CCXXIX. STUDIES ON AMINO-ACID DEHYDROGENASE.

II. ACTIVATOR OF PROLINE DEHYDROGENASE.

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(Received 6 July 1936.)

In a previous communication [Das, 1936] it was shown that there was present in pig kidney a thermostable substance capable of acting as activator of proline dehydrogenase. The present investigation was undertaken with the twofold object of studying the chemistry of the activator and the occurrence of the activator in yeast. Baker's yeast has been found to be a rich source of the activator. In the present investigation *dl*-proline has been used throughout as substrate.

Extraction of the activator.

In the earlier paper [Das, 1936] the activator was demonstrated in the enzyme solution from pig kidney which had been heated at 80° and *pH* 8.0 for 5 min. In the present investigation, in order to ascertain if it were possible to obtain the activator from baker's yeast, 100 g. of the latter (about 80 % moisture) were heated with 200 ml. water at 85° and centrifuged after cooling. The centrifugate was found to activate proline dehydrogenase considerably even in small amount (15.4 mg. solid). It was also proved that the activation was not due to the presence of a donator or to non-enzymic oxidation of proline, Table I.

Table I.

No.	Enzyme solution ml.	Yeast activator ml.	Phosphate buffer ml.	<i>M</i> proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	26.2	57.4	89.1	115.3
2	1.0	0.5	1.0	0.1	87.1	161.8	214.7	249.0
3	1.0	0.5	1.0	—	—	—	—	—
4	—	0.5	1.0	0.1	—	—	—	—

Method of experiment.

The oxidation experiments were carried out in the Warburg-Barcroft apparatus as described in the previous communication [Das, 1936]. The enzyme solution was prepared as already described.

Purification and chemical nature of the activator.

In order to study the chemical nature of the activator attempts were made to obtain it in a relatively concentrated form by methods of precipitation and adsorption. Experimental details are given below and the results are summarized in Table II.

It was found that the activator could be adsorbed on animal charcoal, frankonit and acid clay from slightly acid solution and eluted by treatment with

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Table II.*

Enzyme (pH 7.8) (ml.) ...	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Phosphate buffer (pH 7.8, M/20) ...	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Proline, M ...	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
3 experiments:										
Y (16)	—	0.5	—	—	—	—	—	—	—	—
Y (16) a	—	—	0.5	—	—	—	—	—	—	—
Y (47)	—	—	—	—	0.5	—	—	—	—	—
Y (48)	—	—	—	—	—	0.5	—	—	—	—
Y (67)	—	—	—	—	—	—	—	0.5	—	—
Y (68)	—	—	—	—	—	—	—	—	—	0.5
O ₂ uptake (μl./60 min.)	75.8	89.7	80.5	188.0	242.7	141.5	135.4	416.7	206.3	—
Y (7)	—	0.5	—	—	—	—	—	—	—	—
Y (8)	—	—	0.5	—	—	—	—	—	—	—
Y (9)	—	—	—	0.5	—	—	—	—	—	—
Y (84)	—	—	—	—	—	0.5	—	—	—	—
Y (33)	—	—	—	—	—	—	—	0.3	—	—
Y (37)	—	—	—	—	—	—	—	—	—	0.2
O ₂ uptake (μl./60 min.)	129.4	242.0	248.2	266.5	227.9	291.4	99.1	250.7	242.4	—
2 experiments:										
Y (82)	—	0.5	—	—	—	—	—	—	—	—
Y (83)	—	—	0.5	—	—	—	—	—	—	—
Y (82 + 83)	—	—	—	(0.5 + 0.5)	—	—	—	—	—	—
Y (56)	—	—	—	—	—	0.4	—	—	—	—
Y (61)	—	—	—	—	—	—	0.6	—	—	—
O ₂ uptake (μl./60 min.)	235.1	189.0	220.0	230.4	198.1	254.5	262.3	—	—	—
Y (21)	—	0.5	—	—	—	—	—	—	—	—
Y (26)	—	—	0.5	—	—	—	—	—	—	—
Y (25)	—	—	—	0.5	—	—	—	—	—	—
Y (101)	—	—	—	—	—	0.5	—	—	—	—
Y (102)	—	—	—	—	—	—	0.5	—	—	—
Ash of Y (101)	—	—	—	—	—	—	—	5 mg.	—	—
O ₂ uptake (μl./60 min.)	53.5	105.3	97.3	60.5	160.0	248.0	249.0	155.4	—	—
Proline, M/2	0.1	0.1	0.1	0.1	—	—	—	—	—	—
Proline, M	—	—	—	—	0.1	0.1	0.1	—	—	—
Y (95)	—	0.5	—	—	—	—	—	—	—	—
Y (97)	—	—	0.5	—	—	—	—	—	—	—
Y (100)	—	—	—	0.5	—	—	—	—	—	—
Y (37)	—	—	—	—	—	0.2	—	—	—	—
Y (42)	—	—	—	—	—	—	0.2	—	—	—
O ₂ uptake (μl./60 min.)	111.5	192.5	198.4	177.4	97.8	241.7	80.7	—	—	—

* For description of different fractions see the experimental section.

alkali from acid clay and frankonit but not from animal charcoal. The loss was considerable in the case of frankonit. Attempts to adsorb the activator on kaolin and aluminium hydroxide C_γ (Willstätter) had failed, the activity always remaining in the filtrate. Abortive attempts were also made to adsorb the activator on aluminium oxide (Merck, standardized according to Brockmann) in a "chromatogram" tube.

The activator was precipitated by salts of heavy metals such as mercury and silver. Attempts were made to precipitate with lead acetate but the activity was found to be missing both from the filtrate and the precipitate. Possibly the activator was adsorbed by lead sulphide. The filtrate and precipitate combined also failed to activate proline dehydrogenase.

It is probable that the activator is not dependent on the presence of a primary amino-group for it is not inactivated by nitrous acid. At pH 5.0 the activator is not affected in 8 hours by a current of air at 20° nor is it destroyed by H₂O₂.

Attempts to extract the activator by ether from alkaline solution failed and the whole of the activity was precipitated by alcohol. The activator was destroyed by ashing.

Influence of lactoflavin.

Lactoflavin was incapable of activating proline dehydrogenase showing that the activation was not due to the presence of flavin in the yeast extract (Table III).

Table III.

No.	Enzyme solution ml.	Lacto-flavin (40 γ /ml.) ml.	Phosphate buffer ml.	<i>M</i> proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	43.6	70.3	94.5	111.5
2	1.0	0.5	1.0	0.1	29.2	58.3	84.5	99.1
3	1.0	0.5	1.0	0.1	34.2	59.1	84.0	96.5

Influence of flavin-enzyme.

Formerly it was shown [Das, 1936] that flavin-enzyme [Warburg & Christian, 1932, 1, 2] failed by itself to activate proline dehydrogenase. The possibility was next considered that flavin-enzyme might act in combination with the activator to catalyse the oxidation of proline by the enzyme; this was however not the case (Table IV).

Table IV.

No.	Enzyme solution ml.	Heated enzyme solution ml.	Flavin-enzyme 0.2 g. in 10 ml. water ml.	Phosphate buffer ml.	<i>M</i> proline ml.	μ l. O ₂ uptake after min.			
						15	30	45	60
1	1.0	—	—	1.0	0.1	39.6	92.4	132.0	163.7
2	1.0	—	0.5	1.0	0.1	32.1	75.8	116.6	145.7
3	1.0	0.5	—	1.0	0.1	72.7	140.4	188.0	225.6
4	1.0	0.5	0.5	1.0	0.1	77.6	150.3	206.0	249.7

EXPERIMENTAL.

Preparation of fractions.

1. *Y* (16) *a*. 50 ml. yeast extract, prepared as described above, were shaken with 5 g. of animal charcoal (Merck) at pH 5.0 and left in the refrigerator overnight. Removal of the charcoal by filtration yielded solution *Y* (16) *a*.

Y (16). The charcoal residue was eluted by grinding with 24 ml. phosphate buffer (*M*/20, pH 7.8) and 1 ml. 1% Na₂CO₃ for 30 min. and filtered. 25 ml. of clear solution were obtained.

2. *Y* (48). 40 ml. yeast extract were stirred with 2 g. frankonit at pH 5.0 for 30 min. and filtered.

Y (47). The frankonit residue was eluted by stirring with 16 ml. phosphate buffer (*M*/20, pH 7.8) and 4 ml. 1% Na₂CO₃ for 30 min. and filtered.

3. *Y* (68). 100 ml. yeast extract were agitated with 5 g. acid clay at pH 5.0 for 30 min. and filtered.

Y (67). The acid clay residue was eluted by stirring with 20 ml. 1% Na₂CO₃ for 30 min. The clear filtrate measured 25 ml.

4. *Y* (8). 50 ml. yeast extract [*Y* (7)] were adsorbed with 2 g. kaolin for 120 min. and centrifuged. The clear centrifugate formed fraction *Y* (8).

5. *Y* (9). 50 ml. yeast extract [*Y* (7)] were agitated with 10 ml. suspension of aluminium hydroxide *C γ* (Willstätter) for 120 min. and centrifuged. The clear centrifugate formed fraction *Y* (9).

6. Y (84). 50 ml. yeast extract were completely precipitated with 10% mercuric acetate. The precipitate was washed, suspended in water acidulated with acetic acid and decomposed with H_2S . The clear filtrate [Y (84)] was freed from H_2S by a current of air.

7. Y (82). 100 ml. yeast extract were precipitated with saturated lead acetate at pH 5.0 and centrifuged. The precipitate was washed, suspended in water acidulated with H_2SO_4 and decomposed with H_2S . The clear filtrate [Y (82)] was freed from H_2S by a current of air and by distillation *in vacuo*.

Y (83). The filtrate from the lead acetate precipitate was freed from lead by passing H_2S and excess of the latter was removed by aeration and vacuum distillation. The final fraction measured 90 ml.

8. Y (61). 10 ml. yeast extract [Y (56)] were passed twice through aluminium oxide (Merck, standardized according to Brockmann) in a "chromatogram" column at pH 5.0. The clear filtrate measuring 15 ml. formed fraction Y (61).

9. Y (37). 20 ml. yeast extract [Y (33)] were completely precipitated with 10% $AgNO_3$ and centrifuged. The silver precipitate was decomposed with HCl and centrifuged. The clear centrifugate measuring 5 ml. formed fraction Y (37).

10. Y (25). 50 ml. yeast extract [Y (21)] were made alkaline (pH 10.0) with NaOH and shaken three times with ether, each time with about 10 ml. The ethereal layer was evaporated to dryness and taken up in 10 ml. water.

Y (26). The aqueous layer after ether extraction and freeing from excess of ether formed fraction Y (26).

11. Y (95). 200 g. baker's yeast were extracted as usual with 400 ml. water and the extract was completely precipitated with 10% mercuric acetate solution. The washed precipitate was suspended in water acidulated with acetic acid and decomposed with H_2S . The clear filtrate was freed from H_2S by aeration. This solution, measuring 40 ml., was then agitated twice with 2 g. portions of acid clay and was filtered under suction. The acid clay adsorbate was eluted twice with 10 ml. portions of 1% Na_2CO_3 solution; the eluate measuring 20 ml. formed fraction Y (95).

12. Y (97). 2 ml. Y (95) were treated with 0.5 ml. "perhydrol" (Merck, "perhydrol" 1 vol. and 9 vol. water = 10 vol. 3% H_2O_2), warmed at 40° for 15 min. and then evaporated to dryness in a vacuum desiccator; the residue was taken up in 2 ml. water.

13. Y (100). 2 ml. Y (95) were treated with 5 drops of glacial acetic acid and 5 drops of 25% $NaNO_2$ solution and heated at 90° for 5 min. Presence of excess of HNO_2 was verified by testing with starch-iodide. The solution was then evaporated in a vacuum desiccator and the residue taken up in 2 ml. water.

14. Y (102). 20 ml. yeast extract [Y (101)] were aerated for about 8 hours at room temperature (20°) at pH 5.0.

15. Y (42). 1.5 ml. Y (37) [silver fraction] were precipitated with alcohol and filtered, the residue being washed with more alcohol. The clear filtrate together with the washing was evaporated to dryness under reduced pressure and the residue taken up in 1.5 ml. water.

16. 10 ml. yeast extract [Y (101)] were burnt to ash. Total weight of the ash was found to be 14.8 mg.

SUMMARY.

1. An activator of the proline dehydrogenase of pig kidney has been found in baker's yeast.

2. The activator can be adsorbed on acid clay, frankonit and animal charcoal in slightly acid solution and can be eluted by alkali from acid clay and frankonit. The loss is considerable in the case of frankonit. Attempts to elute the activator from animal charcoal by phosphate buffer (pH 7.8, M/20) and alkali have failed. The activator is not adsorbed on kaolin, aluminium hydroxide C_γ (Willstätter) and on Al_2O_3 in a "chromatogram" column.

3. The activator can be precipitated by silver nitrate and mercuric acetate.

4. The activator is not inactivated by HNO_2 , H_2O_2 or by aeration.

5. The activator is not extracted by ether from alkaline solution and is precipitated by alcohol.

6. Lactoflavin is incapable of activating proline dehydrogenase.

7. Flavin-enzyme is incapable of catalysing the oxidation of proline by the enzyme together with the activator.

The author is grateful to Prof. Hans von Euler for his interest in this work.

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