T H E S I S

on

PANTOTHENIC ACID

ITS DISTRIBUTION IN BIOLOGICAL TISSUES

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INTRODUCTION

In their study of unknown substances which stimulate the growth of yeast, Williams and Truesdail[1] were the first to show that the hypothetical "bios" of Wildiers[2] was not a single substance. By fractional electrolysis it was shown that there were two supplementary factors each having little effect by itself. One of these factors was basic and the other acidic. Previously, yeast #578 (2331) of the American Type Culture Collection, and also Old Process yeast, had been shown to require several different components in order to stimulate their growth,[3,4,5] one of which may be the antineuritic vitamin[6] of Jansen and Donath.

In the case of one strain however, "Gebrude Mayer" yeast, a portion of the contents of one electrolytic cell alone, an acid cell, greatly stimulated the growth of the yeast. No supplementation could be obtained by any combination of the contents of different cells. It thus appeared that the growth of Gebrude Mayer yeast is stimulated by an unknown acidic substance. This acid we have referred to as pantothenic (Greek--from everywhere) acid. The present and subsequent work has shown that it is a single substance and appears to be a universal constituent of living matter.

Electrolysis in a series of cells connected by syphons, as carried out by Williams and Truesdail,[1] was

chosen as the means of investigating whether the active principle in extracts of various tissues is the same chemical compound. This method will not only differentiate between non-electrolytes, acids, bases, and amphoteric substances, but it will also distinguish between weak acids of different strength as will be shown later.

In order to determine the distribution of this unknown growth stimulant in living organisms, tissues from widely different sources were extracted, the extracts electrolyzed, and the various fractions tested for their effect on the growth of the yeast. Representative tissues were chosen from as many of the biological phyla as feasible. These phyla and the tissues selected are as follows: Chordata--beef liver; Arthropoda--crabs eggs; Echinodermata--sea urchin eggs; Mollusca--oysters; Annulata--earth worms; Plathylminthes--planarian worms; Schizophyta--bacillus subtilis; Algae--spirogyra and oscillatoria (mixed); Fungi--aspergillus niger; Spermatophyta--rice bran.

EXPERIMENTAL

In general the extracts were prepared by refluxing the tissue in 80% methanol for one half hour, filtering, and evaporating the filtrate to dryness. If the filtrate contained colloidal matter, it was refiltered using a little kieselguhr. The amount of dried extract used in an electrolysis was usually 100 mg. or less. This

quantity was weighed out and dissolved in 415 cc. of distilled water. Sometimes the solution at this stage contained colloidal material. In that case it was again filtered with a little kieselguhr. The 415 cc. of solution was just sufficient to fill the electrolytic apparatus. After about 24 hours a little water was added to each cell in order to replace that which had evaporated.

The electrolysis apparatus itself consisted of eight cells each of about 50 cc. capacity, connected in series by syphons. The syphons were connected at the top in such a way that they could be filled simultaneously by suction. This also allowed them to be emptied slowly without mixing.

Current for most of the experiments was supplied by an 1800 volt D. C. motor generator. Platinum electrodes were used. At the beginning of an electrolysis the current was usually 5 or 6 milliamperes. This diminished to almost zero at the end of the electrolysis which was generally 48 hours later.

On stopping the current, the pH of the cells was determined immediately in order to obtain the values before the carbon dioxide in the air had time to change the basic cells. The Cullen[7] type quinhydrone electrode was used for this purpose. This type of apparatus has the advantage of requiring only a small portion of a cc. for a determination. After electrolysis the solution in

the central cells was practically unbuffered and of very low conductivity. For this reason a reliable value for the pH was difficult to obtain. In order to overcome the difficulty arising from low conductivity, the vacuum tube was used in the manner described by Goode[8]. This means proved satisfactory. Values of doubtful reliability were omitted from the tables.

A definite fraction of the contents of each cell was then tested for its effect on the growth of Gebrude Mayer yeast. The resulting yeast crops and also the seedings as reported in the tables, were determined by the thermocouple method[9] where-by the turbidity of the yeast suspension is measured.

Rice Bran. The extract was prepared with 60% methanol. The possibility of the purity of the extract having an effect on the accumulation of the activity in any certain cell, was studied in the following way: A series of electrolysis were run taking the most active fractions of one experiment as the material for the following electrolysis and repeating the same process again. The possibility of a higher voltage modifying the results was eliminated at the same time. Table #1 shows the results of an electrolysis in which 500 volts were applied to a solution containing 200 mg. dried extract for a period of 75 hours. Table #2 shows the results of electrolyzing the contents of cells #2,3,4

of the former electrolysis. The voltage was 5000 instead of 500 and was applied for 20 hours.

TABLE I

FRACTIONAL ELECTROLYSIS OF RICE BRAN EXTRACT

Cell Tested	Wt. of dry material in cell (mgs.)	pH of Cell Contents	Amt. dry material used in cultures			Yeast Crops Mgs. of moist yeast per cc.		
			Test #1	Test #2	Test #3	Test #1	Test #2	Test #3
Blanks			0	0	0	.10	.10	.09
#1	31.6	2.6	.25	.5	1.	.13	.125	.13
#2	31.6	3.5	"	"	"	.28	.39	.48
#3	30.5	3.9	"	"	"	.28	.36	.48
#4	25.9	4.1	"	"	"	.24	.33	.48
#5	25.1	4.8	"	"	"	.22	.28	.56
#6	18.5	5.6	"	"	"	.10	.14	.19
#7	15.3	7.2	"	"	"	.09	.12	.24
#8	30.5	9.4	"	"	"	.09	.09	.12

Seeding .006 mg. per cc.

TABLE II

SECOND FRACTIONAL ELECTROLYSIS OF RICE BRAN EXTRACT

Cell Tested	pH of Cell Contents	Percentage of Cell Contents Used in Tests			Yeast Crops Mgs. moist yeast per cc.		
		Test #1	Test #2	Test #3			
Blanks		0	0		.10	.10	
#1	3.0	1	2	4	.36	.52	.68
#2	3.6	"	"	"	.51	.73	.87
#3	4.4	"	"	"	.30	.51	.77
#4	6.0	"	"	"	.12	.12	.17
#5		"	"	"	.10	.12	.13
#6		"	"	"	.11	.11	.11
#7		"	"	"	.11	.11	.13
#8	8.0	"	"	"	.07	.10	.14

Seeding .006 mg. per cc.

Beef Liver. Two grams of finely ground beef liver was extracted with 50 cc. 80% methanol and 100 mg. of the dried extract was electrolyzed in 425 cc. distilled water for 30 hours at 1500 volts.

TABLE III

FRACTIONAL ELECTROLYSIS OF BEEF LIVER EXTRACT

Cell Tested	pH of Cell	Percentages of Cell Contents Used in Tests		Yeast Crops Mgs. moist yeast per cc.	
		Test #1	Test #2	Test #1	Test #2
Blanks		0	0	.12	.11
#1	2.6	2	4	.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

Seeding .007 mg. per cc.

Sea Urchin Eggs. Fresh eggs from the sea urchin (Strongylocentrotus purpuratus) were kindly furnished by Dr. A. R. Moore. These were dried and extracted. A portion of the dried extract (61 mg.) was used for electrolysis which was continued for 48 hours at 1500 volts.

TABLE IV

FRACTIONAL ELECTROLYSIS OF EXTRACT OF SEA URCHIN EGGS

Cell Tested	pH of Cell	Percentage of Cell Contents Used in Tests			Yeast Crops Mgs. moist yeast per cc.		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Blanks		0	0		.10	.10	
#1	2.6	1.	2.	4	.16	.22	.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	.06	.06

Seeding .006 mg. per cc.

Crabs Eggs. The dried eggs from the crab (Cancer productus) contained considerable fatty material which was extracted with a little ether and the ether discarded. Previous tests had shown that ether does not extract the active substance. The eggs were then extracted with 80% methanol as usual. In the electrolysis, 114 mg. dried extract was used. Only about two thirds of this dissolved. In order to clarify the solution it was filtered with kieselguhr.

It will be noticed that the higher dosages do not increase the growth of the yeast as is usually the case. This is due to toxic material in the extract, which counteracts the effect of adding more active material. In this case the toxic material is non-electrolytic as is shown by the fact that it is distributed in all the cells after electrolysis.

TABLE V

FRACTIONAL ELECTROLYSIS OF EXTRACT OF CRAB'S EGGS

Cell Tested	pH of Cell Contents	Percentages of Cell Contents Used in Test			Yeast Crops Mgs. moist yeast per cc.		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Blanks		0	0		.08	.08	
#1	2.4	1.	2.	4.	.47	.56	.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

Seeding .006 mg. per cc.

Oyster. The oyster was first dried and then extracted with ether to remove fats. Extraction was then carried out with 80% methanol and 53 mg. of the extracted material was used in the electrolysis.

TABLE VI

FRACTIONAL ELECTROLYSIS OF OYSTER EXTRACT

Cell Tested	pH of Cell Contents	Percentage of Cell Contents Used in Test			Yeast Crops Mgs. moist yeast per cc.		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Blanks		0	0		.09	.09	
#1	2.6	1.7	3.4	6.8	.10	.12	.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

Seeding .006 mg. per cc.

Earthworms. The worms were dried and the extract prepared in the usual way with 60% methanol. In this experiment 34 mg. of dried extract was electrolyzed.

TABLE VII

FRACTIONAL ELECTROLYSIS OF EXTRACT OF EARTHWORMS

Cell Tested	pH of Cell Contents	Percentage of Cell Contents Used in Tests			Yeast Crops Mgs. moist yeast per cc.		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Blanks		0	0		.06	.06	
#1	2.8	1	2	4	.04	.05	.06
#2	4.1	"	"	"	.26	.54	1.32
#3	5.1	"	"	"	.08	.11	.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

Seeding .006 mg. per cc.

Planarian Worms. The amount of material available was very limited. One gram of moist worms yielded 10 mg. extract. This amount was electrolyzed in the usual way. Dr. Rosalind Wulzen kindly furnished the worms for this experiment.

TABLE VIII

FRACTIONAL ELECTROLYSIS OF AN EXTRACT OF PLANARIAN WORMS

Cell Tested	pH of Cell Contents	Percentage of Cell Contents Used in Test		Yeast Crops Mgs. moist yeast per cc.	
		Test 1	Test 2	Test 1	Test 2
Blanks		0	0	.09	.09
#1	3.4	33	67	.88	1.07
#2	5.1	"	"	.26	.48
#3		"	"	.11	.13
#4		"	"	.10	.11
#5		"	"	.09	.11
#6		"	"	.10	.11
#7		"	"	.06	.07
#8		"	"	.06	.06

Seeding .006 mg. per cc.

Bacillus Subtilis. This organism was grown for three weeks in two liters of broth containing 14 gr. of potato extract and 20 gr. of Bacto-peptone. The medium was decanted and the bacteria washed successively with 1500 cc., 150 cc., and 100 cc. of water. The dried bacteria weighed .412 gr. This yielded 95 mg. of extracted material part of which did not dissolve and was removed with kieselguhr.

TABLE IX

FRACTIONAL ELECTROLYSIS OF BACILLUS SUBTILIS EXTRACT

Cell Tested	pH of Cell Contents	Percentage of Cell Contents Used in Tests		Yeast Crops Mgs. moist yeast per cc.	
		Test #1	Test #2	Test#1	Test#2
Blanks		0	0	.14	.14
#1	3.0	7.	14.	.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

Seeding .007 mg. per cc.

Mold. Aspergillus niger (#1004 Amer. Type Culture Coll.) was chosen as a representative of the fungi. This was grown in a synthetic medium consisting of sugar and various inorganic salts. The crop of mold was dried, extracted, and 50 mg. of the dried extract was electrolyzed. In the first experiment there was not enough acidic material in the extract to give the usual pH gradient. The experiment was repeated later with the help of Dr. Williams, using the same quantity of extract and adding a trace of phosphoric acid. The data for this last experiment is given below.

TABLE X

FRACTIONAL ELECTROLYSIS OF MOLD EXTRACT

Cell Tested	pH of Cell Contents	Percentage of Cell Contents Used in Tests			Yeast Crops Mgs. moist yeast per cc.		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Blanks		0	0		.05	.05	
#1	2.9	1	2	4	.10	.16	.20
#2	3.4	"	"	"	.11	.20	.31
#3	3.7	"	"	"	.16	.26	.38
#4	3.9	"	"	"	.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

Seeding .006 mg. per cc.

Algae. To represent the algae a mixture of Spirogyra and Oscillatoria was obtained from a stagnant pond. The sample was washed repeatedly by decantation after which it was dried and extracted, first with ether and then with 80% methanol. Fifty milligrams of extract was electrolyzed.

TABLE XI

FRACTIONAL ELECTROLYSIS OF ALGAE EXTRACT

Cell Tested	pH of Cell Contents	Percentage of Cell Contents Used in Tests			Yeast Crops Mgs. moist yeast per cc.		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Blanks		0	0		.05	.06	
#1	2.3	1	2	4	.07	.08	.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

Seeding .005 mg. per cc.

Refined Beef Liver Preparation. The results of all of the experiments except the one with beef are consistent. It was suspected that a toxic acid substance was present which migrated to the same place at which the bulk of the active compound accumulated. This would cut down the yeast growth produced by samples from cells with a pH value of about 3.8. That this is what happened is indicated by the following experiment in which a highly refined preparation obtained from beef liver was electrolyzed. The preparation of this concentrate will be described in a later publication. The concentration of the active compound in the solution electrolyzed was in the neighborhood of 100 times greater than in some of the other electrolysis, although only 50 mg. of material was used. At the beginning of the experiment approximately 2500 volts were applied. This was increased to 7500 volts after 24 hours. The duration of the electrolysis was 63¼ hours.

TABLE XII

FRACTIONAL ELECTROLYSIS OF REFINED BEEF LIVER PREPARTION

Cell Tested	pH of Cell Contents	Percentage of Cell Contents Used in Tests			Yeast Crops Mgs. moist yeast per cc.		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Blanks		0	0		.07	.07	
#1	2.7	.032	.065	.13	.14	.26	.48
#2	3.4	"	"	"	.18	.34	.61
#3	3.6	"	"	"	.31	.57	1.03
#4	4.0	"	"	"	.32	.57	1.04
#5	4.9	"	"	"		.08	.08
#6	5.0	"	"	"		.08	.07
#7	5.4	"	"	"		.08	.08
#8	8.6	"	"	"		.07	.07

Seeding .0033 mg. per cc.

DISCUSSION

The accumulation of the active principle in a cell of pH about 3.8 in each electrolysis has important significance in the interpretation of the data. Amphoteric substances such as amino acids are known to show this behavior.[10,1] With this in mind electrolysis experiments were performed in which a small amount of a strong acid was added to the electrolyte in order to test whether the active material would then migrate away from the acidic end (anode). It always failed to do so. To further establish this point the methyl ester and in another experiment, the ethyl ester of the active acidic substance was prepared and electrolyzed[11]. The ester is inactive in its effect on yeast, but it can be evaluated by hydrolysis under controlled conditions. If the substance was amphoteric, the ester should move toward the basic end. Actually it still moved slightly toward the acid end. This shows that the substance cannot be amphoteric, i.e. it has no basic properties.

When an extract of tissue is electrolyzed, a gradient of hydrogen ion concentration is established between the two electrodes and a weak acid moving toward the positive electrode comes into a medium of greater and greater hydrogen ion concentration. The acid will cease to move when the hydrogen ion concentration is great enough so that the major portion of the acid in

question will be in the un-ionized form. By the use of the equation defining the ionization constant it can be calculated that an acid with a constant of 10^{-5} will be a little less than 1% ionized when the pH is 3. It will be 9% ionized when the pH is 4 and 50% ionized when the pH is 5. Moreover it is apparent that the place at which the major part of the acid will stop depends on the ionization constant of that acid. If the ionization constant of the acid taken for illustration had been $5X10^{-5}$ instead of 10^{-5} then at a pH of 4 it would have been 33 1/3% ionized instead of 9% as above. This shows how weak acids of different strength would stop at different points, thus providing a means of distinguishing between them.

Since the material which stimulated the growth of the yeast always migrated to a point of about the same pH, this was considered as evidence that the active material is a single chemical compound, for if different acids were effective, they would not likely have the same ionization constant and would accumulate in cells of different pH.

One reason for including the experiment in which the refined preparation from beef liver extract was electrolyzed was the fact already stated, that the concentration of the active principle in the solution electrolyzed was in the neighborhood of a hundred times

greater than that in some of the other experiments; and yet the activity accumulated in about the same pH range as usual, which is exactly what would be expected according to the theory. This becomes evident when the equation, $\frac{[H^+][An^-]}{M-[An^-]} = K$ where M is the total molarity, is written in the form, $\frac{[An^-]}{M} = \frac{K}{K+[H^+]}$ which shows that if the hydrogen ion concentration is fixed, the proportion of the acid in the ionized form is independent of the total molarity.

The similarity of behavior of the active material on electrolyzing extracts from different source has been shown. Further evidence may also be cited[11] to show that the active principle behaves in the same way with regard to heat stability, hydrogenation, esterification, effect of nitrous acid, and oxidization with ammoniacal silver nitrate. The cumulative evidence leads to the conclusion that a single chemical compound (acid) is responsible for the stimulation of the growth of the yeast in a synthetic medium.

SUMMARY

Extracts of tissues from widely different sources were all shown to contain material which greatly stimulates the growth of Gebrude Mayer yeast. The biological groups represented by tissues extracted are

as follows: Chordata, Arthropoda, Echinodermata, Mollusca, Annulata, Plathylminthes, Schizophyta, Algae, Fungi, and Spermatophyta. In each case, on electrolysis the active material behaved in the same way, accumulating in a cell of about pH 3.8. From this and other supporting evidence, it is concluded that the stimulating effects of these extracts on the growth of the yeast is due to a single acid substance which occurs in all living things.

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