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Nicotinic Acid Oxidation in *Pseudomonas fluorescens.**

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In the determination of nicotinic acid in blood, Allinson[1] first employed a microorganism as the source of an enzyme which specifically destroyed nicotinic acid. The microorganism used was the NC (neutral culture) isolated from soil by Dubos and Miller.[2] Later Koser and Baird[3] made an extensive investigation of bacteria which destroy nicotinic acid during cell multiplication. They found that bacteria of the *Pseudomonas fluorescens* and *Serratia marcescens* groups grew on a synthetic medium in which nicotinic acid was the only organic compound and that they failed to grow when nicotinic acid was replaced by the isomers, isonicotinic acid and picolinic acid.

The nature and mode of action of the bacterial enzyme have not yet been elucidated. The experiments recorded below show that in the case of *Pseudomonas fluorescens* an oxidative mechanism is involved in the enzymic decomposition of nicotinic acid.

Materials and Methods. I. *Culture:* Preliminary tests with strains 1, 2, 3, 4, 12 and 30‡ showed that the latter 2 were most active in decomposing nicotinic acid. *Serratia marcescens* took 7 to 8 days to destroy as much nicotinic acid as was destroyed by *Pseudomonas fluorescens* in 36 hours. The growth of the NC organism was unsatisfactory when the medium was prepared with local tap water or artificial tap water.[4] Since *Pseudomonas fluorescens* 30 was most active and its mineral requirements were easily met by tap water or distilled water plus magnesium sulphate, it was chosen as the test organism. It was grown in media composed of: 2 g Na_2HPO_4; 1.5 g KH_2PO_4; 5 g NaCl; 0.1 g $MgSO_4$; and 2 g nicotinic acid in 1 liter distilled water. The pH was adjusted with sodium hydroxide to 6.8 to 6.9. The temperature was 27°C. The bacteria became well adapted to the medium by repeated transfer of 24 hour cultures. Stock cultures were maintained on agar slopes of nicotinic acid medium and no loss of activity was evident after 6 months. The agar cultures had a bright green color after 18 to 24 hours, which turned to reddish-brown after 48 hours.

To prepare suspensions of the *resting bacteria* transfers were made from the nicotinic acid slopes to 50 ml of medium in 250 ml Erlenmeyer flasks. The flasks were shaken for 24 hours at 25°C after which 10 ml were transferred to 500 ml of medium in a 2-liter flask, the latter being shaken again for 18 to 24 hours. The cells were then separated by centrifuging, washed twice with M/50 phosphate buffer of the pH 7.0, and finally suspended in a volume of sterile water equal to 1/20th the volume of the culture medium. This suspension was stored at 5°C for over 2 months without loss of activity.

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1 Allinson, C. M. J., *J. Biol. Chem.*, 1943, **147**, 785.

2 Dubos, R., and Miller, B. F., *J. Biol. Chem.*, 1937, **121**, 429.

3 Koser, S. A., and Baird, G. R., *J. Infect. Dis.*, 1944, **75**, 250.

‡ The authors wish to express their thanks to Prof. S. A. Koser, Department of Bacteriology, University of Chicago, for the strains 12 and 30 of *Pseudomonas fluorescens* used in this work.

4 Miller, B. F., Allinson, M. J. C., and Baker, Z., *J. Biol. Chem.*, 1939, **130**, 383.

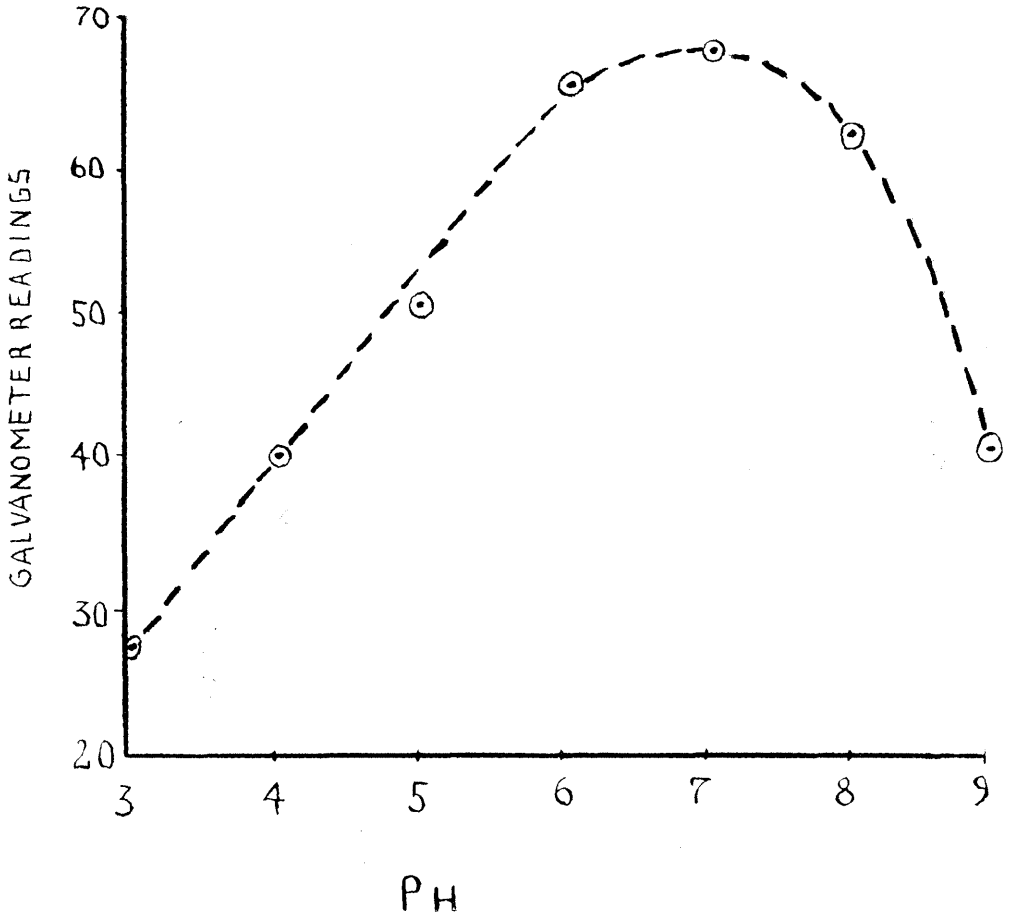

Fig. 1
Relationship of the enzyme activity to pH

II. *Measurement of activity of the bacterial suspension.* A series of tubes was prepared, each containing 5 ml of a standard solution of nicotinic acid (10 μg per ml), 0.5 ml of 0.2 M phosphate buffer of pH 6.0, and 0.05 ml of bacterial suspension. These were allowed to stand at room temperature for 10 to 30 min. and the enzyme was then inactivated by immersion in a water bath at 90°C for 5 min. The tubes were centrifuged for 15 min. at 10,000 r.p.m. and 5 ml of the clear supernatant fluid were analyzed for nicotinic acid by a colorimetric method§ employing cyanogen bromide and p-phenylenediamine. The intensity of the yellow color was measured in a Coleman spectrophotometer at a wave-length of 420 mμ.

§ The procedure used for the estimation of nicotinic acid will shortly be published elsewhere.

Experimental. I. *Activity of the culture.* It was found that 50 μg of nicotinic acid were destroyed in 15 min. at room temperature by 0.05 ml of the bacterial suspension. Under the same conditions there was no appreciable decomposition of nicotinamide.

II. *Effect of pH.* Aliquots of the substrate were adjusted to pH values ranging from 3 to 9. These were inoculated and the amount of nicotinic acid remaining was determined. The results shown in Fig. 1 indicate that the optimum pH was about 7.0.

III. *Attempt to obtain a cell-free enzyme.* Freezing and thawing a sample of the bacterial suspension 8 times in 24 hours destroyed 66% of the normal activity, as is shown in Table I. Experiments are now in progress to obtain cell-free enzymes by other methods.

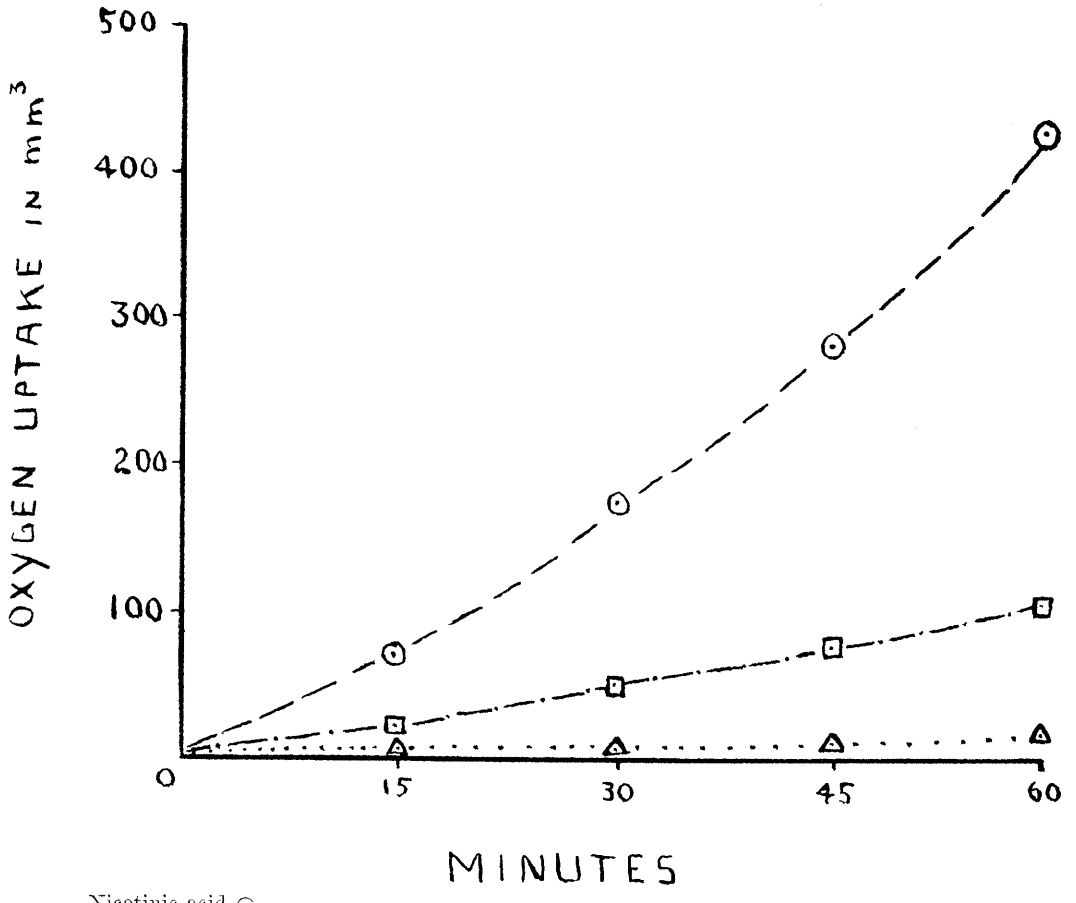

FIG. 2.
Oxygen uptake of *Ps. fluorescens* 30 with nicotinic acid as influenced by sodium azide and hydroxylamine hydrochloride (M/60). Temperature 27°.

TABLE I.
Effect of Alternative Freezing and Thawing on Enzyme Activity.

Time in min.	μg nicotinic acid destroyed by (1) normal cells	(2) treated cells
5	29.6	5.4
10	47.0	9.9
15	50	17.0
30	—	22.3

IV. *Mode of destruction of nicotinic acid.* Warburg experiments showed that the destruction of nicotinic acid was accompanied by oxygen consumption. There was a decrease in the oxygen consumption when the amount of the substrate was decreased. The experiments were conducted at 27°C with M/15 phosphate buffer of pH 7 as medium. The total volume of liquid in the vessels was 2.3 ml; 0.3 ml of bacterial suspension were employed. The center well contained 0.2 ml of 50% KOH.

A nicotinic acid solution containing 615 μg was tipped into the main vessel from one side arm after equilibrium had been reached. After 75 minutes the oxygen uptake was determined and the reaction was stopped by the addition of 0.5 ml of concentrated sulphuric acid from the second side arm. The residual nicotinic acid was determined colorimetrically. For the oxidation of 0.475 micromoles of nicotinic acid 1.42 micromoles were required, *i. e.* 3 moles of oxygen per mole of

nicotinic acid. In a similar experiment 0.0475 micromoles of nicotinic acid were destroyed and 0.13 micromoles of oxygen were taken up. This corresponds to 2.7 moles of oxygen per mole of nicotinic acid.

Sodium azide and hydroxylamine inhibited the oxidation drastically, as is seen in Fig. 2. The manometric measurements were verified with the colorimetric method. In another test, the detergent, lauryl pyridinium chloride‖ in a concentration of 0.0013%, brought about complete inhibition. Octyl alcohol and toluene inhibited the oxidation 68 and 80%, respectively, as indicated with the colorimetric method. There was no oxygen uptake with nicotinamide as substrate.

Summary and Conclusion. The destruction of nicotinic acid by *Pseudomonas fluorescens,* previously described by Koser and Baird[3] was found to be an enzymic oxidation. Utilization of nicotinic acid is accompanied by oxygen uptake at a ratio of about 3 oxygen to 1 nicotinic acid. Nicotinamide is not attacked by the oxidase under our experimental conditions. Two types of inhibitors interfere with the enzymatic destruction of nicotinic acid: inhibitors of heavy metal enzymes, such as sodium azide and hydroxylamine, and surface active agents, such as octyl alcohol, toluene and lauryl pyridinium chloride. The rate of oxygen consumption indicates a destruction of the nicotinic acid molecule. Since nicotinic acid serves as a substrate and not as a co-enzyme it should be noted that this reaction sets it apart from its known function in the co-factors.

‖ We wish to thank the Hooker Electrochemical Corporation, Niagara Falls, N.Y., for a sample of lauryl pyridinium chloride.

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Effect of Age in Guinea Pig on Local Passive Sensitization and Skin Reactions Toward Histamine.

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Young guinea pigs are universally accepted as being more suitable for the study of anaphylactic reactions than old animals. Thomsen,[1] who confirmed the work of others, found old guinea pigs more difficult to sensitize than young guinea pigs. He also studied the effect of age on the passive sensitivity developed after injection of anti-horse rabbit serum. Sensitivity was measured by injecting the antigen intravenously. His results showed that the young guinea pigs became more sensitive than the old guinea pigs.

To determine whether age had a similar effect on the production of local anaphylaxis, a comparison was made of the ability to transfer sensitivity passively to the skin of old and of young animals. The method recently described by Chase[2] for producing local passive sensitization in the skin of the guinea pig was used for making these comparisons.

Since a tissue liberation of histamine or histamine-like substance is thought to occur during anaphylactic reactions,[3,4] the response to intradermal injection of this substance in young and in old guinea pigs was studied.

Material. All of the animals were albinos and were obtained from a single breeding colony. Animals of both sexes were used. The old animals were 2 to 4 years and the young

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4 Lewis, T., *Brit. M. J.,* 1926, **2**, 61.