yeast cells on aneurin was observed by testing auxanographically aneurin solutions which had been in contact with yeast cells for several hours. Except for the longer contact period solutions were treated as in the two previous experiments. The result was a characteristic change in the auxanographic reaction of the supernatant. Auxanograms of successive samples revealed a perfect transition from the compact aneurin response to the diffuse response typical of the aneurin moieties (Pl. 1, fig. 5). A gradual splitting of aneurin had obviously taken place during the prolonged period of contact. Analysis, by the thiochrome method, of the sedimented cells and the supernatant fluid supported this assumption, in so far as a loss of aneurin in the system corresponding to the apparent increase in split products could be demonstrated. The splitting was produced with cells of *Saccharomyces carlsbergensis* or of *S. cerevisiae* but was much more pronounced when certain oxidative yeasts, especially *T. albidus*, acted upon the aneurin solution. In this case first signs of splitting were evident after a contact period of 1 hr. A detailed description of these experiments will be given elsewhere.

DISCUSSION

The vitamin auxanogram has its counterpart in auxanography in the plate method for determination of antibiotic substances. Both methods are based on the same principle: interaction between a susceptible micro-organism, suspended in agar, and an active substance diffusing through this agar. The interaction itself, however, is different in the two cases, and this obviously also affects the diffusion process. In the inhibition response each cell that comes in contact with an effective concentration of the antibiotic is killed or inhibited without much further interaction with the diffusing substance. In the vitamin auxanogram, however, the relatively short contact between cell and growth factor is followed by a period of proliferation, which may continue for several days. This is possible only if a substantial part of the growth-promoting substance is stored by the test organism during the contact period. The capacity of yeast cells to store a large amount of different vitamins has indeed been demonstrated for various yeasts. This means that while in the inhibition auxanogram diffusion proceeds practically undisturbed, in the vitamin auxanogram it is highly modified by processes of absorption, storage, and use in the cells of the test organism. This might explain why a relatively great

shift in concentration of vitamin (twofold to fivefold) is necessary to produce a perceptible change in the diameter of the growth area. The following observation supports this theory. There are differences in the extent of the growth areas when the same concentration of aneurin is applied on test plates containing different numbers of suspended yeast cells; the larger this number the smaller is the growth area.

The modifying influence of absorption and storage is probably also the reason for the differences in size of equivalent auxanograms of aneurin and aneurin split products. If the cells of the test organism are not able to store the moieties as such but have to transform them into aneurin before storage, they may collect a smaller amount of aneurin during a given period of contact than when aneurin itself is available in the surrounding medium. More substance of the two moieties would remain for diffusion, and the resulting growth would consequently be composed of smaller colonies over a larger area. Evidence for a smaller rate of uptake of split products by yeast cells was provided in our experiments with resting cells.

The transparent central areas in the auxanogram of *R. mucilaginosa* which appear usually when the aneurin concentration in the applied drop is higher than $2\mu\text{g./ml.}$ are obviously due to inhibition of growth at this concentration. *R. mucilaginosa* seems to be more sensitive in this respect than, for example, *Tr. cutaneum* which gave homogeneous auxanograms even with $10\mu\text{g./ml.}$ aneurin.

A different explanation is suggested for the reported interference of higher concentrations of aneurin in the case of auxanograms of *T. albida*. Here it is the outer zone of the auxanogram which is less dense than the central part. This condition is characteristic of response to a mixture of aneurin and split products. Evidently with higher concentration of aneurin a considerable part of the surplus aneurin is split, and it is the presence of split products which is responsible for the lack of homogeneity in the auxanogram. That the cleavage should be recognizable with *T. albida* but not with the other two species is not surprising, since *T. albida* is indeed more active in splitting aneurin than are other yeast species.

R. mucilaginosa, in contrast to *Tr. cutaneum* and *T. albida*, is unable to respond in the auxanogram to either diphosphorylated aneurin (cocarboxylase) or the pyrimidine derivative which results from cleavage of aneurin by thiaminase.

It seems that *R. mucilaginosa* lacks enzymes present in the other two species which liberate aneurin or its pyrimidine from combination. Cocarboxylase after treatment with takadiastase is a suitable growth factor for *R. mucilaginosa*, which then gives a typical aneurin response to the product of such treatment.

The use of aneurin auxanogram might be advantageous in several fields. For instance, with this method it is simple to follow the changes which occur in the aneurin content of a substrate during different stages of industrial processing, or to detect the appearance of aneurin split products in metabolic processes. These split products can be characterized to a certain degree and, in the absence of aneurin, may be estimated quantitatively. The pyrimidine

moiety is determined by regular test plates with *R. mucilaginosa*; the thiazole moiety by using *T. albidia* in test plates to which a suitable amount of pyrimidine moiety has been added. The quantitative estimation of aneurin itself is possible if no growth-promoting derivatives of aneurin are present, otherwise the values obtained might be too high. While it is obvious that the degree of accuracy and sensitivity of this simple test cannot reach that attainable by more elaborate methods, it is nevertheless surprisingly high. Moreover, the test permits the observation of subtle interactions between cell and vitamin which might be difficult to detect by other methods.

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EXPLANATION OF PLATE

- Fig. 1. Auxanographic responses of *Rhodotorula mucilaginosa* to equivalent drops of solutions containing ($\mu\text{g./ml.}$): (1) 0.5 aneurin; (2) 0.25 pyrimidine moiety; (3) 0.21 thiazole moiety; (4) a mixture of both moieties in the ratio of their molecular weights and equivalent to 0.5 aneurin; (5) the same mixture as in (4) with the addition of 0.5 aneurin; (6) 0.7 cocarboxylase. (Magnification, $\times 0.7$.)
- Fig. 2. The same as Fig. 1 with *Trichosporon cutaneum* as test organism. Note the absence of response to the pyrimidine moiety in (2) and the response to cocarboxylase in (6). (Magnification, $\times 0.7$.)
- Fig. 3. Border area of the growth response of *R. mucilaginosa* to aneurin. The auxanogram has a dense structure and a well defined boundary. (Magnification, $\times 30$.)
- Fig. 4. Border area of the growth response of *R. mucilaginosa* to a mixture of thiazole and pyrimidine. Loose structure, boundary less distinct. (Magnification, $\times 30$.)
- Fig. 5. Auxanogram of four successively drawn samples of an aneurin solution ($1\mu\text{g./ml.}$) which was maintained in contact with cells of *Torulopsis albidia*. Test organism: *R. mucilaginosa*. Samples were taken after the following periods of contact: (1), 0 (control); (2), 1 hr.; (3), 4 hr.; (4), 22 hr. The auxanograms reveal a gradual splitting of aneurin. (Normal size.)

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G. MILLER & M. ASCHNER—AUXANOGRAPHS WITH YEASTS. PLATE 1