THE EFFECTS OF PANTOTHENIC ACID ON RESPIRATORY ACTIVITY*

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I

INTRODUCTION

Pantothenic acid is known to be present in a wide variety of tissues (1, 2). Its ubiquity suggests that it is a factor essential to cell physiology rather than a compound with a more localized function. Results have been published showing its effects on yeast growth (1) and indicating a relationship with the carbohydrate metabolism of both yeast and green plants (alfalfa) (3, 4). In the experiments here reported it has been found that pantothenic acid has stimulative effects on the respiration of yeasts, on fermentation by non-living enzyme preparations, and on the respiration of certain vegetable tissues.

All of the respiration studies were made with a fourteen unit Warburg-Barcroft microrespirometer. A temperature of 30°C. and an atmosphere of air was used in all cases. The two-vessel method outlined by Dixon (5) was used with the following precautions: (1) Shaking at a rate of 100 oscillations per minute and an amplitude of 4 cm. eliminated any diffusion effects. (2) Buffers (phosphate) were used to maintain constant pH. (3) Manometers were opened frequently (between readings) so that the O_2 pressure was not significantly lowered. (4) Whatman No. 40 "KOH papers" were used and found to give accurate results even when the O_2 consumption was small. (5) The pantothenic acid was added as the calcium salt

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in a preparation which was approximately 25 per cent pure. Much previous work on material of even lower purity indicated that the preparations are biologically pure. The calcium ion was proved not to be responsible for the effects by testing a range of concentrations of $CaCl_2$. This was probably an unnecessary precaution since extremely low concentrations of the pantothenic acid were used. (6) Proper thermobarometric and other controls were run.

II

Effects on the Respiration and Growth of Living Yeasts

In this part of the study the effects of pantothenic acid on yeast respiration and growth were investigated and its effects compared with, and related to the effects of certain compounds known to be biochemically important (6–8).

Two yeasts were studied: (1) Gebrüder Mayer yeast grown on Williams' medium (9) plus a low dosage of pantothenic acid. This is a synthetic medium except for the pantothenic acid which must be added in order to get a crop large enough for the experiments. (2) Fleischmann's yeast used directly from the center of a fresh retail cake. This yeast affords a readily available standard and its respiration was found to be reasonably constant from day to day. The G.M. yeast when grown as described is deficient in all factors which it cannot synthesize. An assay of the pantothenic acid present indicated that it had less than one-tenth as much of the acid as normal yeast. Similar deficient G.M. yeast was used in the previously reported preliminary experiments on the effect of pantothenic acid on CO_2 and O_2 exchange (3).

Three basal synthetic media of increasing degrees of completeness were used: (1) 0.2 molar C. P. sucrose. (Experiments using ordinary sucrose indicated that it contains a respiration stimulant.) (2) Medium 1 plus the inorganic salts and "trace elements" of Williams' medium in twice the concentration in which they are found in that medium (9). (3) Medium 2 plus 10 gm. *l*-asparagine per liter. As used in the respirometer flasks the final medium contained: 1 cc. of one of these three basal media, 1 cc. of KH_2PO_4 solution (4 gm. per liter), and 0.4 cc. of distilled H_2O containing the compound to be studied.

The following compounds were investigated: Pantothenic acid, thiamin (Merck's Betabion), β-alanine, ethanolamine, meso-inositol, indole-3-acetic acid, nicotinic acid, pimelic acid, uracil, and 3,5-dinitro-o-cresol. Liver extract (Lilly's No. 343), representing a crude tissue extract, was also studied. Numerous experiments done with these materials over a range of concentrations showed that pantothenic acid, thiamin, β-alanine, ethanolamine, and the liver extract gave, under the conditions used, definite stimulation, while no appreciable acceleration of the respiratory rate was obtained with any of the other compounds. Since ethanolamine was effective only in relatively large concentrations, and since β-alanine doubtless owed its effect to its intimate structural relation to pantothenic acid (10), it was decided that pantothenic acid, thiamin, and the liver extract should be more intensively investigated.

In all experiments the yeast was washed once by centrifugation from KH_2PO_4 solution and then re-suspended in fresh phosphate solution at a concentration of approximately 4 mg. per ml. for pipetting into the flasks. The actual concentration was determined by the thermocouple method (11). Parallel runs on 2, 4, and 6 mg. of yeast proved that over this range the respiration rate (per milligram) did not vary materially with the weight of yeast present. Respiration was usually measured over a period of about 2 hours and growth was determined by a second reading on the thermocouple after 6 hours shaking in the apparatus. Although at high concentrations certain compounds produce osmotic effects which alter the thermocouple readings, it was found that the small variations in concentration resulting from the differences in the three basal media and from the differences in concentration of the various compounds studied did not alter the thermocouple readings to an appreciable extent. (Corrections were necessary for the liver extract which is colored.) By vigorous mechanical shaking of the yeast suspensions after the 6 hour growth period in the apparatus it was shown that the cells were not clustered enough to significantly affect the opacity of the suspensions.

The pH of 4.8 produced by the KH_2PO_4 buffer was chosen because it is approximately the optimum for yeast (12), and a pH this low reduces preformed CO_2 to a negligible amount (13). It was also found that in this region a small variation in pH does not affect the CO_2 constant of the apparatus. Closer to neutrality CO_2 is much more soluble and a slight variation in pH is more serious.

Table I shows the effects of the three most carefully studied materials on the respiration and growth of the deficient G.M. yeast.

The large number of preliminary experiments that were run made it possible to plan the final series of runs so that they could be completed in a short period thereby eliminating much of the variations resulting from changes in the stock yeast. The data listed within a horizontal column were obtained (except for vertical column F) in one run and fine comparisons among them are valid. In order to make comparisons of the data listed in vertical columns as accurate

TABLE I

Deficient G.M. Yeast

	A	B	C	D	E	F	G
CO_2 evolved—mm.³ per mg. per hour at end of 1 hr.							
Medium 1—sucrose*	23.4	25.8	39.6	22.6	23.7	53.5	129.0
Medium 2—sucrose + salts	35.7	43.0	53.0	38.4	33.2	67.0	150.0
Medium 3—sucrose + salts + asparagine	55.3	73.0	84.0	60.0	76.2	93.0	104.0
O_2 absorbed—mm.³ per mg. per hour at end of 1 hr.							
Medium 1—sucrose*	2.6	2.2	3.2	2.5	2.5	3.0	7.2
Medium 2—sucrose + salts	3.9	6.1	6.3	5.6	4.4	5.1	14.6
Medium 3—sucrose + salts + asparagine	5.5	5.3	8.4	5.3	6.3	5.5	11.0
Ratio of crop to seeding at end of 6 hrs.							
Medium 1—sucrose*	1.0	1.0	1.1	1.0	1.0	3.1	3.5
Medium 2—sucrose + salts	1.0	1.0	1.1	1.0	1.05	3.8	5.8
Medium 3—sucrose + salts + asparagine	1.0	1.4	1.6	1.0	1.1	4.2	7.1

* All three media were buffered with KH_2PO_4.

Vertical column A is with no added stimulant, B is with 0.1 γ pantothenic acid, C is with 10 γ pantothenic acid, D is with 0.1 γ thiamin, E is with 0.1 γ pantothenic acid and 0.1 γ thiamin, F is with 10 mg. liver extract No. 343, and G is with 100 mg. liver extract No. 343.

as possible, a factor was used to eliminate the variation in the controls. Rates of CO_2 evolution and O_2 consumption 1 hour after the end of the equilibration period are given in cubic millimeters per hour per milligram of yeast. Curves plotted from readings over a period of the first 3 hours showed that rate at the end of 1 hour was a fair basis of comparison. Growth is given as ratio of crop to seeding at the end of 6 hours. Ten gammas of pantothenic acid (calculated from the preparation of known purity), 0.1 gamma thiamin, and 100

mg. liver extract were found to be the optimal concentrations. In order to further correlate the effects certain other concentrations as well as combinations of stimulants were studied.

Columns B and C show the great stimulative power of pantothenic acid on CO_2 evolution, O_2 consumption, and growth. It is evident that 10 gammas of pantothenic acid increased the rate of CO_2 evolution in medium 1 about 70 per cent at the end of 1 hour. After 4 hours the CO_2 evolution was stimulated over 150 per cent although little growth occurred. A similar effect was shown in media 2 and 3, except that in the presence of the asparagine there was much more growth. It will be noted that in the absence of pantothenic acid (or the liver extract which contains it and other yeast nutrilites) no growth was evidenced. It is apparent therefore that the respiration and growth stimulative functions of the acid can be separated by the proper choice of media. This is in line with the findings of others (14–16) that respiration and growth may be quite distinct. Still more evidence that this is so is found in column A, where it is shown that adding the inorganic salts to medium 1 or asparagine to medium 2 greatly increases the respiratory rate without appreciably affecting the growth rate.

Under the conditions used the effect of pantothenic acid was far in excess of that of any of the other nine compounds studied. The only compounds which approached its effect are thiamin, ethanolamine (which must be added in relatively high concentrations), and β-alanine, which is intimately related to pantothenic acid (10).

Very little effect was shown by the pantothenic acid on the regular Fleischmann cake yeast. Apparently this yeast already contains approximately the optimal concentration of the acid. This interpretation was substantiated by a series of experiments, not reported in detail, in which it was found that when the Fleischmann yeast was grown in a medium deficient in "growth substances" its respiration was greatly stimulated by pantothenic acid.

The optimal concentration of thiamin has a definite accelerative action on the respiration of the deficient G.M. yeast. It did not stimulate growth, in fact it inhibited the growth stimulative effect of pantothenic acid in medium 3. It also inhibited the respiratory stimulative effect of pantothenic acid in medium 2. Thiamin gave

about the same stimulative effects on the regular Fleischmann yeast as it did on the deficient G.M. yeast. Thus its effects appear to be of contrasting nature to those of pantothenic acid as regards both growth and respiration.

In all three sections of the table the striking effect of the liver extract is obvious. It raised the respiration rate and the growth far beyond the optimum obtainable with pantothenic acid and thiamin. Its activity on the regular Fleischmann yeast was of similar nature and magnitude. Liver extract therefore contains important respiration and growth stimulants other than pantothenic acid or thiamin.

The general agreement of results on the two yeasts becomes more important when it is realized that they are very different in the character and rate of their respiratory activity. The CO_2 evolution of the regular Fleischmann yeast was roughly three times and the O_2 consumption was nearly ten times that of the deficient G.M. yeast. Consequently the respiratory quotient $\left(\frac{CO_2}{O_2}\right)$ of the Fleischmann was approximately one-third that of the G.M. yeast.

III

Effects of Fermentation by Enzyme Preparations from Yeast

Since the effect of pantothenic acid on respiration is not dependent on its effect on growth it was thought desirable to determine if the acid had an effect on fermentation by non-living systems.

A maceration juice prepared according to a procedure adapted from von Lebedev (17) was used. 1 kg. of fresh Fleischmann baker's yeast (pound cakes) was washed 12 hours by allowing tap water to run over it in a large evaporating dish. After filtering at the suction pump the yeast was broken into small pieces, spread out in a thin layer, and dried 48 hours at 30°. Twice during the drying the yeast was screened, first through a 2 mm., then through a 1 mm. screen. An electric fan was used during the last 10 hours of drying. The product (180 gm.) was kept in a desiccator. The maceration juice was prepared by adding 3 volumes H_2O to the dry product and warming at 35° for 2 hours. 3 more volumes H_2O were then added and the cells filtered off (using ordinary filter paper). 1 cc. of the filtrate was pipetted directly, or after dialysis, into the Warburg flasks. Solutions of sucrose, KH_2PO_4, and the pantothenic acid were then added in such volumes and concentrations that the final volume of 2.4 cc. was 0.1 M in sucrose and 0.1 M in KH_2PO_4.

Although extensive experimentation was done no definite effect of pantothenic acid on the undialyzed juice (except inhibition at high concentration) could be detected over a period of several hours. Since pantothenic acid is known to be a small molecule and readily dialyzable (1) it appeared that it might be possible to dialyze out the pantothenic acid known to be present in the maceration juice. Thus it might be possible to obtain an enzyme system deficient in pantothenic acid. Consequently the maceration juice was dialyzed against running tap water for various lengths of time. Both sausage

TABLE II

Net Gas Exchange—CO_2 Evolved Minus O_2 Absorbed

	First 1/4 hr.	Second 1/4 hr.	Third 1/4 hr.
Yeast maceration juice dialyzed 1/2 hr.			
Control	10	11	11
Control + 1/10 γ pantothenic acid	12	13	12
Control + 1 γ " "	14	12	12
Control + 10 γ " "	12	12	13
Yeast maceration juice dialyzed 1½ hr.			
Control	7	8	9
Control + 1/100 γ pantothenic acid	10	10	9
Control + 1/10 γ " "	12	11	10
Control + 1 γ " "	9	9	8

Figures represent pressure differences in millimeters Brodie solution.

casing and cellophane membranes were used with results qualitatively the same.

The results obtained on a typical run with juice dialyzed ½ and 1½ hours are shown in Table II. Results are given directly in terms of pressure changes (millimeters of Brodie solution) for the first three 15 minute periods after the end of the equilibration period. Figures represent net gas exchange (CO_2 evolved minus O_2 absorbed), since preliminary runs showed that the pantothenic acid stimulated chiefly by increasing the CO_2 output making it unnecessary to determine CO_2 and O_2 in separate flasks. Although the data show that fermentation was quite slow, duplicates agreed well for about 1 hour

and a 1 mm. difference is significant. The manometer constants were so nearly equal as to have a negligible effect on the relative values for these small readings.

It is evident from the table that pantothenic acid is a potent stimulant for the enzymatic fermentation in the dialyzed maceration juice. Particularly striking is the effect shown by only 1/100 gamma of pantothenic acid on the juice dialyzed $1\frac{1}{2}$ hours. The action occurs immediately and lasts less than an hour. A much larger per cent stimulation is shown if the first reading is taken after only 5 minutes instead of 15. It also seems probable that the effect could be still further increased by adding the pantothenic acid from the sidearm at the end of the 10 minute equilibration period.

Fermentation is known to be a very complex process requiring many "factors," some of which are dialyzable. Consequently it seems probable that the enzyme system is deficient in other essential factors besides pantothenic acid. Evidence supporting this viewpoint was obtained by adding $MgSO_4$, $MnSO_4$, $(NH_4)_2SO_4$, and acetaldehyde in concentrations calculated from those used by Warburg in some of his work on similar systems (18). The effective concentration of these compounds was an entirely higher order of magnitude than the concentration of pantothenic acid. The addition of these compounds increased the rate of fermentation 10–20 per cent and the further increase in rate by adding pantothenic acid to this modified control was essentially the same as on the untreated control.

Corroboration of these results was obtained in numerous experiments on the effects of pantothenic acid on zymin (the "acetone yeast" of Harden (19)). As was to be expected from the work of others (20–22) it was found that various organic and inorganic compounds stimulated the fermentation by zymin. The large effects of these compounds, together with the fact that relatively large concentrations of the pantothenic acid were required to give the maximum rate of fermentation, made it difficult to establish the effect of pantothenic acid itself. However, it was proved that the free acid (as well as the calcium salt) markedly supplemented the effects of the other compounds and made it possible to obtain a higher maximum rate of fermentation than was obtainable without the addition of pantothenic acid.

IV

Effects on the Respiration of Potato and Apple Tissue

Since the importance of pantothenic acid to the respiration of yeasts and enzyme preparations from yeast seemed well established, the next step was to determine if the acid had an influence on the respiration of higher plants.

The procedure used in this part of the investigation was adapted from that used by Lemmon in his careful study of the effects of pH on the respiration of potato tissue (23). Nine cylinders of tissue (three from each of three potatoes or apples) approximately 4 by 10 mm. were cut as rapidly as possible and placed in each Warburg

TABLE III

	First 1/4 hr.	Second 1/4 hr.	Third 1/4 hr.
Oxygen consumption of potato tissue			
Control	16.0	17.0	16.5
Control + 1/10 γ pantothenic acid	18.5	18.5	18.0
Control + 1 γ " "	18.0	18.5	16.0
Oxygen consumption of apple tissue			
Control	5.5	10.5	10.0
Control + 1/10 γ pantothenic acid	9.0	11.5	10.5
Control + 1 γ " "	7.5	11.5	8.5

Figures represent pressure differences in millimeters Brodie solution.

flask in 2 cc. of 0.1 M phosphate buffer at pH of 5.3. The pantothenic acid was added in 0.4 cc. H_2O. The potatoes and apples were kept at 30° overnight before use. Duplicates were always run and the results averaged. The duplicates usually agreed to within less than 5 per cent during the first hour.

In Table III are given a typical set of results for apple tissue and a similar set for potato tissue. The numerous preliminary experiments showed that pantothenic acid did not appreciably affect the respiratory quotient $\left(\frac{CO_2}{O_2}\right)$. Therefore the work was expedited by determining the O_2 consumption only. As in the previous section the figures

represent millimeters pressure change for the three 15 minute periods immediately following the end of the equilibration period. The larger per cent stimulation by the pantothenic acid on the apple tissue may be due to the fact that the work on potato tissue was done first and the acid was added before the flasks were put on the manometers, while in the work on apple tissue the pantothenic acid was added from the sidearm of the flasks at the end of the 10 minute equilibration period.

The table shows that, as in the case of the enzyme preparations, the pantothenic acid has a strong stimulative action which begins immediately and lasts less than an hour. It is, therefore, evident that pantothenic acid is a potent stimulant for the respiration of these tissues. It is noteworthy that these tissues were not made "deficient" as was done with the living yeast and also with the enzyme preparations.

Considerable work has been done to determine if pantothenic acid has a stimulative effect on the respiration of sliced rabbit muscle and homogenized (and dialyzed) rabbit brain. Positive results have been indicated, but there are large experimental variations. It may be that larger effects were not obtained because pantothenic acid is tied up in the tissues (24). If pantothenic acid is present in optimal amounts it would be difficult to obtain a deficient tissue on which its effect could be demonstrated. It is planned to continue the investigation in this direction.

SUMMARY

Experiments using the Warburg-Barcroft apparatus led to the following results and conclusions: (1) Two yeasts in three different media were strikingly stimulated in their respiration by minute amounts of pantothenic acid. (2) Nine other compounds (vitamins and other biologically important substances) were tested and found in all cases to have on the deficient G.M. yeast, lesser and in some cases no appreciable stimulative effect. Thiamin was the most effective of these compounds. Its action was shown to be different and in some ways antagonistic to that of pantothenic acid. (3) Liver extract (Lilly's Number 343) contains substances capable of speeding up respiration (and growth) to a much higher level than

seems possible with known compounds. (4) Pantothenic acid was found to have a definite stimulative effect on fermentation by dialyzed maceration juice from yeast. (5) It likewise stimulated respiration of apple and potato tissue and indications of a similar effect on certain animal tissues were obtained.

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