[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF THE UNIVERSITY OF OREGON AND OREGON STATE COLLEGE]

"Pantothenic Acid," A Growth Determinant of Universal Biological Occurrence

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In our study of the substances involved in yeast growth stimulation carried out through a period of years, we first investigated extensively the nutrilite requirements of yeast number 578 (2331) of the American Type Culture Collection, and found that several unknown components must be present in order to stimulate its growth[1,2,3] one of which may be the anti-neuritic vitamin[4] of Jansen and Donath.

Preliminary studies had been made upon a number of yeasts[5] and we attempted to find other "strains" of *Saccharomyces Cerevisiae* which might have simpler requirements. The original strain used by Wildiers in his discovery of "bios" was investigated and the hypothetical "bios" was found beyond question to consist of two substances which were separable by fractional electrolysis, one an acid substance and the other basic.[6]

Our work is now centered upon the requirements of the "Gebrüde Mayer" strain of *Saccharomyces Cerevisiae.* The work here reported indicates that a single acid substance is capable of stimulating its growth in a remarkable way and that this acid substance is a very widespread if not universal constituent of living matter.

To assure ourselves that we would be utilizing a seed yeast which functions as a single type of yeast, the usual bacteriological methods were used to separate the different types if present. Sub-cultures growing on the surface of agar plates, slightly below the surface (yellowish), and wholly beneath the surface (which appeared whiter), were tested in comparison with the original culture, and all were found to behave identically, within experimental errors, toward nutrilite preparations.

Effect as a Growth Determinant.—With a modification of the thermocouple method for determination of the amount of yeast in suspensions[7] whereby we use a cell of 4 mm. thickness for heavier suspensions and a cell of 52 mm. thickness for more dilute suspensions, we are now able to determine directly with satisfactory accuracy amounts of moist yeast ranging from about 0.0001 mg. per cc. up to 4 mg. per cc. This makes possible the demonstration of the remarkable stimulating effects with which we are concerned. The results of the experiment listed in Table I are self-evident.

(1) Williams, *J. Biol. Chem.*, **38**, 465 (1919).
(2) Williams, Wilson and von der Ahe, THIS JOURNAL, **49**, 227 (1927).
(3) Williams and Bradway, *ibid.*, **53**, 783 (1931).
(4) Williams and Roehm, *J. Biol. Chem.*, **87**, 581 (1930).
(5) Williams, Warner and Roehm, THIS JOURNAL, **51**, 2764 (1929).
(6) Williams and Truesdail, *ibid.*, **53**, 4171 (1931).
(7) Williams, McAlister and Roehm, *J. Biol. Chem.*, **83**, 315 (1929).

TABLE I

EFFECT ON YEAST MULTIPLICATION

Yeast seeding, 0.00004 mg. per cc.

Amt. (mg.) of rice bran extract added to 1 cc. of synthetic medium	Yeast crops, mg. per cc. after 18 hrs.	Fold increase	Amt. (mg.) of rice bran extract added to 1 cc. of synthetic medium	Yeast crops, mg. per cc. after 18 hrs.	Fold increase
0.00	0.00045	11	0.64	0.104	2,600
.00	.00052	12	1.28	.111	2,775
.02	.0025	60	2.56	.143	3,560
.04	.007	175	5.0	.285	7,125
.08	.017	425	10.0	.46	11,500
.16	.023	560	20.0	.49	12,250
.32	.058	1450	40.0	.64	16,000

The extract used in this experiment was unrefined material obtained directly from rice bran and hence contained a variety of different substances. Our results as reported later in this paper indicate, however, that substantially the whole of the effect of the rice bran extract should be attributed to a single substance which probably constitutes a very minute portion of the crude extract. Indeed we have reason to think that the purified substance if available would have a more striking effect (and in vastly lower doses) than the crude material. This is because the crude material gives indications of having in it substances which are toxic to yeast even when used in doses of the order of 0.05 mg. per cc. of medium. In our routine tests we always use two or more dilutions of the active substance in order to detect the presence of toxic substances. Unless doubling the dosage results in approximately a doubling of the yeast growth, we attribute the effect to toxic material. Materials which are relatively free from toxicity show up in the tests on some of the electrolytic preparations from sea urchin eggs, earthworms, mold, etc., detailed later.

It is to be noted that even with the rice bran extract which contains a small amount of toxic material, the maximum growth has by no means been reached in the experiment cited. Parenthetically it may be remarked that this particular strain of yeast probably grows more rapidly on a synthetic medium than any other yeast we have investigated.

Electrolysis and Testing of Diverse Tissue Extracts.—Previous experience led us to suspect that the nutrilite for this strain of yeast would be found widespread in nature. We desired, however, to study its distribution further and particularly to find out if the same substance is the responsible agent present in different tissues.

The method of fractional electrolysis, which not only separates substances with different isoelectric points but differentiates electrolytes from non-electrolytes, impressed us as a useful method by which we could test the identity of the active material from different sources. By its use we had been able for the first time to separate Wildiers "bios" into two fractions which were almost entirely without effect when used separately.

From the materials enumerated in Table II we attempted to obtain a water soluble extract with as little colloidal matter as possible. In general the procedure was to extract with 80% methanol (making allowance for the water in the tissue if necessary) for a half hour under a reflux. The filtered extract was evaporated to dryness, taken up with water and filtered clear with kieselguhr if necessary. In several cases, especially if the material to be extracted was of fatty character, the material was first extracted with a little ether and the ether-soluble material discarded. In several cases the ether-soluble material was tested for nutrilite and was always found to be negligibly weak.

TABLE II

MATERIALS FOR ELECTROLYTIC EXPERIMENTS

Material extracted	Treatment previous to electrolysis	Dry weight material electrolyzed, mg.	Equivalence in moist tissue, approx. g.	Length of electrolysis, hours
Rice bran	60% methanol used	200	2	75 hrs. (500 v.)
Beef liver	Dried, ether ext. discarded	100	2	30
Crab eggs (*Cancer productus*)	Dried, ether ext. discarded	80	0.8 (dry)	47
Sea urchin eggs (*Strongylocentrotus purpuratus*)	Dried	61	0.75 (dry)	48
Oyster	Dried, ether ext. discarded	53	1.5	48
Earthworms		34	3.0	48
Planarian worms		8	1	48
Slime mold (*Physarum polycephalum*)	Dried	14	0.09 (dry)	48
Bacteria (*B. subtilis*)	Dried	80	0.4 (dry)	
Mold (*Aspergillus niger*)	Trace of H_3PO_4 added	50	4	48
Algae (*Spyrogyra* and *Oscillatoria* mixed)	Dried, ether ext. discarded	50	1	48
Milk	Ext. dried, abs. MeOH used	109	15	48
Egg white		50	8.0	48

A specified amount of the water-soluble material (usually less than 100 mg.) was finally dissolved in 415–425 cc. of water and the solution used to fill exactly an 8-cell electrolysis system similar to the 4-cell battery used in an earlier study.[6] A 1500 volt d. c. motor generator was used to bring about most of the electrolyses and current was passed for thirty to forty-eight hours, during which time the conductivity became very low. At the start the current passing was from 1–6 milliamperes, depending upon the material and the concentration used.

At the end of the electrolysis period the P_H values of the different cells were determined and in some cases dry weights were obtained. For determining P_H values the quinhydrone electrode was generally used. In some of the cells there was a very low concentration of electrolyte present and the solutions were practically unbuffered. In order to overcome the

lack of conductivity of the solutions the vacuum tube was utilized according to the method of Goode[8] with excellent results. There were a few solutions, however, which were so lacking in buffering substances that the *P*H values were inconstant and meaningless. These values are omitted from the tables. In some of the later experiments the *P*H values were determined only colorimetrically. These values and any others which were somewhat in doubt are indicated in parentheses.

The contents of each of the eight cells after electrolysis were tested for their effect on the growth of the yeast, according to the method developed in this Laboratory. The proportion of the total cell contents used in each culture is indicated in each case, as well as the amount of the yeast crops obtained (Tables III–XV).

TABLE III

FRACTIONAL ELECTROLYSIS OF RICE BRAN EXTRACT[a]

Cell tested	Dry material in cell, mg.	*P*H cell contents	Dry material used in 12 cc. cultures, mg. Test 1	Test 2	Test 3	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2	Test 3
Blanks	..	...	0	0	0	0.10	0.10	0.09
1	31.6	2.6	0.25	0.5	1	.13	.125	.13
2	31.6	3.5	.25	.5	1	.28	.39	.48
3	30.5	3.9	.25	.5	1	.28	.36	.48
4	25.9	4.1	.25	.5	1	.24	.33	.48
5	25.1	4.8	.25	.5	1	.22	.28	.56
6	18.5	5.6	.25	.5	1	.10	.14	.19
7	15.3	7.2	.25	.5	1	.09	.12	.24
8	30.5	9.4	.25	.5	1	.09	.09	.12

[a] This electrolysis was for seventy-five hours at 500 volts. A re-electrolysis of the contents of cells Nos. 2, 3, 4 and 5 using 5000 volts for twenty hours caused the active substance to accumulate most in a cell with a *P*H of 3.6.

TABLE IV

FRACTIONAL ELECTROLYSIS OF BEEF LIVER EXTRACT

Percentages of cell contents used in tests (12 cc.): none in blanks; 2 in Test 1; 4 in Test 2.

Cell tested	*P*H of cell	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2
Blanks	..	0.12	0.11
1	2.6	.51	.73
2	3.0	.49	.69
3	3.4	.52	.64
4	3.4	.49	.60
5	(4.6)	.18	.26
6	(4.1)	.10	.12
7	(4.45)	.12	.14
8	7.9	.10	.14

TABLE V

FRACTIONAL ELECTROLYSIS OF EXTRACT OF CRAB'S EGGS

Percentages of cell contents used in tests (12 cc.): none in blanks; 1 in Test 1; 2 in Test 2; 4 in Test 3.

Cell tested	*P*H of cell contents	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2	Test 3
Blanks		0.08	0.08	
1	2.4	.47	.56	0.52
2	3.3	.70	.68	.67
3	4.1	.76	.72	.72
4	5.2	.10	.16	.18
5	5.25	.08	.08	.07
6	..	.09	.08	.07
7	5.4	.09	.09	.08
8	9.1	.05	.05	.05

(8) Goode, THIS JOURNAL, **47**, 2483 (1925).

TABLE VI

FRACTIONAL ELECTROLYSIS OF EXTRACT OF SEA URCHIN EGGS

Percentages of cell contents used in tests: none in blanks; 1 in Test 1; 2 in Test 2; 4 in Test 3.

Cell tested	PH of cell contents	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2	Test 3
Blanks	...	0.10	0.10	..
1	2.6	.16	.22	0.30
2	3.2	.25	.39	.62
3	3.8	.29	.47	.78
4	5.8	.08	.08	.10
5	6.0	.08	.08	.10
6	6.1	.09	.09	.10
7	6.8	.10	.10	.10
8	8.3	.05	.08	.06

TABLE VII

FRACTIONAL ELECTROLYSIS OF OYSTER EXTRACT

Percentages of cell contents used in tests: none in blanks; 1.7 in Test 1; 3.4 in Test 2; 6.8 in Test 3.

Cell tested	PH of cell contents	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2	Test 3
Blanks	...	0.09	0.09	..
1	2.6	.10	.12	0.20
2	3.8	.26	.41	.58
3	4.0	.23	.35	.52
4	...	.12	.19	.26
5	4.9	.12	.20	.31
6	5.3	.15	.23	.35
7	...	.17	.27	.42
8	7.9	.13	.17	.22

TABLE VIII

FRACTIONAL ELECTROLYSIS OF EXTRACT OF EARTHWORMS

Percentage of cell contents used in tests: none in blanks; 1 in Test 1; 2 in Test 2; 4 in Test 3.

Cell tested	PH of cell contents	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2	Test 3
Blanks	...	0.06	0.06	..
1	2.8	.04	.05	0.06
2	4.1	.26	.54	1.32
3	5.1	.08	.11	0.21
4	5.2	.06	.07	.08
5	...	.06	.06	.07
6	...	.06	.06	.065
7	6.5	.06	.06	.06
8	8.4	.05	.05	.05

TABLE IX

FRACTIONAL ELECTROLYSIS OF AN EXTRACT OF PLANARIAN WORMS

Percentage of cell contents used in tests: none in blanks; 33 in Test 1; 67 in Test 2.

Cell tested	PH of cell contents	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2
Blanks	...	0.09	0.09
1	3.4	.88	1.07
2	5.1	.26	0.48
3	...	.11	.13
4	...	.10	.11
5	...	.09	.11
6	...	.10	.11
7	...	.06	.07
8	...	.06	.06

TABLE X

FRACTIONAL ELECTROLYSIS OF SLIME MOLD EXTRACT

Percentage of cell contents used in tests: none in blanks; 1 in Test 1; 2 in Test 2; 4 in Test 3.

Cell tested	PH of cell contents	Yeast crops Test 1	Test 2	Test 3
Blanks	...	0.06	0.06	..
1	2.8	.09	.11	0.13
2	3.2	.07	.09	.15
3	3.5	.10	.16	.24
4	3.7	.13	.20	.30
5	...	.06	.06	.06
6	...	.06	.06	.06
7	5.9	.06	.06	.06
8	7.9	.06	.06	.06

TABLE XI

FRACTIONAL ELECTROLYSIS OF B. SUBTILIS EXTRACT

Percentage of cell contents used in tests: none in blanks; 7 in Test 1; 14 in Test 2.

Cell tested	PH of cell contents	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2
Blanks	...	0.14	0.14
1	3.0	.21	.28
2	4.2	.18	.24
3	4.7	.14	.14
4	5.4	.12	.13
5	5.5	.12	.125
6	5.7	.12	.125
7	6.0	.12	.13
8	7.7	.07	.07

TABLE XII

FRACTIONAL ELECTROLYSIS OF MOLD EXTRACT

Percentage of cell contents used in tests: none in blanks; 1 in Test 1; 2 in Test 2; 4 in Test 3.

Cell tested	PH of cell contents	Yeast crops Test 1	Test 2	Test 3
Blanks	...	0.05	0.05	..
1	2.7	.10	.16	0.20
2	3.2	.11	.20	.31
3	3.5	.16	.26	.38
4	3.7	.26	.42	.60
5	(4.9)	.05	.05	.05
6	(5.7)	.05	.05	.05
7	5.2	.05	.05	.05
8	7.9	.05	.06	.05

TABLE XIII

FRACTIONAL ELECTROLYSIS OF ALGAE EXTRACT

Percentage of cell contents used in tests: none in blanks; 1 in Test 1; 2 in Test 2; 4 in Test 3.

Cell tested	PH of cell contents	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2	Test 3
Blanks	...	0.05	0.06	..
1	2.3	.07	.08	0.08
2	2.8	.08	.10	.12
3	3.8	.10	.16	.27
4	4.7	.08	.08	.09
5	5.0	.08	.09	.09
6	5.2	.08	.08	.08
7	8.5	.08	.08	.07
8	9.0	.07	.07	.06

TABLE XIV

FRACTIONAL ELECTROLYSIS OF MILK EXTRACT

Percentage of cell contents used in tests: none in blanks; 1 in Test 1; 2 in Test 2; 4 in Test 3.

Cell tested	PH of cell	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2	Test 3
Blanks	...	0.04	0.03	..
1	2.8	.12	.16	0.26
2	(3.2)	.17	.31	.40
3	(4.0)	.22	.34	.46
4	...	.04	.04	.06
5	...	.05	.05	.04
6	...	.04	.04	.04
7	(7.0)	.05	.04	.06
8	(7.4)	.04	.03	.06

TABLE XV

FRACTIONAL ELECTROLYSIS OF EXTRACT OF EGG WHITE

Percentages of cell contents used in tests: none in blanks; 1 in Test 1; 2 in Test 2; 4 in Test 3.

Cell tested	PH of cell	Yeast crops, mg. of moist yeast per cc. Test 1	Test 2	Test 3
Blanks	...	0.07	0.07	..
1	(2.8)	.23	.21	0.26
2	(4.3)	.24	.35	.40
3	...	.19	.26	.36
4	...	.08	.09	.09
5	...	.07	.07	.08
6	...	.06	.06	.07
7	(7.0)	.06	.08	.06
8	(8.0)	.06	.06	.06

We are indebted to Mr. John C. Queen of Marshfield, Oregon, for collecting and drying the crab eggs, to Dr. A. R. Moore for the sea urchin eggs and the slime mold culture, and to Dr. Rosalind Wulzen for some of the planarian worms used in her nutrition studies.[9] The bacteria culture was grown by us in a potato extract peptone broth medium and the organisms were washed three times centrifugally. The mold was also grown in our laboratory on the synthetic medium which had been used previously for mold culture.[10]

The electrolytic experiments reported were supplemented by a number of others which are not described for lack of space. Results similar to those reported were obtained on extracts of tomatoes and potatoes. Several of

(9) Wulzen and Bahrs, *Physiol. Zoöl.*, **4**, 204 (1931).

(10) Williams and Honn, *Plant Physiol.*, **4**, 629 (1932).

the electrolyses were repeated under slightly different conditions. All gave concordant results.

After most of these experiments had been completed, we obtained a few grams of tubercle bacilli through the kindness of the McDermott Foundation of Seattle, Washington. These were found to be rather rich in yeast growth stimulant. The extract was not electrolyzed, as this seemed superfluous in view of the numerous other electrolytic tests that had been made.

The somewhat increased growth promoting power shown in cells 6 and 7, Table VII, is significant rather than accidental because the same results were obtained in two different experiments in which oyster extract was electrolyzed. This point will be mentioned later.

Interpretation and Extension of the Electrolysis Experiments.—In accordance with the theory underlying the fractional electrolysis,[6,11] we at first assumed that the behavior of the unknown substance in becoming concentrated during electrolysis at a definite P_H value was due to its arrival at its isoelectric point, from which position it did not migrate. Attempts were made to check this conclusion by causing the substance to migrate *from the acid* end of the system by making the solution acidic enough so that theoretically this should happen readily if the unknown amphoteric substance had an isoelectric point of about P_H 3.6. In none of our experiments, however, was there an appreciable tendency to migrate from the most acidic cells even when these cells had a P_H value as low as 1.95.

We sought to find the reason for the behavior by subjecting to electrolysis mixtures containing, in one case, the methyl and another case the ethyl ester of the unknown substance. The formation of these esters is discussed below. As such the esters have no effect on yeast growth, but they can be evaluated by hydrolyzing the contents of each cell under controlled conditions and then testing for the effect on yeast growth. The results showed that in neither case was there any migration of the ester toward the basic end of the system. In fact there was still an appreciable migration *toward the acidic end.* The conclusion seemed inevitable that the substance in question is not amphoteric, since its esters appear to have no basic properties.

A calculation showed that the behavior we had observed in electrolyzing the various extracts is exactly what should be expected in the case of an alcohol-acid with no basic groups. From the equation for the ionization constant of an acid

$$\frac{[H^+][A\bar{n}]}{M - [A\bar{n}]} = K$$

where M is the total molar concentration of the acid, we obtain

$$[A\bar{n}] = KM/(K + [H^+])$$

(11) R. R. Williams and Waterman, *Proc. Soc. Expil. Biol. Med.*, **27**, 56 (1929)

from which, dividing each side by M, we get

$$[A\bar{n}]/M = K/(K + [H^+])$$

It is obvious, therefore, that the proportion of the acid in the ionized form, $[A\bar{n}]/M$, at a given hydrogen-ion concentration is constant and independent of the total concentration of the acid substance. Thus an acid with ionization constant of 2×10^{-5} would be 0.67 in the ionized form at P_H 5, 0.17 at P_H 4, 0.02 at P_H 3 and 0.002 at P_H 2. In each case this would be true regardless of the total concentration of the acid.

A weak acid in the presence of buffering substances should migrate, in an electric field, regardless of its concentration to the same region of acidity where its ionization is too low to be effective. It should always accumulate in the same position provided the electrolysis is carried out in the same way. It is obviously impossible to carry out electrolyses in exactly the same way because the presence of foreign substances influences the character of the results. The character of the P_H gradient is presumably determined almost wholly by the substances with which the acid in question is associated because the concentration of the acid itself is probably negligibly small.

The slight migration of the ester toward the acidic end should be expected in the case of a polyhydroxy acid. We have demonstrated that under the conditions of our experiments glucose (in pure water solution) migrates slightly toward the acidic end. The non-amphoteric character of the acid is further indicated by an experiment in which adsorption by fuller's earth was measured at different P_H values. It was found that the absorption increased in a regular fashion from P_H 6.5 to 0.9. If the acid is amphoteric, its isoelectric point should lie in this range, in which case one would expect a break in the adsorption curve at this point rather than the regular increase actually observed.

Certainly there is nothing in our electrolysis results (except in the case of the oyster extract) to suggest the presence of more than one substance which is active in the stimulation of the growth of the yeast. In the case of the oyster the effect of the second substance was comparatively small. We do not, however, regard the results as definite and conclusive enough to constitute strong proof of the identity of the stimulating principle from different sources. The results may be explained by the existence of various similar growth stimulating acids, no one of which may be present in all of the tissues examined.

We therefore sought to obtain more definite evidence by subjecting materials from diverse sources to a number of different treatments and tests to see if the active principle behaves in the same way regardless of its sources. These experiments had also another important purpose, namely, to find out the chemical nature of the active substance (or substances).

Diffusion Experiments.—Since we are able to obtain quantitative data with regard to the amount of yeast growth stimulant in a solution, we made studies on the rate of diffusion of the active substance, in order to throw light on its molecular weight and the constancy of this value as determined when different sources were used.

The determinations were made in the apparatus used by Friedman and Klemm[12] and we are indebted to these colleagues for their help in making the determinations. The diffusion constants were calculated from the equation

$$D = \frac{\log\left(1 + \frac{C_o}{C_i}\right) - \log\left(1 - \frac{C_o}{C_i}\right)}{Kt}$$

where C_i and C_o are the concentrations inside and outside the cell, respectively, when diffusion ended. K is the cell constant as determined previously by potassium chloride diffusion, and t is the time of the diffusion. The results are given in Table XVI.

TABLE XVI

DIFFUSION DATA

Extract used	Cell constant	Time of diffusion, hours	$\frac{C_o}{C_i} \times 100$ Test 1	Test 2	Test 3	Test 4	Av.	Diffusion constant at 25°
Rice bran	0.002145	31.4	5	5.5	5.7		5.4	0.725
Oyster	.00322	39.4	6.86	10.2	20.4		12.5	(.858)
Beef liver No. 1	.002145	39.06	6.21	8.11	8.86		7.72	.803
Beef liver No. 2	.002145	24	4.4	4.3	(3.2)		4.38	.738
Aspergillus niger	.00322	24	6.46	8.13	6.93	7.25	7.19	.809

These results are more reliable than the somewhat lower values given in our preliminary communication[13] because most of the earlier values were obtained by taking samples from time to time during diffusion, a procedure which was later found to be faulty. However, the method is not susceptible to a high degree of accuracy and, in addition, we have the uncertainties involved in the biological assay of the substance in question. The values obtained indicate that the same substance is the effective constituent of the different extracts and that its molecular weight is about 150. We hope to get more accurate values by preparing material for diffusion which will be freer from toxic material than any we have used so far.

When toxic material is present and diffuses at a rate different from the active substance, the quantitative determinations are thrown off badly. This was partly responsible for the very irregular results shown in the case of the oyster extract, though the second growth stimulating substance in the oyster extract was a complicating factor.

Diffusion was also studied using an egg white extract as the starting material. The results in this case were surprisingly low and irregular, the

(12) Friedman and Klemm, THIS JOURNAL, **54**, 2637 (1932).

(13) *Ibid.*, **54**, 3462 (1932).

substance diffusing more slowly than many colloids. We suspected that proteins (ovomucoid, etc.) might be interfering with the passage of the substance through the sintered glass diaphragm. Unfortunately the apparatus had been dismantled before we were able to clear the matter up, but a study of diffusion from colloid-free extracts through cellophane showed that the active material from egg white diffused so rapidly that at the end of twelve hours at room temperature equilibrium was nearly reached. Similar results were obtained in four tests. Evidently the substance from egg white is capable of diffusing readily as in the case of the other materials.

Hydrogenation Experiments.—In order again to throw light on the chemical nature of the growth stimulating substance (or substances) and in anticipation of the possible presence of reactive groups, we carried out hydrogenation experiments on extracts from various sources. In two preliminary experiments a 1% methanol solution of rice bran extract was hydrogenated using the catalyst of R. Adams.[14] In both experiments the weight of the catalyst used was one-seventh that of the extracted material to be hydrogenated. In the first experiment the catalyst was used alone for one hour; in the second it was activated with oxygen from time to time and ferrous chloride equal to one-sixth the weight of the catalyst was introduced. The material was left in contact with three atmospheres' pressure of hydrogen for nineteen hours. In both experiments the activity of the material was recovered undiminished; in fact there was a very slight increase in activity which we attributed to the destruction of toxic substances by the hydrogenation process.

Later experiments were performed on four other extracts with a freshly prepared batch of catalyst. We anticipated that the extracts or the methanol might possibly poison the catalyst and render it relatively inert. In order to avoid this possibility we studied the hydrogenation of cinnamic aldehyde in acid-free methanol in the presence of 1% of our rice bran extract. We followed the progress of the hydrogenation of the olefin double bond by iodine number determinations and the hydrogenation of the aldehyde group by parallel Fehling's solution reductions. Our experiments showed that during forty minutes, without ferrous chloride present, a considerable amount of the cinnamic aldehyde was reduced at the double bond but that the catalyst was actually poisoned somewhat. After a further reduction for thirty-five minutes in the presence of ferrous chloride, tests showed that nearly all the aldehyde group was reduced. In these experiments the ratio of catalyst to total aldehyde to be reduced was 1:20.

In order to avoid inactivation through poisoning of the catalyst we decided to carry out the additional hydrogenation experiments on 0.2% solu-

(14) Adams, Voorhees and Shriner, "Organic Syntheses," Coll. Vol. I, 1932, p. 452.

tions of the concentrates and using a large amount of catalyst equal in weight to two-thirds that of the material to be reduced. The reductions were run for forty-five minutes in the absence of ferrous chloride and then forty-five minutes longer in the presence of ferrous chloride. These conditions seemed to be sufficient to ensure complete reduction of all substances in the extract which are capable of being reduced with this catalyst.

The results of the additional hydrogenation experiments are listed in Table XVII.

TABLE XVII

HYDROGENATION EXPERIMENTS

Extract used	Amounts, mg.			Yeast crops, mg./cc. before hydrogenation			Yeast crops, mg./cc. after hydrogenation		
Beef liver	0, 0			0.04	0.05				
	0.5	1.0	2	.64	1.56	1.90	62	1.01	1.50
Aspergillus niger	0, 0			.05	0.05				
	.5	1.0	2.0	.33	.49	0.70	0.34	0.40	0.54
Crab's eggs	0, 0			.06	.06				
	.5	1.0	2.0	.26	.48	.92	.22	(.03)	.63
Earthworm	0, 0			.13	.13				
	.5	1.0	2.0	.17	.22	.36	.25	.36	.52

It is apparent that a large part of the activity is recovered after hydrogenation in each one of these experiments, whereas the conditions were such as to reduce completely all substances capable of reduction by this means. The loss of a portion of the activity by adsorption should be expected because of the very large amount of catalyst used. A number of hydrogenation experiments other than these reported have been carried out. Whenever the catalyst has not been used in extremely large excess, we have always recovered substantially the whole of the original activity after hydrogenation and at the same time the appearance and odor of the solution indicated that reduction had taken place. We have no explanation to offer for the appreciable increase in activity in the case of the earthworm extract, unless it be due to the destruction of interfering substances.

Oxidation Tests.—The hydrogenation experiments seem to show that the active substance is not olefinic nor aromatic nor does it contain an ordinary aldehyde or ketone group. However, according to our experience as well as previous experiments of others, glucose is not reduced in the presence of platinum catalyst. We sought to perform an oxidation test which would oxidize any sugar groups if present and thus settle the question whether our active substance possesses one of these groups.

Solutions containing 10 mg. per cc. of extracts of rice bran, beef liver, mold and crab eggs were placed in test-tubes. To these were added equal volumes of ammoniacal silver solution (made up of 2 volumes of $N/6$ NH_4OH and 1 volume of 2% $AgNO_3$ solution) and heated at 65–70° for ten minutes. Under these conditions we found that glucose in dilute solu-

tion is completely oxidized. Other samples of extracts were treated in exactly the same manner except that they were not heated. One-fifth of their volume of $N/6$ hydrochloric acid was then added to each of the tubes, the precipitated silver chloride digested in the slightly acid medium at 60–70°, and the solution finally filtered clear. The results of the tests are given in Table XVIII. It is clear that there is no substantial reduction of activity by this treatment; therefore it may be concluded that the active substance does not possess a sugar group. The absence of a sulfhydryl group is also indicated.

TABLE XVIII

OXIDATION TESTS WITH AMMONIACAL SILVER SOLUTION

Extract tested	Amt. used, mg.		Yeast crops (unheated)		Yeast crops (heated)	
Blanks	0	0	0.06	0.06		
Rice bran	0.8	1.6	.77	1.34	0.56	1.50
Beef liver	.8	1.6	1.39	1.91	1.49	1.91
Mold	.8	1.6	0.85	1.16	0.84	0.98
Crab eggs	.8	1.6	.49	0.92	.49	.80

Heat Stability in Acid, Neutral and Basic Media.—The heat stability of the active substance (or substances) was tested principally to ascertain whether preparations from different sources would give similar results. This should throw light on the possible identity of the growth stimulating substances from diverse sources. One per cent. solutions of the extracts were mixed with double their volume of 2 M $NaOH$–H_3PO_4 buffers with P_H values 5, 7 and 9, respectively. These solutions were autoclaved for four hours at 119° and tested in comparison with a neutral buffered sample which was sterilized at 100° for a few minutes. All solutions were brought to neutrality before being used in the tests. The results are tabulated in Table XIX.

TABLE XIX

TESTS ON HEAT STABILITY

Extract used	Amt., mg.		Yeast crops: Untreated P_H 7		Heated 119° P_H 5		Heated 119° P_H 7		Heated 119° P_H 9	
Blanks			(0.06)	(0.06)						
Rice bran	1.0	2.0	.48	.89	0.67	1.2	0.62	1.13	0.08	0.10
Beef liver	1.0	2.0	.90	1.13	.91	1.60	.68	1.10	.29	.52
Aspergillus niger	1.0	2.0	.38	.48	.40	0.67	.32	0.46	.06	.07
Crab eggs	1.0	2.0	.37	.48	.28	.46	.18	.24	.07	.28
Egg white	1.0	2.0	.44	(.26)	.35	.60	.23	.24	.06	.08

It is evident that nearly all of the activity in each case is destroyed by prolonged heating in weakly alkaline medium but that heating in weakly acid or neutral medium for four hours at 119° causes very little destruction. In fact the activity is increased slightly in some cases. We have observed slight increases of this sort many times, especially with slight acid treatment (also very mild alkali treatment) and suspect that the effect may

be due to the hydrolysis of an inactive lactone or other ester form in which the active acidic substance may partly exist. Other experiments using rice bran extract have shown clearly that when it is autoclaved at 119° for four hours at P_H 3 or less, a considerable part of the activity is destroyed.

Treatment with Nitrous Acid.—One-tenth per cent. extracts from the sources indicated in Table XX were subjected to treatment with nitrous acid by passing N_2O_3 (generated by dropping concentrated sulfuric acid into 20% sodium nitrite solution) into the cold solutions and then warming. This procedure was repeated once and the solution brought finally to boiling for fifteen minutes.

TABLE XX

TREATMENT WITH NITROUS ACID

Extract used	Amt. used, mg.		Untreated		Yeast crops treated	
Blanks	0	0	0.08	0.08		
Rice bran	0.5	1.0	.42	.80	0.10	0.10
Liver	.5	1.0	.70	1.27	.17	.26
Mold	.5	1.0	.37	(1.57)	.08	.07
Crab eggs	.5	1.0	.29	.52	.10	.11
Egg white	.5	1.0	.20	.34	.06	.06

The uniform destruction of the active substance contributes evidence toward its identity in the different extracts, but we are inclined to believe, in view of other definite evidence, that the action with nitrous acid does not involve any basic nitrogenous group.

Esterification Experiments.—Before a large portion of the work reported in this paper was done, we attempted to concentrate the active substance by esterifying, separating the ester and distilling in high vacuum. While we failed to accomplish the distillation, we obtained some significant results which are reported in brief.

We found that esterification was readily accomplished and that the best conversion was obtained when we allowed the substance to stand for about a half-hour at 30° in a methanol solution to which had been added sulfuric acid to make it half normal. The ester formed (the conversion is not, however, complete) is inactive toward yeast but by heating at 100° for thirty minutes in 0.1 *N* sulfuric acid solution, a considerable amount of activity is recovered. The activity could be recovered also by heating with dilute alkali, but increased destruction was apparent.

Under the conditions mentioned we were never able to recover more than about 20% of the original activity from hydrolysis of the ester. Long standing in acid solution causes much unrecoverable loss of activity. Hydrogen chloride under these conditions seemed to be especially destructive. We obtained evidence to indicate that part of the destruction which seemed inevitable was due to oxidation, and that the ester could be pro-

tected to a very appreciable extent by antioxidants, of which hydroquinone was most effective.

The esters (methyl and ethyl) unlike the free acid are somewhat soluble in ether, but even so are more soluble in water. We were unable to find any solvent that would extract the esters satisfactorily from water. This ready solubility in water we attribute to the presence of several hydroxyl groups in the acid. The failure of the methyl ester to distil at 100° and 1.4×10^{-3} mm. pressure we also attribute to the presence of hydroxyl groups. We are now attempting, however, the distillation of the free acid in a molecular still. The electrolysis experiments on the esters are mentioned earlier in this paper.

Subsequent to these studies we performed experiments with extracts from mold, liver and egg white. In each case there was apparent esterification under the conditions outlined above, and a subsequent recovery of a portion of the activity by acid hydrolysis. In these cases water solutions of the ester mixtures were used, again showing that the ester is water soluble. From the similarity in behavior toward esterification we again conclude that the same substance is probably concerned in the different extracts.

Discussion

Probability of a Single Compound as the Responsible Agent.—The results of all our experiments with the extracts of very diverse biological origin, point very strongly to the existence of a single acid substance in these tissues (and presumably all tissues) which is capable of stimulating yeast growth in a very striking way and over a very wide range. The behavior of the active substance from different sources when subjected to various tests and treatments, despite its admixture with different types of impurities, is so nearly uniform that it seems very unlikely that different substances could be responsible for the growth stimulating action. If several different acids are involved, they must have very similar properties and molecular weights and must be of a single narrowly defined type. For present purposes we can safely regard the activity as due to a single acid. Since this acid appears to be of very widespread occurrence, possibly more widespread than any known substance, we have tentatively called it "pantothenic" acid. The name is derived from the Greek meaning *from everywhere.*

Properties of the Acid.—Our experiments indicate that the substance is a distinct acid (probably carboxylic) with molecular weight about 150; that it does not contain in its structure any olefin double bond, aldehyde or ketone group, aromatic nucleus, sugar group, sulfhydryl group, amino or other basic nitrogenous group; that it does contain probably more than one hydroxyl group and under some conditions is easily susceptible to oxidation. As yet we have made only moderate progress in concentrating the

substance with the idea of isolating it chemically and none of the materials used in this study represent anything more concentrated than a crude extract. This problem is being actively pursued, now that we feel convinced that the substance is of wide importance biologically. The substance is apparently not a hydrolysis product of proteins because we have again prepared a casein digest by the action of pancreatin and found the resulting mixture to be devoid of activity as a yeast growth stimulant. While we have not tested exhaustively, it seems very unlikely that the substance is any well-known compound. We are now proceeding to test various synthetic compounds which are available or may be prepared.

We have indications that the acid sometimes exists partly in an inactive lactone or ester form which can be activated by heating in dilute acid or alkali. In the oyster electrolysis we have evidence of a second substance which has some yeast growth stimulating activity. While we have no experimental evidence on this point, it seems likely that the second substance may be a derivative of the widely occurring acid. Finally, in the case of egg white, we have evidence that the active substance may sometimes be intimately associated with colloidal material, in which case the active substance is nearly non-diffusible through a sintered glass diaphragm.

Origin and Function in Nature.—While the origin of this acid in nature is obscure, except that it is produced by *Aspergillus niger* (in soils, for example),[15] we are led to suspect that it is one of the unidentified water-soluble vitamins. In fact several of its properties (heat stability under different conditions and destruction by acidic alcohol) suggest a close relationship or identity to vitamin G. This point requires further study.

There is little question that yeasts other than the Gebrüde Mayer strain require it as one of the components of a satisfactory medium, and its occurrence in all cells suggests that it may function universally as a growth catalyst. The fact that it is without doubt produced by microorganisms in soil suggests the possibility that it may not be synthesized by green plants.

We wish to express our thanks to those who have furnished us materials for this study; to the institutional Research Council for grants of funds, and to Standard Brands Inc., successors to the Fleischmann Co., who have for a period of years supported this work with a fellowship grant.

Summary

1. Extracts of very diverse tissues representing many different biological groups, Chordata, Arthropoda, Echinoderms, Molluscs, Annulata, Plathylminthes, Myxomycetes, Bacteria, Fungi, Algae, Spermatophytes, all contain material which is capable of stimulating in a very striking way the growth of Gebrüde Mayer yeast.

(15) Todd, *Science*, **76**, 464 (1932).

2. Based upon the similarity of behavior in fractional electrolysis experiments, diffusion experiments and experiments involving hydrogenation, oxidation, treatment with nitrous acid, tests of heat stability in acid, neutral and alkaline media and esterification, we conclude that the ability of these extracts to stimulate yeast growth is due to the presence of a single acid substance which appears to be of universal biological occurrence. We have tentatively designated it as "pantothenic" acid, the name being derived from the Greek, meaning *from everywhere.*

3. Pantothenic acid appears to have a molecular weight of about 150; to be a distinct acid without amphoteric properties, to have several hydroxyl groups in its structure, but no olefin double bond, aldehyde, ketone, sulfhydryl, basic nitrogen, aromatic or sugar group.

4. Pantothenic acid is synthesized by *Aspergillus niger.* The probability is suggested that it may be produced by microörganisms in the soil and may function very widely in nature as a growth catalyst. Several similarities suggest its close relationship to vitamin G(B_2).

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The Use of Gamma-Iodopropyl Chloride as a Synthetic Reagent. The Synthesis of Certain Alpha-Phenyl Dibasic Acids

BY FRANCIS H. CASE

γ-Iodopropyl chloride, prepared by Henry[1] from trimethylene chlorobromide and sodium iodide, may be conveniently prepared in large quantities by the action of phosphorus and iodine upon trimethylene chlorohydrin. A study of its reactions with sodium cyanide and the sodium derivatives of phenylacetonitrile, ethyl malonate and ethyl phenylmalonate is here reported.

In the reaction with an equimolar quantity of sodium cyanide the sole product is γ-iodobutyronitrile, the absence of chlorine being probably due to the interaction of the γ-chlorobutyronitrile, initially formed, with the sodium iodide produced in the primary reaction. Whether this is the true mechanism of the reaction could be ascertained by converting an unsymmetrically substituted chlorohydrin such as 3-chloro-1-phenylpropanol-1, $HOCH(C_6H_5)CH_2CH_2Cl$ (whose preparation is described), to the corresponding chloro-iodide, treating it with one mole of sodium cyanide, and identifying the acid formed by hydrolysis.

γ-Iodobutyronitrile reacts with the sodium derivative of ethyl phenylmalonate suspended in toluene to form an ester which on hydrolysis and

(1) Henry, *Bull. soc. chim.*, [3] **17**, 93 (1897).