LXXXIV. ON THE VITAMIN D.

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THE well-known fact, first described by Wildiers, that yeast cells when in low concentration fail either to ferment sugar or to grow, but may be induced to do so by the addition of "bios," is as yet imperfectly understood. The need of yeast cells for "bios" depends clearly on dilution; for yeast, when in adequate initial concentration, is able to synthesise this substance indefinitely from the ingredients of a simple medium. It is an organic substance, soluble in water or alcohol, dialysable, and thermostable; it appears therefore to belong to the group of the vitamins. It seems, however, to be more thermostable than the vitamin which promotes the growth of rats [Souza and McCollum, 1920]; it is more thermostable also than the co-enzyme of zymase [Tholin, 1921]. The capacity for activating yeast in this manner has been used nevertheless by Williams [1919] and others [see Sherman, 1921], as a measure of the richness of any material in the vitamin B; and there is no doubt that in many cases, in yeast itself for instance, this vitamin and "bios" are closely associated.

Now the comparative value of the various organs of animals, for preventing the onset of polyneuritis in pigeons fed on polished rice, has been determined by Cooper [1914], who places them in the following order: liver, heart, cerebrum, cerebellum, muscle; 0.5 g. of yeast being equivalent for this purpose to 0.9 g. of liver, 1.2 g. of brain, or 5.0 g. of muscle (dried weights in each case). In respect of promoting the growth of young rats, Osborne and Mendel [1918] place the tissues in a similar order. The distribution of "bios" among the organs of animals, therefore, seemed of interest as a test of the closeness of association between it and this vitamin; and a comparison in this respect between the organs of normal animals and those of animals suffering from vitamin-B deficiency, as an indication how far this deficiency can be attributed to a shortage of "bios." Such an investigation forms the subject of the experiments to be described.

METHOD.

Two methods have been adopted for measuring "bios," or the substance which activates yeast; the one depends upon the rate of multiplication of yeast cells, the other upon the rate of fermentation, as indicated by the evolution of CO₂. The majority of workers have preferred to study growth,

as being a less erratic phenomenon than that of CO_2 production. The number and complexity of the factors which influence growth, however, are likely to be no less great than those which influence fermentation; and the fermentation method has been used in the present experiments, since it has the advantages of convenience and speed, and allows therefore many estimations to be made and an average drawn.

The medium employed has been Nägeli's solution, omitting ammonium nitrate. Ammonium nitrate of course is necessary for the growth of yeast, and leads also to more rapid fermentation; but where the rate of fermentation by a given quantity of yeast is alone in question, a factor which only promotes growth is a source of error; and results without it, while less sensitive, seemed likely to be more accurate than in its presence. The composition of the medium used, then, was:

Cane sugar	...	...	10 g.
Potassium biphosphate	...	...	0.5 g.
Magnesium sulphate	...	...	0.25 g.
Calcium phosphate	...	...	0.05 g.
Distilled water to	...	...	100 cc.

To this medium is added 0.01 % of fresh brewer's yeast (1 cc. of a 1 % suspension to 100 cc.), a quantity in itself insufficient to produce any fermentation whatever in 24 hours: this constitutes the standard yeast-Nägeli medium. To 10 cc. in each of a series of tubes is added a graduated quantity of a watery extract of the substance under investigation. The mixtures are transferred with a serum-syringe to a series of 3 cc. glass ampoules, such as are used for vaccines, two ampoules from each tube. The experiments are performed in duplicate, so that four determinations are made for each concentration of the activating substance. The ampoules are then inverted over a receptacle, and incubated at 33° for 24 hours. As fermentation occurs, fluid is displaced, and drips out of the neck of the ampoule into the receptacle. Each ampoule is weighed empty (*A*), full (*B*), and at the end of the experiment (*C*). The difference, $B - C$, represents the cc. of CO_2 produced in $B - A$ cc. of solution; from which the percentage CO_2 formation may be calculated.

Results found to be most reliable are obtained when the fermentation amounts to about 5–10 %; the yeast tends to adhere to the glass as the fluid recedes, vitiating results when larger amounts of fermentation have occurred. And the progressive emptying of the ampoule constantly diminishes the quantity of fluid in which the recorded fermentation is taking place. Other influences, moreover, which may affect the rate of fermentation, such as the presence of amino-acid or of phosphate, cause an increasingly greater percentage error. The suggestion has indeed been made that such influences completely vitiate the test. At these concentrations of yeast, however, the error that they produce is not large. Table I shows the fermentation produced in the medium, in the absence of a specific activating substance, by various influences of this kind.

Table I. *Showing absence of fermentation in standard yeast-Nägeli medium, in absence of specific activating substance.*

Added to 10 cc. yeast-Nägeli medium				05	.1	.15	.2 cc.
				Percentage fermentation			
Sodium phosphate 2 %	...	...	...	0	0	1	1
Commercial glucose 2 %	...	...	...	1	1	1	1
Dried blood 2 % extract	...	...	...	1	1	1	2

Table II shows, further, that when an extract of dried yeast, which is an efficient activating substance, is added to the medium, the further addition of phosphate makes very little difference.

Table II. *Showing absence of effect of sodium phosphate on fermentation produced in activated yeast-Nägeli medium.*

10 cc. yeast-Nägeli medium, with 0.075 cc. of 2 % extract of dried yeast				Fermentation
Without sodium phosphate	...	...	...	4.5
With sodium phosphate	...	...	...	4.25

A more searching test, however, as to whether the activation of minimal quantities of yeast is or is not the effect of a single substance, and whether the fermentation thereby produced is or is not a satisfactory measure of such substance, is afforded by a comparison of the fermentation curves obtained when the activating substance is derived from various sources. The following have been used for this purpose: cow's milk, a 10 % solution of "Glaxo," a 2 % extract of the desiccated spleen of the calf (Armour), and a 2 % extract of dried yeast. Table III represents the average of a large number of determinations of the fermentation produced in the standard medium, after addition of these four substances in increasing quantity.

Table III. *Average fermentation obtained in standard yeast-Nägeli medium, after addition of increasing amounts of various activating substances.*

Amount added to 10 cc. yeast-Nägeli medium				05	.075	.1	.125	.15	.175	.2	.225	.25	.3 cc.
Substance added				Percentage fermentation in 24 hours									
Cow's milk	...	...	...	2.5	4.6	8.6	11.8	16.5	16.9	23.4	—	—	—
10 % Glaxo	...	...	...	—	—	4	—	7	—	8.5	—	15	24
2 % dried yeast	...	...	...	1.5	3.3	7.7	11.6	16.2	24.2	—	—	—	—
2 % desiccated spleen	...	...	...	—	2.5	4.4	6.5	12	14.5	18.1	21.5	—	—

It is clear from this table that while the activating power of cow's milk is almost exactly the same as that of 2 % yeast extract, it is considerably greater than that of either 10 % Glaxo or 2 % spleen. If, however, the activating powers be calculated from the above figures, of 17 % Glaxo and of 2.6 % spleen respectively, and the results plotted on a curve, it is found that such fermentation curves, for the four activating substances used, almost exactly coincide (Fig. 1). This seems to indicate that the activating substance is a single and definite entity, and that the method is a fair one for estimating its quantity in any material.

In the following experiments the curve shown in Fig. 1 has been taken as a standard, indicating the fermentation produced by a 2 % extract of dried yeast. The organs of various animals have been air-dried at 33°, and powdered, and their activating power determined in 2 % watery extract. By comparison with Fig. 1 (or Table III) it is possible to say how much dried yeast is the equivalent in activating power of unit weight of the material investigated. This, for convenience, I have called the "yeast-equivalent" of the substance. From Table III, for instance, it will be seen that the yeast-equivalent of this preparation of desiccated spleen is 0.8.

Fig. 1. Curve of fermentation in yeast-Nägeli medium, induced by addition of various activating substances.

Abscissae: Percentage addition to the medium of the various substances. Ordinates: Percentage fermentation in 24 hours at 33°. Crosses: 17 % Glaxo¹. Squares: Cow's milk. Hollow circles: 2 % dried yeast. Solid circles: 2.6 % desiccated spleen¹.

¹ Calculated from observations with 10 % Glaxo and 2 % spleen respectively.

RESULTS.

(1) *Pigeons*. The organs were taken and dried under the same conditions from a series of birds, being (1) normal pigeons, and (2) pigeons which on an exclusive diet of polished rice had developed avian polyneuritis, and had either died from this condition, or had been killed at the point of death. It was thought that if the substance which activates yeast were the same as that whose absence causes polyneuritis, the organs of polyneuritic birds should show a deficiency of this substance; and this deficiency should be most marked in the brain, where the symptoms are so nearly exclusively localised. Funk [1912], as is well known, has shown a deficiency in the N- and P-content of the brains of birds in this condition.

Because the symptoms of avian polyneuritis are markedly cerebellar in type, the cerebral hemispheres and cerebellum were dried and estimated separately. In order to illustrate the degree of consistency of the results, the figures obtained in the case of the brain (cerebral hemispheres) are given in detail in Table IV. Table V is a summary of results from all organs, the average alone being given in each case, together with the calculated "yeast-equivalent."

Table IV. *Details of experiments showing percentage fermentation obtained in yeast-Nügel medium by addition of 2 % extracts of dried cerebral hemispheres of pigeons.*

Pigeon	1	2	3	4	Av.	1	2	3	4	Av.
I. Polyneuritic										
P	2.5	6	2.5	7.5	4.9	11	11	11.5	9	10.5
Q	4.5	3.5	4	4.5	4.1	9	7	8	4.5	7.1
O	3	3	1.5	1.5	2.2	5	5	8.5	6	6.1
Y	8	4	3.5	5.5	5.2	8	9	10	10	9.2
Z	4	8	4	5	5.2	9	11	10	12	10.5
B	3	2	1.5	3.5	2.5	6	9	10	6	7.7
X	3.5	3	4.5	4	3.8	8	5	7.5	4	6.1
Average	4.1	4.2	3.1	4.9	4.0	8	8.1	9.4	7.4	8.2
II. Normal										
D	5	3	5.5	4	4.4	8	11.5	11	6	9.1
H	5	3	5	5	4.5	9	12	10	5	9.0
A	4	5	6	4	4.7	11	10	9	11	10.2
Average	4.7	3.7	5.5	4.3	4.5	9.3	11.2	10	7.3	9.5

Tables IV and V show, firstly, that the power to activate yeast is approximately the same in all the organs of pigeons that were examined, and that this power is not very much less than that of dried yeast itself: the liver, which is slightly more active than other organs, being scarcely at all inferior to yeast. This offers a striking contrast with the figures of Cooper, already referred to, showing the relative efficiency of the organs in preventing the development of polyneuritis.

So marked a contrast seems to show quite clearly, not only that the yeast-activating substance is not identical with the antineuritic vitamin, but that the two are not associated together in constant relative proportions.

Table V. *Showing average fermentation produced in standard yeast-Nägeli medium by addition of 2 % extracts of dried organs of pigeons, and "yeast-equivalents" calculated therefrom.*

Organ	... 2 % cerebrum			2 % cerebellum			2 % liver			2 % kidney			2 % heart			2 % muscle			
Cc. added to 10 cc. yeast-Nägeli medium	...	.1	.15		.1	.15		.1	.15		.1	.15		.1	.15		.1	.15	
Pigeon		% Ferm.	Yeast equiv.		% Ferm.	Yeast equiv.		% Ferm.	Yeast equiv.		% Ferm.	Yeast equiv.		% Ferm.	Yeast equiv.		% Ferm.	Yeast equiv.	
I. Polyneuritic																			
<i>P</i>		5	10.5	.8	1.5	4	.4	9.5	14.5	1.0	7	12	.9	8	12	.95	5	11	.8
<i>Q</i>		4	7	.7	5	12	.85	4.5	12	.8	4	8	.75	5	9	.75	5.5	11	.85
<i>O</i>		2	6	.5	4	9	.75	8.5	14	1.0	4.5	10	.8	4	8	.75	4	6	.7
<i>Y</i>		5	9	.8	5.5	11	.85	7	14	.9			.85						
<i>Z</i>		5	10.5	.8				8	9.5	.9									
<i>B</i>		2.5	7.5	.6				8	12	.9	6	10.5	.85	6	8	.8	5.5	9.5	.8
<i>X</i>		4	6	.6	3.5	8	.7	6.5	8.5	.8									
Average		4	8.2	.7	4	9	.75	7.5	12	.9	5.4	10	.8	5.7	9	.8	5	9.5	.8
II. Normal																			
<i>D</i>		4.5	9	.8	5	10	.8	8.5	25	1.1	4	8	.75	6.5	11	.85	4	5	.6
<i>H</i>		4.5	9	.8	4	8	.75				8								
<i>A</i>		4.5	10	.8				5	7.5	.7	8	14	1.0	4.5	9	.8	5	8	.7
Average		4.5	9.3	.8	4.5	9	.8	6.7	16.3	.9	6	11	.85	5.5	10	.8	4.5	6.5	.7

The tables show, moreover, secondly, that the activating power of the organs of polyneuritic pigeons is only very slightly less than that of the normal organs. This fact serves to strengthen the conclusion that deficiency of the substance which activates yeast is not the deficiency which causes polyneuritis.

There might indeed be a possibility that the organs of polyneuritic animals, while retaining their percentage of this activating substance, suffered a loss in their total content, by diminution in their whole weight. However, as Abderhalden has shown [1921], such a diminution in the weight of individual organs in polyneuritis occurs only in the case of the liver and voluntary muscles, not in that of the brain. This observation was confirmed in the foregoing experiments, as is seen in Table VI.

Table VI. *Showing weights of normal and of polyneuritic pigeons, and of their organs.*

Pigeon	Total body weight	Weights of organs		
		Cerebrum	Cerebellum	Liver
I. Polyneuritic				
P	150	0.98	0.22	4.52
Q	165	1.05	0.20	7.78
O	145	1.13	0.22	3.15
Y	150	1.12	0.23	5.22
Z	165	1.01	0.26	6.10
B	224	1.11	0.27	8.67
X	199	0.94	0.27	5.85
Average	171	1.05	0.24	5.90
II. Normal				
D	270	1.08	0.28	6.39
H	—	1.03	0.25	—
A	323	1.15	0.24	6.86
Average	296	1.09	0.26	6.68

(2) *Rats*. It follows from the above experiments, that the yeast-activating substance cannot be identified with the antineuritic vitamin, its amount being very nearly as great in the organs of polyneuritic as in those of normal pigeons. Similar experiments were performed on rats, the organs of normal animals being compared with those of others, which had been either killed when moribund, or had died, as the result of a diet free from water-soluble vitamin; and with those of a third series which, as the result of such a diet, had ceased to grow for several weeks, but had been killed before any other symptoms had manifested themselves. The diet consisted of the usual mixture of purified caseinogen, starch, sugar, butter, and salts. Upon this diet growth ceased at once; but if the initial weight of the animals was not less than about 70 g., they remained active and in good condition for many weeks; eventually dying with or without irregular nervous symptoms. The course of this condition, as has often been observed, is in striking contrast with the regular and rapid onset and constant symptoms of polyneuritis in birds. Table VII gives the summary of the average results of experiments on the content of the organs of these rats in the substance which activates yeast.

It will be seen that in the case of normal rats, the "yeast-equivalents" approximate very closely to those found in pigeons; but that in the rats which had died as the result of deprivation of water-soluble vitamin, this "equivalent" has diminished considerably. The rats which were killed after a shorter period (six weeks) of vitamin starvation occupy in this respect an intermediate position.

Table VII. *Showing average percentage fermentation produced in standard yeast-Nägeli medium by addition of 2 % extracts of dried organs of rats, with "yeast-equivalents" calculated therefrom.*

Organ Cc. added to 10 cc. yeast-Nägeli medium	2 % brain				2 % liver				2 % kidney				2 % heart			
	.1	.15	.2	Yeast equiv.	.1	.15	.2	Yeast equiv.	.1	.15	.2	Yeast equiv.	.1	.15	.2	Yeast equiv.
Rat number	fermentation				fermentation				fermentation				fermentation			
I. Normal rats																
11	5.5	13	—	0.85	5.5	17	—	0.9	14	28.5	—	1.3	5	10	16	0.8
6	5	11	19.5	0.8	6	14	—	0.9	7	17	—	0.9	5.5	9	15	0.8
7	8	20	—	1.0	3.5	6	—	0.6	5.5	16	—	0.9	5	7	16	0.75
Average	6	15	—	0.9	5	12	—	0.8	9	20.5	—	1.0	5	9	16	0.75
II. Vitamin-free diet six weeks. No symptoms																
8	4.5	6	13	0.7	5	10	15	0.8	6	9	14	0.8	3.5	4.5	6	0.6
9	2	3	9	0.5	4	9	13	0.75	7.5	9	16	0.85	1.5	2.5	6	0.45
10	2	4	8	0.5	2.5	4	8	0.5	6	11	14	0.8	3	7	10	0.6
Average	3	4.3	10	0.6	4	8	12	0.7	6.5	9.7	15	0.8	2.7	4.7	7	0.5
III. Vitamin-free diet till death or moribund																
1	1	—	8.5	0.5	1.5	—	11	0.6	2.5	—	7.5	0.5	1	—	2.5	0.3
2	2	—	10.5	0.55	1	—	5	0.4	1	—	5	0.4	2	—	7.5	0.5
3	1.5	—	10	0.55	1.5	—	7.5	0.45	4	—	11	0.55	1	—	6	0.45
4	1.5	—	6.5	0.45	3	—	7.5	0.5	—	3	8	0.5	—	1	6	0.4
5	—	6	9	0.55	—	7	9	0.55	—	9.5	15.5	0.8	—	3	4.5	0.4
Average	1.5	6	9	0.55	1.5	—	8	0.55	2.5	6	9.5	0.6	1.5	2	5.3	0.4

There is therefore a considerable difference between the condition of these rats and that of polyneuritic pigeons. This is due, no doubt, to the long duration of the diet; for pigeons on a diet of polished rice are invariably dead from polyneuritis within 28 days. But it shows at any rate, that rats suffering from deficiency of water-soluble vitamin are depleted of a substance which polyneuritic pigeons still possess; since death occurs before this second deficiency is manifested.

These findings afford support to the conclusions of Funk and Dubin [1921], that there are two water-soluble vitamins present in yeast: it is the absence of one of these, vitamin B, which causes polyneuritis in birds; while the other, which these authors call vitamin D, is the "bios" of Wildiers, necessary to the activity of low concentrations of yeast cells; both of them are necessary for the proper growth of rats. The prevailing uncertainty as to the identity of the antineuritic and the growth-promoting vitamins is due, therefore, to the fact that the latter includes the antineuritic substance, but contains also another essential factor, the vitamin D.

So far as yeast is concerned, the "bios" effect indicates that the vitamin D alone is necessary for the activation of low concentrations of yeast cells, and leads not only to fermentation but also to growth. This must not be taken to imply, however, that fermentation and growth are identical phenomena; nor that vitamin D is the only requisite for the manifestation of either or both of them. The fact that vitamin B is present in yeast cells is a proof to the contrary; and other vitamins, it may be, are responsible for various functions in the life-history of the yeast-plant. The "bios" effect signifies merely that vitamin D is one of the substances necessary to the life of the cell, and that it differs from most other such substances in undergoing dilution by diffusion into the surrounding medium.

SUMMARY.

(1) The substance which activates minimal concentrations of yeast is a definite chemical entity, though obtainable from many sources. It may be measured fairly satisfactorily by the fermentation to which it gives rise.

(2) It is present, in approximately the same amount, in the following organs, both of pigeons and rats: cerebrum, cerebellum, liver, kidney, heart, and voluntary muscle; its amount in any of these is about 80 % of its amount in dried yeast. This distribution is contrasted with that of the antineuritic vitamin.

(3) In the organs of pigeons rendered polyneuritic by a diet of polished rice, it is present in the same amount as in those of normal pigeons.

(4) In the organs of rats fed on a diet purified of water-soluble vitamin, its amount progressively diminishes, and when death occurs is only about one-half the normal.

(5) For these reasons this activating substance cannot be identified with

the antineuritic vitamin B. The deprivation of water-soluble vitamin from rats is held to involve a double deficiency; a deficiency of vitamin B in the first place, and of the yeast-activating substance in the second. This is the substance which Wildiers called "bios," and Funk the vitamin D.

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