[Contribution from the Chemical Laboratory of the University of Oregon]

THE USE OF FRACTIONAL ELECTROLYSIS IN THE FRACTIONATION OF THE "BIOS" OF WILDIERS

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Received July 28, 1931 Published November 5, 1931

When Wildiers discovered that yeast requires some unknown factor for growth stimulation, he assumed it to be a single substance and gave it the name "bios." Attempts to purify "bios" by Devloo and others who were in close touch with Wildiers' work resulted in complete failure, as judged by their reported results in light of more recent developments.

The more recent work of Eddy and Kerr,[1,2] W. Lash Miller and his associates,[3,4] Fulmer,[5] Narayanan,[6] etc., has in no case been concerned with the "bios" described by Wildiers, as our work shows that the yeasts which they used differ materially in their behavior from Wildiers' yeast. The yeast used by Narayanan appears to react toward yeast nutrilites more nearly like Wildiers' yeast than any of the others. As was shown in a previous paper[7] the results on fractionation of yeast nutrilites obtained by various investigators including those from this Laboratory do not apply to Wildiers' yeast and hence are not concerned with the "bios" studied by Wildiers.

In the present report we are presenting evidence to show that by a procedure which has hitherto not been used in this connection, namely, fractional electrolysis, it is possible to separate the "bios" of Wildiers into two distinct factors. Either of these factors alone is almost completely ineffective but the two together give striking results, as are shown in accompanying curves.

Experimental

I. Toxic Material in Rice Bran Extract.—Rice bran has proved to be an excellent and cheap source of yeast nutrilites and has been used by us in recent studies. In working with Wildiers yeast,[7] however, we found that a toxic material develops in the course of a few weeks, when either the rice bran itself or an extract of it, is kept. This is clearly shown in Fig. 1, in which an extract was tested on the various dates indicated. The extract was made by extracting a sample of freshly milled rice bran (obtained a week or so previous to the first test) with 60% methanol and evaporating to dryness. Experiments showed that the toxic material developed in

[1] W. H. Eddy, R. W. Kerr and R. R. Williams, This Journal, **46**, 2846 (1924).
[2] R. W. Kerr, *Proc. Soc. Expil. Biol. Med.*, **25**, 344 (1928).
[3] W. L. Miller, *Science*, **59**, 197 (1924).
[4] E. V. Eastcott, *J. Phys. Chem.*, **32**, 1094 (1928).
[5] E. I. Fulmer and Nelson, *J. Biol. Chem.*, **51**, 77 (1922).
[6] B. T. Narayanan, *Biochem. J.*, **24**, 6 (1930).
[7] R. J. Williams and E. M. Bradway, This Journal, **53**, 783 (1931).

the rice bran itself at about the same rate as it did in the extract. It will be noted in the graphs that as the toxic material accumulates, the effect of larger doses of the extract is to diminish growth. We were able to show definitely that the loss of activity was not due to a destruction of the growth-promoting principles but to the production of a toxic substance, because by electrolysis the toxic material moved to the anode, leaving behind material which promotes growth very satisfactorily.

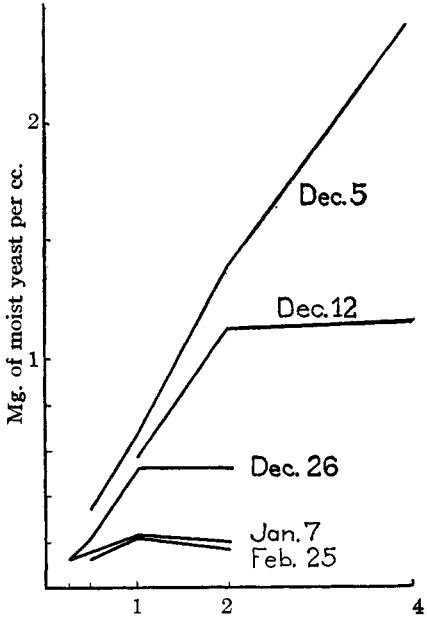

Fig. 1.

The electrolysis apparatus (Fig. 2) which we used for the study of these extracts was suggested by the work of Williams and Waterman.[8] It consisted of a series of four cylinders about 5 cm. in diameter and 5 cm. high connected by siphons as indicated. The siphons were filled by suction and emptied at the end of the electrolysis by opening the stopcocks.

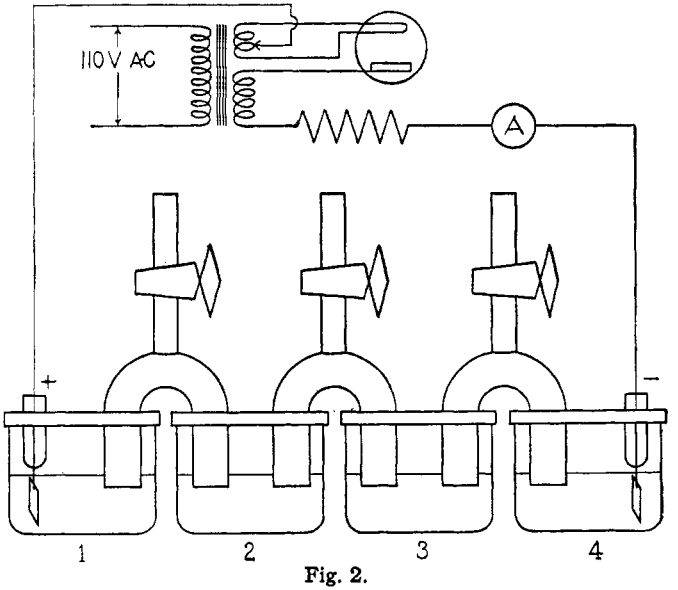

Fig. 2.

[8] R. R. Williams and R. E. Waterman, *Proc. Soc. Exptl. Biol. Med.*, **27**, 56 (1929).

Figure 3 shows graphically the results of a short electrolysis of 2 g. of the material extracted from rice bran dissolved in 100 cc. of water. A potential of 500 volts was used and 6 milliamperes passed for eighty minutes. At the end of this time the P_H values in the four cells were <3.6, 4.0, 9.4 >9.6, respectively, beginning with the anode cell. This electrolysis was performed on January 5 and it will be noted that although the original material was toxic in the higher doses, the cathode cell, particularly, is free from any such effect, whereas the anode cell and the one next to it show the toxic effect very markedly. It is apparent that the toxic material travels rapidly to the anode.

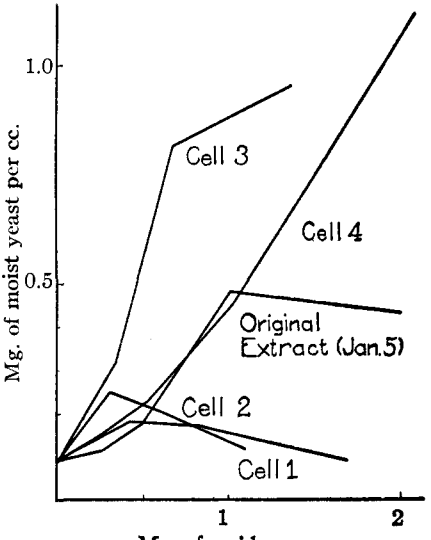

Fig. 3.

Separation of Activity into Two Factors.—Table I gives data concerning a more prolonged electrolysis (twenty-four hours) and Fig. 4 shows graphically the growth-promoting powers of each of the residues and various combinations taken two at a time. In this experiment it is clearly indicated that the stimulating material has been divided by the electrolysis into two portions (in cells 2 and 4) each of which is ineffective alone, but which together are very effective.

TABLE I

RESULTS OF TWENTY-FOUR-HOUR ELECTROLYSIS

Cell	Appearance of solution	P_H	Dry residue, mg.	Residue designation
1	Extremely cloudy, yellowish	3.6	63.8	R_{72}
2	Cloudy, yellowish	3.8	76.6	R_{73}
3	Clear, colorless	7.2	96.4	R_{74}
4	Clear, yellowish	9.6	70.2	R_{75}

An even more complete separation of the two factors was accomplished by performing two preliminary electrolyses each of one-hour duration, and discarding the contents of cells 1 and 2 in each case. Most of the toxic material was thus removed while the greater part of the activity remained. For the third and final electrolysis, instead of diluting the contents of cells 3 and 4 back to the starting volume (100 cc.), they were mixed and introduced into cells 1 and 2, while cells 3 and 4 were filled with distilled water. The final electrolysis ran for seventy-two hours using an e. m. f. of 1000

volts. At the start the current was 10 milliamperes, but after about twenty-four hours the value dropped to 0.1 milliamp. The P_H values at the end of the run were <3.6, 7.3, 8.2, >9.6, respectively. Figure 5 shows the effects produced by the contents of cells 1 and 4 taken separately and together. The original material used in this experiment (February 18) showed practically no activity until the toxic material had been removed by preliminary electrolysis. The contents of cells 2 and 3 showed very little activity in any combination.

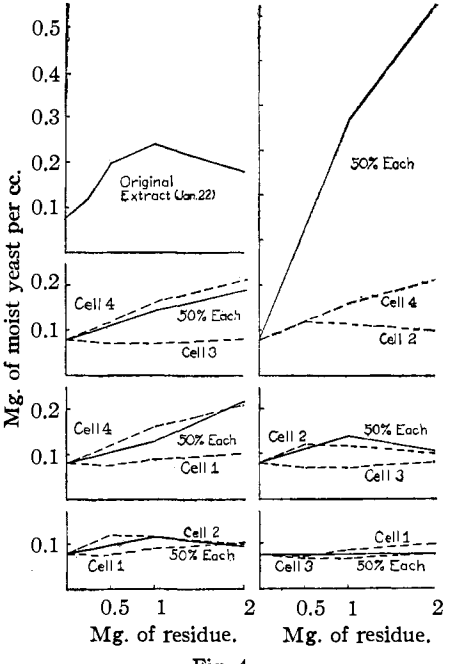

Fig. 4.

Use of Other Materials.—In order to see whether the same results are obtainable when materials other than rice bran are used for extraction, we carried out experiments on malt sprouts, yeast and milk, which are reported in brief below.

Malt sprouts were extracted with 60% methanol, 0.215 g. of the residue was dissolved in 100 cc. of water and electrolyzed for seventy hours using 500 volts. The yeast growths produced by the contents of cells 2 and 4 taken together and separately are given in Table II. The seeding used was 0.154 mg. of moist yeast per cc.

TABLE II

FRACTIONATION OF MALT SPROUT EXTRACT

Addition to synthetic medium	Yeast crops, mg. of moist yeast per cc.	Δ above blank
Blank	0.08	
1 mg. from cell 2 (P_H 3.6)	.075	−0.005
2 mg. from cell 2	.05	− .03
1 mg. from cell 4 (P_H 9.6)	.12	.04
2 mg. from cell 4	.13	.05
1 mg. from cells 2 and 4 (50% of each)	.35	.27
2 mg. from cells 2 and 4 (50% of each)	.74	.66

A similar extract of dried baker's yeast was made with 60% methanol and 0.300 g. of the residue was dissolved in 100 cc. of water and electrolyzed for forty-five hours. The contents of cells 2 and 3 were then diluted to 100 cc. and reëlectrolyzed for forty-two hours. The tests of the contents

of cells 2 and 4 taken separately and together are outlined below (Table III). The seeding used was 0.0133 mg. of moist yeast per cc.

TABLE III

FRACTIONATION OF YEAST EXTRACT

Addition to synthetic medium	Yeast crops, mg. of moist yeast per cc.	Δ above blank
Blank	0.10[a]	...
1 mg. from cell 2 (P_H 3.8)	.15	0.05
2 mg. from cell 2	.24	.14
1 mg. from cell 4 (P_H 9.2)	.12	.02
2 mg. from cell 4	.20	.10
1 mg. from cells 2 and 4 (50% each)	.26	.16
2 mg. from cells 2 and 4 (50% each)	.70	.60

[a] The blank in this particular experiment was lost but we were able to judge its value very closely from numerous previous runs.

We found it more difficult to separate the two factors completely when yeast extract was used, than when rice bran extract was used. This fact will be commented upon in the discussion.

In a similar way an extract of milk was electrolyzed. To whole milk was added 150% of its volume of absolute methanol. The mixture was warmed for thirty minutes on a water-bath and after flocculating with a few drops of aluminum sulfate solution, the solution was filtered clear and evaporated to dryness. About 0.200 g. of the residue was dissolved in 100 cc. of water and electrolyzed for forty-one hours. The results are shown in Table IV. The seeding was 0.0128 mg. of moist yeast per cc.

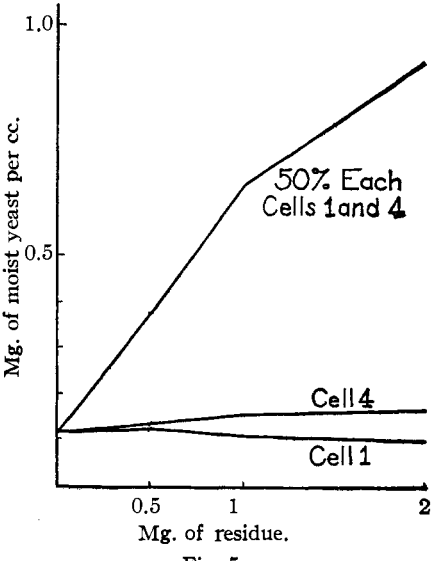

Fig. 5.

In the milk extract, again, as in the case of the yeast extract a single electrolysis did not effect a complete separation. Nevertheless, in the above experiment a definite supplementary action is shown between the contents of cells 2 and 4. While 1 mg. of the contents of cells 2 and 4 produced increments of growth of 0.03 and 0.02, respectively, the two together produced a growth increment of 0.09. Whereas 2 mg. of numbers 2 and 4 separately produced increased growths of 0.08 and 0.05, the two together produced an increment of 0.19. While this effect is not very striking it was

TABLE IV

EXPERIMENT WITH MILK

Addition to synthetic medium	Yeast crops, mg. of moist yeast per cc.	Δ above blank
Blank	0.13	...
2 mg. extract of milk (untreated)	.32	0.19
4 mg. extract of milk	.65	.52
1 mg. from cell 2	.16	.03
2 mg. from cell 2	.21	.08
4 mg. from cell 2	.32	.19
1 mg. from cell 4	.15	.02
2 mg. from cell 4	.18	.05
4 mg. from cell 4	.17	.04
2 mg. from cells 2 and 4 (50% each)	.22	.09
4 mg. from cells 2 and 4 (50% each)	.32	.19

sufficient to convince us that milk contains two factors which are separable by the electrolytic method.

Characteristics of the Two Factors.—Using collodion bags dried in 40, 60 and 80% alcohol after the manner outlined by Brown,[9] we found the acid factor, that is, the one which accumulates in the anode cell, to be readily dialyzable through all of the membranes. The basic factor on the other hand was found to dialyze very slowly through the bag which had been treated with 40% alcohol, and very little if any through the others. These results are in accordance with Wildiers' observation that "bios" is dialyzable, because the undialyzable material *by itself* has practically no effect on yeast growth, whereas the material which passes through is likely to have some of both factors in it and thus show growth stimulating activity.

That the active materials are both organic in nature is indicated by the fact that on ignition each loses its activity. We found, however, that the ash from the factor derived from yeast improved our synthetic medium slightly for Wildiers' yeast and will investigate this point further.

Effect on Different Yeasts.—Having convinced ourselves of the existence of separable factors in various food materials, which stimulate the growth of Wildiers' yeast, we next turned to the question of what the effect of these preparations might be on yeast other than Wildiers'.

In Table V is shown the effect of two particular electrolytic preparations R_{91} ($P_H < 3.6$) and R_{94} ($P_H > 9.6$) obtained from rice bran essentially as outlined before, on five different yeasts.

It will be observed that Wildiers' yeast stands out as the exception in that it is the only one for which the particular preparations show a supplementary action. (It should be pointed out, however, that supplementary factors prepared in other ways have been reported for all except one of the yeasts, *i. e.* Gebrüde Mayer, listed in the table.)

[9] W. Brown, *Biochem. J.*, **9**, 591 (1915).

TABLE V

COMPARISON OF DIFFERENT YEASTS

Addition to synthetic medium	Yeast crops, mg. of moist yeast per cc. Wildiers'	578	O. P.	Gebrüde Mayer	W. L. Miller's
Blank	0.12	0.11	0.12	0.25	0.25
0.5 mg. R_{91}	.13	.27	.25	1.27	1.40
1.0 mg. R_{91}	.115	.47	.40	1.96	0.81
0.5 mg. R_{94}	.14	.08	.04	0.24	.22
1.0 mg. R_{94}	.16	.06	.03	.17	.47
0.5 mg. (50% R_{91}–50% R_{94})	.38	.19	.08	.60	.70
1.0 mg. (50% R_{91}–50% R_{94})	.67	.29	.12	.94	1.08
Seeding used	.0142	.008	.009	.0146	0.0116

Subsequent to carrying out the above experiments we were able to obtain through the kindness of Dr. J. C. Drummond of University College, London, a culture of the yeast (No. 2190 of the National Collection of Type Cultures (London)) with which the recent experiments of Narayanan were carried out. He was able to concentrate the active factor for this yeast but observed no supplementary relationships between any of his preparations.

Our first results using rice bran extract led us to think that the same factors which are concerned in the growth of Wildiers' yeast are also effective for Narayanan's yeast. Later experiments throw doubt on this point though there remains no doubt that Narayanan's yeast is affected by factors which can be separated by fractional electrolysis.

The supplementary action of two pairs of electrolytic rice bran preparations for Narayanan's yeast is shown in Table VI. The seedings were 0.014 and 0.0084 mg. of moist yeast per cc., respectively.

TABLE VI

SUPPLEMENTARY FACTORS FOR NARAYANAN'S YEAST

Addition to synthetic medium	Yeast crops, mg. of moist yeast per cc.	Δ above blank
Blank	0.03	
0.5 mg. A_7 (P_H 4.6)	.03	0
1.0 mg. A_7	.03	0
0.5 mg. B_7 ($P_H > 9.6$)	.05	0.02
1.0 mg. B_7	.06	.03
0.5 mg. (50% A_7 + 50% B_7)	.07	.04
1.0 mg. (50% A_7 + 50% B_7)	.21	.18
Blank	.03	
1 mg. A_4 (P_H 4.0)	.03	0
1 mg. B_4 ($P_H > 9.6$)	.04	.01
1 mg. (50% A_4 + 50% B_4)	.36	.33

The factors for Narayanan's yeast appear not to be completely identical with those for Wildiers' yeast because the preparation A_7 (referred to above) by itself stimulated the growth of Wildiers' yeast very appreciably, while it

had no stimulating effect on Narayanan's yeast. It would appear that Narayanan's yeast needs one factor not required by Wildiers' yeast. However, we have not investigated the case sufficiently to draw any definite conclusion except as to the effectiveness of two supplementary preparations for Narayanan's yeast. The value of the electrolytic method is demonstrated in the study of the nutrilites for this yeast.

Discussion

Theory of the Electrolytic Method.—The general theory underlying the method of separation has been outlined by Williams and Waterman.[8] A gradient of P_H values is established between the anode cell and the cathode cell and an ampholyte tends to move toward the cell possessing the P_H value which approximates the isoelectric point of the ampholyte. In a solution which has a lower P_H than its isoelectric point, the ampholyte ionizes as a base and travels toward the negative electrode. In a solution which has a higher P_H than its isoelectric point the ampholyte ionizes as an acid and travels to the positive electrode. When the ampholyte reaches that portion of the circuit which is at its isoelectric point, no further movement should take place.

In carrying out numerous electrolyses we have made a number of observations. Of these perhaps the most important is that relatively complete separations are impossible unless one electrolyzes dilute solutions of the extracts and uses high potential to force appreciable amounts of current through. We attribute this behavior primarily to the presence of inorganic salts which, when present, cause the anode cell to become very strongly acid and the cathode cell very strongly alkaline with the result that there is not a suitable gradient between them. When the solution is very dilute, however, these acidities and alkalinities do not develop.

In the experiments of Williams and Waterman[8] in which they electrolyzed a solution containing quinine sulfate, anthranilic acid and valine, we note that the more nearly neutral substances were segregated less successfully. We attribute this to the fact that most of the conductance of the solution was due to the H^+, OH^- and $SO_4^=$ ions and the approximately neutral ampholytes carried very little of the current. From our experiments we judge that the elimination of the extraneous ions and the use of a high potential on the dilute solution would have resulted in a much sharper separation.

We also observed a marked difference in the ease of electrolytic separation of the yeast nutrilites when rice bran and yeast extracts are used. We were inclined at first to conclude that yeast extract contains a single nutrilite which is effective by itself, *in addition* to the two supplementary factors. Later experiments throw doubt on this interpretation, however, since the difference may be explained in terms of the differences between the salts and ampholytes with which the potent material is associated in the

two materials. When a very dilute solution of yeast extract was used a good separation was effected.[10]

It seems reasonable to assume that if the potent ampholyte is present in the medium in very minute amounts and is unaccompanied by other ampholytes with similar P_H values, there is small chance that the potent substances will be segregated in any one compartment, unless the number of compartments used is large.

Possible Applications.—In view of our results it appears that the tool to which Williams and Waterman have called attention, has possibilities not only in the way of studying in a preliminary way the characteristics of physiologically potent substances, as they suggest, but also in their actual separation and concentration, provided the materials are potent in minute amounts and are capable of being previously concentrated by some other method. In other words, the method is not one which is adapted to working over large amounts of crude material but may be of value in dealing with small amounts of highly concentrated material.

We believe that the method may find very important applications in the concentration and study of vitamins, hormones, enzymes and other physiologically active principles. A study of its possibilities and limitations is being continued in this Laboratory.

Number of Yeast Nutrilites.—When work dealing with the concentration of "bios" was started in this Laboratory several years ago, it was assumed that "bios" was a single substance which affected growth of various yeasts and probably other organisms as well. A continued study of this question using cultures of yeast obtained from most of the investigators in this field has convinced us that the situation is much more complex. For yeast No. 578 of the American Type Culture Collection (new catalog number 2331) we have shown[7] the existence of four separable nutrilites, three of which supplement one another and the fourth is interchangeable with one of these three. For the yeast used in the laboratory of W. L. Miller, we have confirmed the finding that at least two supplementary substances are effective. As well as we are able to judge these factors are distinct from those required by yeast 578, though one factor may be in common. As reported in the present paper, the "bios" of Wildiers is proved to be at least two supplementary factors. At least one of these is distinct from the factors required by the yeast of W. L. Miller. Furthermore, the yeast used by Narayanan likewise requires two (and probably three) factors one of which appears from incomplete evidence to be different from those required by Wildiers' yeast. The only yeast so far investigated which appears to require only one nutrilite is Gebrüde Mayer yeast. We are investigating this further.

[10] However, a recent private communication from Dr. Drummond leads us to suspect that our first interpretation mentioned above may be correct.

From the above discussion it is clear that experimental work which has been carried on in this Laboratory indicates that there are probably not less than seven yeast nutrilites, which are effective for what has been considered one species of yeast. The indications are that all of these nutrilites are effective in very high dilutions. We have never observed an effect from any amino acid or amino acid mixture, "alpha bios," "beta bios," inositol (Bios I) or any other pure substance (with the exception of Jansen and Donath's antineuritic vitamin), which has been comparable to the effects produced by impure concentrates.

Significance for Vitamin Research.—If one takes the position that research on single-celled organisms should throw light on the behavior of more complex organisms, there are some inferences which can be drawn from the results of investigations on yeast nutrilites. If a single type of yeast is affected, as indicated above, by minute quantities of as many as four different nutrilites of a water soluble and heat stable type, and different yeasts which are morphologically indistinguishable and which have been regarded as belonging to the same species, require *different* nutrilites, it would seem unlikely, for example, that a single definite chemical substance is *solely* effective in the prevention of polyneuritis in the numerous forms of birds and mammals which are known to carry on a varied metabolism. It has already been demonstrated that carotene is one of the two substances which can function as vitamin A and that more than one irradiated sterol is effective as vitamin D. It seems likely that in nature, in the case of various vitamins (and perhaps hormones), substitutionary substances are available and that the vitamin (or hormone) potency of a given material may, in general, be due to the presence of several substances rather than a single substance. Perhaps in these cases nature prefers not "to carry all her eggs in one basket," and it is possible that research in the purification of vitamins and hormones might progress faster in some cases if investigators considered this as a serious probability.

We wish to express our indebtedness to Standard Brands, Inc., successors to the Fleischmann Co., etc., and to the Research Council of the University of Oregon for generous support of this work.

Summary

1. Most of the previous work dealing with yeast nutrilites has been concerned with the nutrilite requirements of yeasts which differ physiologically from Wildiers' yeast.

2. By use of fractional electrolysis of extracts of rice bran, yeast, malt sprouts and milk, the "bios" of Wildiers has been divided into two fractions which are supplementary.

3. The theory and possible applications of the electrolytic method to the study and concentration of vitamins, hormones and enzymes, are discussed.

4. The complex situation with regard to yeast nutrilites suggests the probability that the specific vitamin (or hormone) potency of a given material may be due to several substances which perform the same function, rather than one single substance.

EUGENE, OREGON

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

A NEW METHOD FOR THE PREPARATION OF SYRINGIC ALDEHYDE[1]

BY W. M. MCCORD

RECEIVED JULY 31, 1931 PUBLISHED NOVEMBER 5, 1931

During the progress of research in the Sterling Laboratory dealing with the synthesis of some new aromatic ethers related in structure to thyroxine, it became necessary to develop a method for synthesizing syringic aldehyde, which would be free from experimental difficulties encountered in applying methods hitherto described and recommended by other investigators. Graebe and Martz[2] were the first to prepare the aldehyde by condensing 2,6-dimethoxyphenol with chloroform in alkaline solution. Their method is not productive of the aldehyde in good yields. Mauthner[3] developed later a method of synthesis by condensing 2,6-dimethoxyphenol with ethyl mesoxalate, and then converting his condensation product to syringic aldehyde by hydrolysis. Pauly and Strassberger[4] recently have improved on Mauthner's technique by substituting chloral hydrate for ethyl mesoxalate and prepared the aldehyde from 2,6-dimethoxyphenol according to the patented procedure recommended for the manufacture of vanillin.[5] Both methods of operating require a supply of 2,6-dimethoxyphenol, which is a reagent that is not easily prepared in quantity with ordinary laboratory equipment.

Späth[6] applied the Rosenmund technique[7] for a syringic aldehyde synthesis by reducing catalytically carbethoxysyringic acid chloride in the presence of palladium but here again the yield is small and the purification of the aldehyde is difficult. We now find that the Späth method can be modified and syringic aldehyde prepared easily from syringic acid without

[1] Constructed from a dissertation presented by the author in June, 1931, to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy. (T. B. Johnson.)

[2] Graebe and Martz, *Ber.*, **36**, 1031 (1903).

[3] Mauthner, *Ann.*, **395**, 273 (1912).

[4] Pauly and Strassberger, *Ber.*, **62**, 2279 (1929); Pauly and Schanz, *ibid.*, **56**, 979 (1923).

[5] U. S. Patent 1,536,732 (1924).

[6] Späth, *Monatsh.*, **41**, 278 (1920).

[7] Rosenmund, *Ber.*, **51**, 598 (1918).