The Inhibition of Growth and Cozymase Synthesis in Bacteria by Halogen-substituted Nicotinic Acids

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(Received 13 January 1954)

Among the non-metallic elements which form single covalent bonds, fluorine most nearly approaches hydrogen in atomic weight and size. The effect of the substitution of fluorine for hydrogen in molecules of essential metabolites is therefore of interest. This is especially so since it has been shown that mono-fluoroacetate is transformed into fluorocitrate which in its turn inhibits aconitase and thus blocks the tricarboxylic acid cycle (Peters, 1952). Fluoro-tyrosine and fluorophenylalanine have been shown to act as antagonists to tyrosine and phenylalanine in *Neurospora* (Mitchell & Nieman, 1947). More recently, Halvorson & Spiegelman (1952) have shown that these two fluoro analogues inhibit adaptation in bacteria. The halogen-substituted nicotinic acids examined in this paper were synthesized for test as therapeutic agents (Minor, Hawkins, Vanderverf & Roe, 1949; Hawkins & Roe, 1949). One of them, namely 5-fluoronicotinic acid was shown to inhibit bacterial growth. It seemed likely that this inhibition was due to the inhibition of cozymase synthesis. This idea has been tested by growth tests as well as by studies of the effect of the analogues on cozymase synthesis in washed suspensions of bacteria as described in a previous paper (Hughes & Williamson, 1952a). The anticipated inhibition of cozymase synthesis by 5-fluoronicotinic acid has been found in three species of bacteria, but it has not been possible to establish definitely the precise point at which the synthesis is blocked.

EXPERIMENTAL

Organisms

Lactobacillus arabinosus 17-5 was maintained by daily subculture on the enzymic casein hydrolysate medium described by Nossal (1951). *Staphylococcus aureus*, National Collection of Type Cultures (N.C.T.C.) Strain 7663, *Bacterium coli*, N.C.T.C. Strain 86 and *Proteus vulgaris* I (a locally isolated strain) were maintained by subculture on agar slopes containing 4% yeast extract and 1% glucose.

Growth tests

The effect of nicotinic acid and its derivatives on the growth of *Lb. arabinosus* was studied in the supplemented casein hydrolysate medium of Barton-Wright (1946).

Double-strength medium for *Staph. aureus* was made from stock solutions (Barton-Wright, 1946) as follows: hydrolysed casein, 50 ml.; inorganic salts A and B, 5 ml.; cystine, 50 ml.; tryptophan, 50 ml.; pyridoxine, 1.0 ml.; riboflavin, 1.0 ml.; biotin, 4.0 ml.; thiamine, 1.0 ml.; glucose, 10 g. were dissolved in 500 ml. of water and the pH was adjusted to 7.6 with NaOH. The medium for *Prot. vulgaris* and *Bact. coli* was prepared as follows: KH_2PO_4 , 4.5 g.; NH_4Cl , 1.0 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g.; salts B, 5.0 ml.; hydrolysed casein, 10 ml.; pyridoxine, folic acid, pantothenic acid, thiamine, all 1.0 ml.; biotin, 4.0 ml.; glucose, 10 g. or lactic acid 2.5 g. These were dissolved in 800 ml. of water, the pH was adjusted to 7.6 with NaOH, and then simmered for 10 min., cooled and filtered. All the media were stored at 2° over CHCl_3 and sterilized at 10 lb./sq.in. for 10 min. before test. In growth tests with *Lb. arabinosus* and *Staph. aureus* the medium (2.0 ml.) and the solutions under test (2.0 ml.) were mixed in $6 \times \frac{1}{2}$ in. test tubes which were covered with aluminium caps (Oxo Ltd., London) and then sterilized. Cozymase was sterilized by filtration through a Seitz filter and added to the sterilized medium aseptically; no significant amounts of cozymase were absorbed by the filter pad. After sterilization the test tubes were inoculated with one drop of a washed suspension of the cells which had been diluted to contain approximately 10 000 cells per ml. In tests with *Prot. vulgaris* and *Bact. coli* the final volume of medium was 10 ml. and 50 ml. conical flasks were used in the place of test tubes. The temperature of incubation was 37° except with *Bact. coli*, which was incubated at 30°. Growth was measured turbidimetrically in a EEL Nephelometer (Evans Electro Selenium Ltd.) when test tubes were used, or by eye when flasks were used. *Lb. arabinosus*, which adhered to the side of the test tube during the first hours of growth, was evenly suspended by blowing a sterile air stream through the medium before turbidimetric measurements.

Preparation and handling of washed suspensions

To study the effect of the inhibitors on the catalytic function and synthesis of cozymase, deficient cells were produced as follows: *Lb. arabinosus* 17-5 was grown for 22-44 hr. on the supplemented casein hydrolysate medium (Barton-Wright, 1946) containing 2×10^{-7} M nicotinic acid (see Hughes & Williamson, 1952a). *Staph. aureus*, 6773, was grown for 18-20 hr. in Roux bottles on the solid medium described by Krebs (1937); these cells were deficient in thiamine as well as cozymase (see p. 491), but it was found that the addition of thiamine either to the medium or to washed suspensions made no difference to the rate of cozymase synthesis. *Prot. vulgaris* was grown on the vitamin and casein supplemented salt medium already described to which had been added 2×10^{-7} M nicotinic acid. After

growth, the *Lb. arabinosus* and *Prot. vulgaris* were collected by centrifuging and washed once in 0.9% NaCl. *Staph. aureus* was washed from the surface of the agar with 0.9% NaCl, the washings strained through glass wool, the cells collected by centrifuging and washed once again. After washing, the organisms were suspended in Krebs & Henseleit (1932) saline to give suspensions containing 2–10 mg. dry wt./ml. Dry weights were estimated by heating samples at 110° to constant weight and subtracting the dry weight of an equal volume of saline.

The effect of nicotinic acid and its analogues on glycolysis by washed suspensions was followed manometrically in the Warburg apparatus by measuring the evolution of CO₂ from a bicarbonate buffer as described by Hughes & Williamson (1952*a*). O₂ uptake by *Prot. vulgaris* was also followed manometrically; each Warburg cup was filled with 5–10 mg. dry wt. of cells suspended in 1.8 ml. Krebs & Henseleit (1932) saline containing 0.025*M* glucose in the main compartment. The centre well contained 0.2 ml. of 2*M*-KOH and a folded paper to absorb CO₂. When manometric data were not required cozymase synthesis was carried out in inverted 'Y' tubes (Hughes & Williamson, 1952*b*). The reaction was stopped by placing the vessel in an ice-salt freezing mixture. The cells were collected and washed by centrifuging as described previously (Hughes & Williamson, 1952*a*).

Nicotinic acid derivatives

The nicotinic acid, nicotinamide and cozymase were the specimens described in a previous paper (Hughes & Williamson, 1952*a*). The halogen-substituted nicotinic acids, and a small sample of 5-fluoronicotinamide synthesized according to Hawkins & Roe (1949), were kindly given by Dr K. Rohrman, Ely Lilly International Corporation, Indianapolis, U.S.A. The 2-, 5- and 6-monofluoronicotinic acid (2-FNA, 5-FNA, 6-FNA) showed no contamination with nicotinic acid on paper chromatography (see below), and were therefore not purified further. The 5-chloro- and 5-bromo-nicotinic acids (5-ClNA, 5-BrNA) had been found to be contaminated with nicotinic acid and were purified as follows: 324 mg. of the crude 5-BrNA were dissolved by warming in 20 ml. of the *n*-butanol phase of the butanol acetic solvent of Partridge & Westall (1948). The butanol solution was transferred to a powdered cellulose column (1.5 cm. wide by 50 cm. long) which had been previously washed with the butanol solvent until no material absorbing at 200 m μ . was detected in the eluate. The chromatogram was developed with the butanol:acetic solvent and 5 ml. fractions were collected in an automatic fraction collector. From each fourth fraction 0.1 ml. was taken and tested with CNBr and NaOH for 5-BrNA. The bulk of the substance was in fractions 18–26 and these were therefore pooled, and

evaporated to dryness at 60°, *in vacuo*. The residue was recrystallized twice from an ethanol:water mixture. The crystals were finally washed with water, dried over P₂O₅ and weighed 165 mg.; no nicotinic acid could be detected in this material. The 5-ClNA was purified similarly.

Identification of nicotinic acid and derivatives

The reaction with CNBr and NaOH (Hughes & Williamson, 1953) and ultraviolet absorption were used to identify nicotinic acid derivatives on chromatograms. After treatment with CNBr and NaOH the absorption spectra of nicotinic acids substituted at carbon-atom 5 were similar to that of nicotinic acid; the absorption maxima were between 370 and 380 m μ . The molecular extinction coefficients measured at 372 m μ . (ϵ) were, however, lower than those of nicotinic acid (Table 1, column 2); 2-FNA and 6-FNA did not react with CNBr. Solutions of nicotinic acid and the halogeno derivatives in *N*-HCl or *N*-NaOH had absorption spectra between 230 and 300 m μ ., similar to other pyridine derivatives; the maxima were between 260 and 280 m μ . (Table 1).

The detection of nicotinic acid in halogeno nicotinic acids by paper chromatography

Aqueous solutions of the halogeno acids (50 μ l. of 0.1 *M*) were placed on Whatman no. 1 paper strips with an Agla syringe and the spots dried in a warm air stream. The chromatograms were developed in a descending butanol:acetic mixture (Partridge & Westall, 1948) for 16 hr., dried at 60–80° in an air stream and photographed on to reflex paper in ultraviolet light as described by Markham & Smith (1949). Spots containing 5 μ l. of 0.03 *M* nicotinic acid were just visible by this method. The spots were approximately circular with a diameter of 2–3 cm. The *R_F* value of nicotinic acid (0.72) was sufficiently different from that of the halogeno nicotinic acids (0.8–0.9) to make a good separation possible. To isolate the nicotinic acid derivatives from the paper, the positions of the spots were marked with pencil and the paper corresponding to the spots was cut out. Each piece of paper was then extracted with 3 ml. water by heating for 10 min. in a boiling water bath. The growth-promoting activity of the extracts was estimated by microbiological assay. Two separate growth-promoting fractions were obtained from 5-BrNA nicotinic acid, one whose *R_F* corresponded to that of nicotinic acid (0.78) and the other whose *R_F* was less than that of nicotinic acid (0.38), only one spot whose *R_F* corresponded to that of nicotinic acid was obtained in chromatograms of crude 5-ClNA. In each case the main absorbing spot on the chromatogram, which was assumed to be that of the halogen-substituted nicotinic

Table 1. Spectrophotometric data of nicotinic acid and halogeno nicotinic acids

Compound	Absorbancy after reaction with CNBr $\epsilon_{372 \text{ m}\mu}$ ($\times 10^{-4}$)	Absorbancy of solution in 0.1 <i>N</i> -HCl		Absorbancy of solution in 0.1 <i>N</i> -NaOH	
		$E_{\text{max.}}$ (<i>m</i> μ .)	$\epsilon_{\text{max.}}$ ($\times 10^{-3}$)	$E_{\text{max.}}$ (<i>m</i> μ .)	$\epsilon_{\text{max.}}$ ($\times 10^{-3}$)
Nicotinic acid	3.02	260	4.36	260	3.10
2-Fluoronicotinic acid	No reaction	266	4.42	264	5.86
5-Fluoronicotinic acid	1.44	270	4.84	270	4.00
6-Fluoronicotinic acid	No reaction	262	3.70	263	3.70
5-Chloronicotinic acid	1.62	275	4.04	270	3.96
5-Bromonicotinic acid	1.25	280	3.32	270	5.36

acids, was without growth-promoting activity. As judged by the growth-promoting activity of the spots from the chromatograms, the amount of nicotinic acid in the crude 5-CINA was 0.004% (w/w), while the combined spots from the crude 5-BrNA corresponded to a contamination of 0.025% (w/w). This degree of contamination was sufficient to explain the apparent growth-promoting activity found in the crude compounds.

Spectrophotometric determination of cozymase

This method consists of measuring the increase in optical density of extracts of the cells at 340 m μ . after reduction with crystalline alcohol dehydrogenase and ethanol as described by Racker (1949). Spectrophotometric measurements were made in the micro-cells of a Spekker Absorptiometer (1 cm. light path, 2 mm. wide and 8 mm. deep) fitted to a micro-attachment constructed as described by Lowry & Bessey (1946), but adapted to a Unicam Spectrophotometer (Hughes, 1951). The cozymase was extracted by heating the cells suspended in 0.5 ml. of water for 20 min. at 75°, the solid residue was separated by centrifuging and 0.3 ml. of the clear supernatant pipetted into a 2 $\times$ $\frac{3}{8}$ in. test tube. A solution (0.04 ml.) containing 0.5 M sodium pyrophosphate and 0.1 M ethanol was added, and the mixture was then transferred with a dropping pipette to the absorptiometer cell, and the optical density at 340 m μ . ($E_{340\text{m}\mu}^{1\text{cm}}$) measured.

The solution was then returned to the test tube, and 0.01 ml. of a solution of alcohol dehydrogenase was added. After a thorough mixing, $E_{340\text{m}\mu}^{1\text{cm}}$ was measured when it had reached equilibrium (3–5 min.). The concentration of enzyme was such that 40 μ m-mole of cozymase was reduced in approximately 2 min. The cozymase content of the sample was calculated from the difference between the two readings, using the value for the molecular extinction coefficient ($\epsilon = 6.22 \times 10^3$) of Horecker & Kornberg (1948). Parallel estimations on extracts of *Lb. arabinosus* and *Staph. aureus* indicated that the spectrophotometric method gave values, some 10–15% lower than the fermentation method used previously (Hughes & Williamson, 1952a). This suggests that either nicotinamide riboside, or reduced cozymase are also present in the cells.

RESULTS

Growth tests

Effect of halogeno nicotinic acids on the growth of Lb. arabinosus. The five halogeno nicotinic acids inhibited to varying degrees the growth of *Lb. arabinosus* (Table 2). In all cases the magnitude of the inhibition was dependent not only on the concentration of the inhibitor but also the concentration of the nicotinic acid: i.e. the inhibition was competitive over the range tested (10^{-3} to 10^{-5} M) (cf. Tables 2 and 3). Differences between the inhibitors were most marked at the early stages of growth. As judged by inhibition indices the order of effectiveness was as follows: 5-FNA > 5-CINA > 5-BrNA > 2-FNA = 6-FNA. After 5 days of incubation,

Table 3. *Effect of 5-fluoronicotinic acid upon the growth of Lb. arabinosus stimulated by nicotinic acid, nicotinamide or cozymase*

For conditions see text.

Growth factor added ($\text{M} \times 10^5$)	5-Fluoro-nicotinic acid (conc.)	Growth Nephelometer readings after		
		16 hr.	23 hr.	60 hr.
Nicotinic acid				
5.0	None	46	51	87
0.5	None	42	57	56
0.05	None	38	47	50
5.0	$\text{M} \times 10^{-3}$	45	58	82
0.5	$\text{M} \times 10^{-3}$	20	86	73
0.05	$\text{M} \times 10^{-3}$	4	5	40
Nicotinamide				
5.0	None	49	88	88
0.5	None	42	59	88
0.05	None	20	26	25
5.0	$\text{M} \times 10^{-3}$	44	96	82
0.5	$\text{M} \times 10^{-3}$	12	56	23
0.05	$\text{M} \times 10^{-3}$	5	5	40
Cozymase				
5.0	None	48	78	88
0.5	None	34	38	88
0.05	None	11	14	17
5.0	$\text{M} \times 10^{-3}$	29	90	91
0.5	$\text{M} \times 10^{-3}$	8	55	87
0.05	$\text{M} \times 10^{-3}$	5	5	9

Table 2. *Effect of halogeno nicotinic acids upon the growth of Lb. arabinosus stimulated by varying concentrations of nicotinic acid*

For conditions see text. The concn. of inhibitor was 2.5×10^{-3} M in all cases.

Inhibitor	Nicotinic acid (M $\times 10^5$)	Growth Nephelometer* readings after		
		16 hr.	23 hr.	72 hr.
None	None	5	6	7
	250	19	56	82
	25	20	56	74
	2.5	22	52	72
	0.25	14	48	68
5-Fluoronicotinic acid	250	6	20	60
	25	4	10	58
	2.5	5	5	10
	0.25	6	6	6
2-Fluoronicotinic acid	250	28	51	84
	25	10	44	78
	2.5	16	41	76
	0.25	6	16	62
6-Fluoronicotinic acid	250	22	54	78
	25	14	52	78
	2.5	20	52	80
	0.25	10	36	62
5-Chloronicotinic acid	250	14	50	66
	25	14	52	66
	2.5	16	56	72
	0.25	8	52	66
5-Bromonicotinic acid	250	19	54	82
	25	16	54	74
	2.5	20	56	72
	0.25	9	52	68

* Nephelometer reading of 100 is equal to 2.8–3.0 mg. dry wt. bacteria/ml.

maximal growth was found in all tubes except where 5-FNA was present. Thus 5-FNA is the most effective antagonist of nicotinic acid. As cozymase synthesis is the only known reaction utilizing nicotinic acid in this organism (Hughes & Williamson, 1952a) and as the inhibition is competitive, it may be inferred that the conversion of nicotinic acid into cozymase is inhibited, and the question arises as to which step is affected. To decide this, the effect of nicotinamide and cozymase in reversing the inhibition by 5-FNA was examined. Their effect in overcoming the inhibition by 5-FNA was no greater than that of nicotinic acid (Table 3) despite the fact that both are readily available for growth (Ellinger & Abdel-Kader, 1949). The reversal was also competitive and this might be explained by the assumption that it was the function of cozymase that was inhibited rather than its synthesis. That this is not so is shown by the experiments with washed suspensions described below.

Effect of halogeno nicotinic acids on the growth of Staph. aureus. The early growth of *Staph. aureus* was also inhibited by the halogen-substituted nicotinic acids (Table 4); the magnitude of the inhibition was dependent not only on the concentration of the inhibitor but also on the concentration of nicotinic acid (Table 4). Inhibition indices after 18 hr. growth indicated that as in *Lb. arabinosus*, 5-FNA was the most effective antagonist of nicotinic acid. In contrast to *Lb. arabinosus* and *Prot. vulgaris*, the inhibition was not completely reversed in *Staph. aureus* after 4 days' growth, even in the highest concentrations of nicotinic acid (Table 4). This effect was most marked in the case of 5-FNA acid. As judged by their effects on the early growth, nicotinamide and cozymase were less readily available for growth than nicotinic acid; likewise they were both less effective in reversing the inhibition caused by 5-FNA.

Effect of halogeno nicotinic acids on the growth of Prot. vulgaris. All of the halogeno nicotinic acids were less effective in inhibiting the growth of *Prot. vulgaris* than they were for the other two organisms (Table 5), but again 5-FNA acid was the most effective. The inhibition was competitive and was completely reversed in the higher concentrations of nicotinic acid ($2.5 \times 10^{-6}M$) and 5-FNA ($10^{-3}M$). Nicotinamide, although readily available for growth, was no more effective in reversing the inhibition than was nicotinic acid. Cozymase was less readily available for growth and less effective than the other two growth factors in reversing the inhibition.

Effect of halogeno nicotinic acids on the growth of Esch. coli. Of the halogeno nicotinic acids, only 5-FNA inhibited the growth of *Esch. coli*. The inhibition was marked in the early growth (16–18 hr.) and was competitively reversed by nicotinic acid. Later

Table 4. *Effect of halogeno nicotinic acids upon the growth of Staph. aureus stimulated by varying concentrations of nicotinic acid*

For conditions see text. The concn. of inhibitor was $2.5 \times 10^{-3}M$ in all cases.

Inhibitor	Nicotinic acid ($M \times 10^8$)	Growth Nephelometer readings after		
		16 hr.	23 hr.	74 hr.
None	None	8	9	8
	250	34	48	86
	25	34	44	86
	2.5	36	48	84
	0.25	12	15	88
5-Fluoronicotinic acid	250	12	21	36
	25	10	18	36
	2.5	10	10	40
	0.25	9	9	10
2-Fluoronicotinic acid	250	22	37	56
	25	18	47	69
	2.5	20	48	70
	0.25	12	12	35
6-Fluoronicotinic acid	250	18	26	40
	25	18	26	45
	2.5	14	28	45
	0.25	10	10	16
5-Chloronicotinic acid	250	20	26	42
	25	18	28	44
	2.5	20	28	44
	0.25	11	10	40
5-Bromonicotinic acid	250	21	28	42
	25	20	26	42
	2.5	16	23	43
	0.25	12	10	43

Table 5. *Effect of halogeno nicotinic acids on the growth of Proteus vulgaris at varying concentrations of nicotinic acid*

For conditions see text. The concn. of inhibitor was $10^{-3}M$ in all cases.

Inhibitor	Nicotinic acid concn. ($M \times 10^8$)	Growth Nephelometer readings after	
		15½ hr.	24 hr.
None	None	7	7
	10	60	91
	1.0	60	96
	0.1	37	55
5-Fluoronicotinic acid	10	63	93
	1.0	24	63
	0.1	6	17
2-Fluoronicotinic acid	10	60	96
	1.0	47	79
	0.1	9	91
6-Fluoronicotinic acid	10	60	90
	1.0	45	90
	0.1	12	50
5-Chloronicotinic acid	10	63	87
	1.0	34	83
	0.1	31	67
5-Bromonicotinic acid	10	64	90
	1.0	60	93
	0.1	41	85

(24–30 hr.) the cells grew out fully in the highest concentrations of the inhibitor and without the addition of nicotinic acid. This is presumably because this organism, unlike the previous three, can synthesize its own nicotinic acid (Table 6).

Experiments with washed suspensions

Effect of halogeno nicotinic acids on glycolysis in Lb. arabinosus. Glycolysis in washed suspensions of *Lb. arabinosus* was not inhibited by any of the halogeno nicotinic acids in concentrations up to 5×10^{-3} M. In contrast to this the acceleration of glycolysis which occurs on the addition of nicotinic acid, nicotinamide and nicotinamide riboside was inhibited by 5-FNA (Fig. 1), but not by the other analogues. The magnitude of the inhibition was dependent not only on the concentration of the 5-FNA added but also on the concentration of the added nicotinic acid or its derivative; i.e. the inhibition was competitive. The acceleration of glycolysis by cozymase and nicotinamide ribonucleotide was not inhibited by 5-FNA. These results suggest that it is the synthesis and not the uptake and function of cozymase that is inhibited. The inactivity of the other halogen analogues was not unexpected because their effect on growth is transitory and they are at least 100-fold less efficient antagonists of nicotinic acid than the 5-fluoro analogue.

Effect of halogeno nicotinic acids on cozymase synthesis and uptake in Lb. arabinosus. The synthesis of cozymase from nicotinic acid by washed suspensions of *Lb. arabinosus* was inhibited to some extent by each of the five halogeno nicotinic acids (Table 7). In a concentration of nicotinic acid which permitted maximal rate of synthesis (5×10^{-5} M),

Fig. 1. Effect of 5-fluoronicotinic acid on the acceleration of glycolysis by nicotinic acid and cozymase in *Lb. arabinosus*. For conditions see Experimental, page 486. ○—○, no nicotinic acid; ×—×, nicotinic acid (5×10^{-5} M); ●—●, nicotinic acid (5×10^{-5} M) and 5-fluoronicotinic acid (2.5×10^{-4} M); △—△, cozymase (5×10^{-5} M) and 5-fluoronicotinic acid (2.5×10^{-4} M).

Table 6. Effect of 5-fluoronicotinic acid on the growth of *Esch. coli*

Growth conditions were as described in the text. + + + + to + Indicates gradation of growth from full growth to just detectable growth. 0 indicates no growth.

Addition to the medium	Growth after			
	16 hr.	20 hr.	24 hr.	36 hr.
None	+	++	+++	++++
5-FNA (5×10^{-3} M)	0	0	+	++++
5-FNA (5×10^{-3} M) and nicotinic acid (10^{-6} M)	+	++	++	++++
5-FNA (5×10^{-3} M) and nicotinic acid (5×10^{-7} M)	0	+	+	++++
5-FNA (5×10^{-3} M) and nicotinic acid (10^{-8} M)	?	?	+	++++
Nicotinic acid (10^{-6} M)	+	++	+++	++++

Table 7. Inhibition of cozymase synthesis by halogeno nicotinic acids in *Lb. arabinosus* and *Staph. aureus*

Washed cells were incubated in the glucose-saline mixture containing nicotinic acid (5×10^{-5} M) and inhibitor. After 15 min. incubation at 37° cozymase was estimated as described.

Inhibitor	Inhibitor concn. (M $\times 10^3$)	<i>Lb. arabinosus</i>		<i>Staph. aureus</i>	
		Cozymase synthesized (μm-mole)	Inhibition (%)	Cozymase synthesized (μm-mole)	Inhibition (%)
None	—	6.6	—	10.2	—
5-FNA	2.5	2.6	70	0	100
	0.25	3.6	45	30	100
	0.05	4.8	26	30	24
2-FNA	2.5	4.6	30	7.1	30
	0.25	6.5	None	10.6	None
6-FNA	2.5	5.7	14	6.5	37
	0.25	6.6	None	10.3	None
5-CINA	2.5	5.2	21	10.5	None
	0.25	6.6	None	10.8	None
5-BrNA	2.5	5.3	21	6.4	33
	0.25	6.6	None	10.2	None

inhibition (26 %) was found with 5-FNA ($5 \times 10^{-5} \text{ M}$), whilst no inhibition was found under these conditions with the other analogues. Thus, as would be expected from the previous experiments on growth and glycolysis, the 5-fluoro analogue is by far the most effective inhibitor of cozymase synthesis. The magnitude of the inhibition of the synthesis from nicotinamide and nicotinamide riboside by 5-FNA was the same as that from nicotinic acid (Table 8). The inhibition of the synthesis from nicotinamide ribonucleotide was variable, in some experiments being the same but in others only 30–40 % of that from nicotinic acid. The uptake of cozymase was not usually inhibited when cozymase and inhibitor were added simultaneously (Table 8). In concentrations of nicotinic acid from 10^{-3} to 10^{-6} M the magnitude of the inhibition of cozymase synthesis by 5-FNA (2×10^{-3} to 10^{-5} M) was greatest in the lowest concentrations of nicotinic acid and least in the highest concentration. The inhibition was of a competitive type, although in most batches of cells the reversal of the inhibition was never complete in the highest concentration of nicotinic acid. Similar results were found with nicotinamide over the same range of concentrations (Table 9), and with nicotinamide riboside over a smaller range of inhibitor (5×10^{-4} to 10^{-5} M) and metabolite (10^{-5} to 10^{-6} M) concentrations tested. In the foregoing experiments the inhibitor and nicotinic acid derivative were added simultaneously. When the inhibitor was added before the metabolite, the magnitude of the inhibition was no longer dependent on the concentration of the metabolite but mainly on the length of time of the pre-incubation with the inhibitor

Table 8. *Effect of 5-fluoronicotinic acid on cozymase synthesis from nicotinic acid and its derivatives and the uptake of cozymase in Lb. arabinosus*

Washed cells (3.0 mg. dry wt.) were incubated in glucose-saline and with the additions indicated below. Cozymase was estimated after 20 min. incubation at 37° . The concn. of growth factor was $2.5 \times 10^{-5} \text{ M}$ in all cases.

Growth factor added	5-Fluoronicotinic acid concn. ($\text{M} \times 10^3$)	Cozymase synthesized ($\mu\text{m-mole}$)
Nicotinic acid	None	8.5
	2.5	5.1
	0.25	7.4
Nicotinamide	None	9.2
	2.5	3.3
	0.25	5.0
Nicotinamide riboside	None	10.2
	2.5	3.3
	0.25	6.5
Nicotinamide ribonucleotide	None	10
	2.5	3.0
	0.25	6.5
Cozymase	None	5.0
	2.5	5.2
	0.25	5.0

(Table 10). Under these conditions the effectiveness of 5-FNA, when judged by the lowest concentration at which inhibition was found, was increased some 100- to 1000-fold by the prior incubation (Table 11). The nature of the inhibition also appeared to change and was no longer competitive when the inhibitor was added before the metabolite (Table 12). It is also noteworthy that in two of five experiments in which cozymase was added after a prior incubation of cells with 5-FNA, the uptake was inhibited (20–30 %), whereas no inhibition of uptake was found when cozymase and inhibitor were added simultaneously. If, after a pre-incubation of cells with inhibitor, the excess of inhibitor was removed

Table 9. *The inhibition by 5-fluoronicotinic acid of cozymase synthesis from varying concentrations of nicotinic acid and nicotinamide in washed suspensions of Lb. arabinosus*

Washed cells (9.5 mg. dry wt.) suspended in 1.0 ml. glucose-saline were added to 1.0 ml. water containing 5-fluoronicotinic acid ($5 \times 10^{-4} \text{ M}$) and nicotinic acid in one experiment and nicotinamide in another experiment. After 10 min. incubation at 37° cozymase was estimated in the cells as described in the text.

Nicotinic acid concn. ($\text{M} \times 10^6$)	Cozymase synthesized ($\mu\text{m-mole}$)		Inhibition (%)
	Without inhibitor	With inhibitor	
10	12.4	7.0	44
5	10.9	6.2	44
1.0	12.9	3.9	68
0.5	7.0	1.0	86
Nicotinamide concn. ($\text{M} \times 10^5$)			
10	20	19.6	2
5	20.2	17.9	10
1.0	18.6	10.2	40
0.5	7.9	1.5	80

Table 10. *Effect of time of incubation with 5-fluoronicotinic acid on the inhibition of cozymase synthesis in Lb. arabinosus*

Washed cells (4 mg. dry wt.) were incubated in the usual glucose-saline mixture with 5-fluoronicotinic acid ($2.5 \times 10^{-4} \text{ M}$ final concn.). Nicotinic acid ($2.5 \times 10^{-5} \text{ M}$) was then added and after another 10 min. the cozymase content of the cells was estimated.

Time of incubation (min.)	Cozymase content of cells ($\mu\text{m-mole}$)		Inhibition (%)
	Without FNA	With FNA	
0	14	None	—
0	14	12	14
10	14	11	21
20	12	5	59
40	11	1.5	82

by washing the cells in saline, cozymase synthesis was still found to be inhibited without the addition of further inhibitor (Table 13). Thus, not only was the effect of 5-FNA as an inhibitor of cozymase synthesis increased by the prior incubation but it was also taken up and retained by the cells on washing. The uptake and retention of 5-FNA is dependent on the presence of a concomitant glycolytic reaction (Table 13), suggesting that energy is needed either for its passage into the cell or for the retention.

Table 11. Effect of pre-incubation with varying concentrations of 5-fluoronicotinic acid on cozymase synthesis in washed suspensions of *Lb. arabinosus*

Washed cells (11.5 mg. dry wt.) suspended in 1.0 ml. of glucose-saline mixture were added to tubes containing the 5-fluoronicotinic acid as indicated below. Set A contained nicotinic acid ($5 \times 10^{-5} \text{ M}$) and were incubated for 15 min. at 27° . Set B contained no nicotinic acid but after the first 15 min. nicotinic acid was added and incubation continued for a further 15 min. Cozymase was extracted and estimated as described in the text.

5-Fluoronicotinic acid concn. ($\text{M} \times 10^5$)	Cozymase synthesized ($\mu\text{m-mole}$)		Inhibition (%)	
	A	B	A	B
250	9.7	9.0	52	59
25	15.2	12.2	25	43
2.5	20	15.8	2	26
0.25	21	15.8	0	26
None	20.2	21.4	—	—

Table 12. Effect of pre-incubation of cells with 5-fluoronicotinic acid on cozymase synthesis from varying concentrations of nicotinic acid and nicotinamide

Washed cells of *Lb. arabinosus* (8.5 mg. dry wt.) were incubated at 37° for 10 min. in the usual glucose-saline mixture and with and without 5-fluoronicotinic acid ($2 \times 10^{-4} \text{ M}$). Nicotinic acid and nicotinamide were then added as indicated below and the incubation continued for a further 15 min. Cozymase was extracted and estimated as described in the text.

Nicotinic acid concn. ($\text{M} \times 10^5$)	Cozymase synthesized ($\mu\text{m-mole}$)		Inhibition (%)
	Without 5-FNA	With 5-FNA	
10	13.8	5.9	56
5	11.8	5.4	54
1	12.7	5.5	56
0.5	9.8	5.0	49

Nicotinamide concn. ($\text{M} \times 10^5$)	Cozymase synthesized ($\mu\text{m-mole}$)		Inhibition (%)
	Without 5-FNA	With 5-FNA	
10	15	5.9	60
5	15	5.4	63
1	13	5.8	55
0.5	10	5.4	46

Effect of halogeno nicotinic acids on glycolysis and cozymase synthesis in *Staph. aureus*. The addition of nicotinic acid (5×10^{-5} to $5 \times 10^{-6} \text{ M}$) to glycolysing washed suspensions of *Staph. aureus* stimulated their rate of glycolysis by some 20–40 % (Fig. 2). Similar concentrations of nicotinamide and cozymase were usually without effect. This result agrees with the findings of the growth tests in suggesting that in this organism nicotinamide and cozymase are not so readily available as nicotinic acid.

Table 13. Effect of glucose on the inhibition of cozymase synthesis by 5-fluoronicotinic acid in *Lb. arabinosus*

2.5 mg. dry wt. of cells were incubated with the additions shown below. They were then separated and washed twice in 0.9 % saline and resuspended in 1.8 ml. of the usual glucose-saline mixture. After 5 min. incubation at 37° nicotinic acid ($5 \times 10^{-5} \text{ M}$ final) was added and cozymase estimated after a further 15 min. incubation.

Time of first incubation (min.)	Addition	Cozymase content of cells ($\mu\text{m-mole}$)	Inhibition (%)
10	None	8.3	—
10	Glucose ($2.5 \times 10^{-2} \text{ M}$)	8.5	—
10	5-FNA ($2.5 \times 10^{-4} \text{ M}$)	8.1	None
10	Glucose ($2.5 \times 10^{-2} \text{ M}$) and 5-FNA ($2.5 \times 10^{-4} \text{ M}$)	4.1	49
30	None	8.9	—
30	Glucose ($2.5 \times 10^{-2} \text{ M}$)	7.1	—
30	5-FNA ($2.5 \times 10^{-4} \text{ M}$)	6.5	27
30	Glucose ($2.5 \times 10^{-2} \text{ M}$) and 5-FNA ($2.5 \times 10^{-4} \text{ M}$)	2.1	70

Fig. 2. Effect of 5-fluoronicotinic acid on the acceleration of glycolysis by nicotinic acid in *Staph. aureus*. For conditions see page 486. ●—●, no nicotinic acid; x—x, nicotinic acid ($5 \times 10^{-6} \text{ M}$) and 5-fluoronicotinic acid (10^{-2} M); O—O, nicotinic acid ($5 \times 10^{-5} \text{ M}$).

5-FNA inhibited the stimulation of glycolysis by nicotinic acid but did not inhibit glycolysis before the addition of nicotinic acid. Cozymase synthesis in *Staph. aureus* was, as in *Lb. arabinosus*, inhibited to some extent by all of the halogeno nicotinic acids (Table 6), but again 5-FNA was the most effective inhibitor. The inhibition of the synthesis from nicotinic acid by 5-FNA was competitive over the range tested (5×10^{-4} to 5×10^{-6} M). Synthesis of cozymase from nicotinamide was slower than from nicotinic acid but was also inhibited competitively (Table 14). The rate of uptake of cozymase was also slower than its synthesis from nicotinic acid but the uptake was not inhibited by 5-FNA. Prior incubation of the cells with the inhibitor increased the

Table 14. *The inhibition by 5-fluoronicotinic acid of cozymase synthesis from varying concentrations of nicotinic acid and nicotinamide in washed suspensions of Staph. aureus*

Washed cells (7.5 mg.) suspended in glucose-saline (1.0 ml.) were added to 1.0 ml. aqueous solutions of nicotinic acid and nicotinamide as indicated below and 5-FNA (5×10^{-4} M). Cozymase content of the cells was estimated after 15 min. incubation at 37°.

Nicotinic acid ($\text{M} \times 10^4$)	Cozymase synthesized ($\mu\text{m-mole}$)		Inhibition (%)
	Without 5-FNA	With 5-FNA	
40	17.3	16.8	3
20	16.4	14.7	10
1	11.7	6.7	43
0.5	6.7	1.5	75
Nicotinamide ($\text{M} \times 10^4$)			
40	9.7	7.7	20
20	8.1	4.4	46
10	4.7	2.0	57
5	4.8	None	100

Table 15. *Effect of sodium fluoride on the inhibition of cozymase synthesis by 5-fluoronicotinic acid in Staph. aureus*

Washed cells (8.0 mg. dry wt.) were incubated 10 min. at 37° in the usual glucose-saline mixture and with 5-fluoronicotinic acid (2.5×10^{-4} M final concn.). They were then washed twice in 0.9% saline and resuspended in 1.0 ml. glucose-saline and incubated with nicotinic acid (5×10^{-5} M). Cozymase was estimated in the cells after 20 min.

Sodium fluoride ($\text{M} \times 10^3$)	Cozymase content of cells ($\mu\text{m-mole}$)		Inhibition (%)
	Without 5-FNA	With 5-FNA	
None	14	None	100
25	17.5	17.0	3
2.5	13.5	4.1	70
0.25	11.6	2.0	90

inhibitory effect of 5-FNA; this was independent of the presence of glucose in most batches of cells. The addition of fluoride (5×10^{-2} to 5×10^{-4} M) inhibited the uptake of 5-FNA as judged by the amount of cozymase synthesized after the excess of both inhibitors had been removed by washing (Table 15). It seems likely therefore that, as in *Lb. arabinosus*, 5-FNA is taken up and retained in cells of *Staph. aureus* and that this reaction depends on the presence of an energy-yielding reaction, such as glycolysis.

Effect of 5-fluoronicotinic acid on oxygen uptake and cozymase synthesis in Prot. vulgaris. The oxygen uptake of washed suspension of *Prot. vulgaris* grown for 40–44 hr. in medium containing nicotinic acid (10^{-5} to 10^{-7} M) was followed manometrically. Oxygen uptake of the cells grown in the lower concentrations of nicotinic acid (1.0 to 2.0×10^{-7} M) was stimulated (six- to eight-fold) by the addition of nicotinic acid, nicotinamide, nicotinamide ribonucleotide or cozymase (5 to 0.5×10^{-5} M): oxygen uptake in cells grown on the higher concentrations was not stimulated. 5-FNA did not inhibit oxygen uptake before the addition of nicotinic acid and its derivatives but did inhibit the stimulation by nicotinic acid, nicotinamide and nicotinamide ribonucleotide; the stimulating effect of cozymase was not inhibited (Fig. 3). The cozymase content of the cells ($0.05 \mu\text{m-mole/mg. dry wt.}$) was too low to be measured by the present methods (see also Jackson & Copping, 1952) and the nicotinamide nucleotide in the cells was therefore estimated by the acetone condensation method of Levitis, Robinson, Rosen, Huff & Perlzweig (1945). The apparent nicotinamide nucleotide content of the cells increased from 0.02 to $0.25 \mu\text{m-mole/mg. dry wt.}$ upon incubation with nicotinic acid and glucose; this increase was abolished by the addition of 5-FNA. It is likely

Fig. 3. *Effect of 5-fluoronicotinic acid on the acceleration of oxygen uptake by nicotinic acid and cozymase in Prot. vulgaris.* For conditions see page 486. ○—○, no nicotinic acid; ×—×, nicotinic acid (5×10^{-5} M); ●—●, nicotinic acid (5×10^{-5} M) and 5-fluoronicotinic acid (2.5×10^{-3} M); △—△, cozymase (5×10^{-5} M) and 5-fluoronicotinic acid (2.5×10^{-3} M).

therefore that in *Prot. vulgaris*, 5-FNA inhibits cozymase synthesis from nicotinic acid, nicotinamide and nicotinamide riboside but does not inhibit the uptake nor the function of cozymase.

DISCUSSION

The mechanism of the inhibition of cozymase synthesis in bacteria by 5-fluoronicotinic acid. Available evidence (Hughes & Williamson, 1952a) suggests the following route for cozymase synthesis:

From consideration of this scheme and according to the concepts of inhibition analysis (Shive & Roberts, 1946) it was expected that 5-FNA would inhibit at stage 1 competitively, and that synthesis from nicotinamide and its derivatives would not be markedly inhibited: any inhibition of synthesis from nicotinamide would be expected to be non-competitive. It was in fact found that the inhibition of synthesis from nicotinic acid was competitive over a reasonable range. Unexpectedly, synthesis from nicotinamide and nicotinamide riboside was inhibited to approximately the same extent as that from nicotinic acid and this inhibition was also competitive; synthesis from nicotinamide ribonucleotide was generally less markedly inhibited. The order of addition of inhibitor (5-FNA) and metabolite changed the competitive into non-competitive inhibitions and markedly increased their magnitude. Thus the action of the inhibitor (5-FNA) appears to be more complicated than the expected inhibition of the single stage leading to the formation of nicotinamide from nicotinic acid. It seems likely that either steps 1, 2 and 3 are all inhibited to some extent, or that step 3 alone rather than step 1 is inhibited. 5-FNA might bring about such inhibitions because (a) structural similarity between it

and the presumed cozymase intermediates is sufficiently close to permit competition in each reaction, (b) 5-FNA is metabolically transformed into a more active inhibitor or inhibitors. That 5-FNA inhibits neither the deamidation of nicotinamide by *Lb. arabinosus* (Hughes & Williamson, 1953) nor cozymase-catalysed reactions suggests that mechanism (a) is unlikely. The changes which occur on incubation of inhibitor with glycolysing cells greatly favour the intervention of mechanism (b). Some other inhibitions are shown in Table 16 in which an analogue of an essential metabolite undergoes metabolic transformation. Of particular interest is the action of isonicotinic acid hydrazide which undergoes an exchange reaction and is transformed into an analogue of cozymase. This does not depend on an energy-yielding reaction and thus differs from the other reactions but can be considered as forming a link between mechanism (a) and (b). The inhibitions may be considered as belonging to the type recently characterized by Peters (1952) as 'lethal synthesis', following studies of the inhibition of acetate oxidation by monofluoroacetate. The inhibition of cozymase synthesis by 5-FNA has the following points in common with inhibition by 'lethal synthesis':

(1) It is competitive when inhibitor and metabolite are added simultaneously, but non-competitive when the inhibitor is added before metabolite.

(2) The magnitude of the inhibition is increased by incubation of inhibitor with actively metabolizing cells.

(3) The inhibition takes place at several points in a metabolic sequence or at a point higher in a synthetic sequence than would be expected from structural similarity between inhibitor and metabolite.

(4) The inhibitor is taken up and retained by the cells. It seems feasible therefore that the inhibition of cozymase synthesis by 5-FNA might be a type of 'lethal synthesis' in which the inhibitor enters into the pathway of cozymase synthesis from nicotinic acid and blocks it at several points. If this were so it

Table 16. *Some synthetic transformations of analogues to form inhibitors of enzymic reactions ('lethal synthesis')*

Analogue	Inhibitor	Synthetic system	System inhibited	Reference
Deoxypyridoxine	Deoxypyridoxine phosphate	<i>Strep. faecalis</i>	Tyrosine decarboxylase	Umbreit & Waddell (1949)
Monofluoroacetate	Fluorocitrate	Mammalian liver	Aconitase	Peters (1952)
Ethionine	? Triethylcholine	Intact rats	Rat growth	Stekol & Weiss (1950)
Isonicotinic acid hydrazide (INH)	INH analogue of cozymase	Neurospora DPNase	Mammalian DNPase	Zatman, Kaplan & Colowick (1953)
8-Azaguanine	? Nucleic acids	Intact rat tumour tissue and plant tissue	Rat growth, tumour growth and plant virus	Matthews (1953)

would be expected that derivatives of 5-fluoronicotinamide would appear in the cells during incubation with 5-FNA. Some attempts were therefore made to isolate such products (see Hughes, 1952a), and material tentatively identified as 5-fluoronicotinamide was isolated from large-scale experiments (5 g. dry wt. of cells of *Staph. aureus*). Full proof of the identity of this material as a fluorine-substituted pyridine derivative must await isolation in larger quantities. Because of the small amounts of inhibitor taken up by the cells (c. 1–2 μ m-moles/mg. dry wt.) and because large amounts of deficient cells have to be used, such isolation presents many difficulties. Experiments have shown that in the more readily available baker's yeast, 5-FNA inhibits cozymase synthesis and is taken up and bound by the cells. It seems likely therefore that baker's yeast will be suitable material in which to study the metabolism of 5-FNA.

The significance of 'lethal synthesis' in the design of anti-bacterial agents. The advocacy by Fildes (1940) of a 'rational approach to chemotherapy' has led to the synthesis of a host of analogues of essential metabolites, but few have been of use as chemotherapeutic agents. This is probably because, as pointed out by Rydon (1949), the chemical approach has been based on pictorial likeness without knowledge of the enzymic mechanisms involved. In addition, the biological approach has been based on finding a 'nutritional weak point' in the bacteria and too often this has also been a 'weak point' in the host also. The simplicity of the antagonism between *p*-aminobenzoic acid and sulphonamide (Woods, 1940) has also led to a rigidity in interpretation along what Woolley (1952) has called the 'most favoured hypothesis', i.e. a competition between analogue and metabolite for a single enzyme site. However, Woolley (1952, p. 99), in discussing the effects of ethionine, has suggested the possibility that 'the action of a metabolite analogue... might thus not be due to a simple direct effect but rather to an accumulation of slight hindrances exerted along a chain of metabolic reactions'. This idea has been given further importance by the discoveries by Peters (1952) of the mechanism of fluoroacetate poisoning to which he applied the term 'lethal synthesis'. As shown in Table 16 some other inhibitors may also be considered as belonging to this type. It seems likely that potent inhibitors may be obtained when their design is based upon metabolites which normally enter into synthetic sequences, and if the inhibitors themselves will undergo some, if not all, of the synthetic transformations. For such inhibitory analogues to be successful chemotherapeutic agents they would by preference be based upon metabolites involved in synthetic sequences carried out by pathogens and not by the host organism. For instance, precursors of vitamin

B₁₂ might be considered as starting points since most bacteria synthesize their own supplies of this vitamin whilst higher animals cannot. Such considerations would tend to concentrate to a much greater extent than in the past, on the differences between synthetic ability of parasite and host. The choice of substituent groups in the design of such inhibitors must be governed by the experience gained in the last decade (see Woolley, 1952). It is well known, for instance, that the substitution of hydrogen by such groups as CH₃, NH₂, OH, Cl or Br often yields potent inhibitors (Albert, 1952). However, because of its small atomic size and because of the stability of the C—F bond, fluorine appears to be preferable to these groups when considering the design of compounds to take part in a 'lethal synthesis'. Additional evidence that fluoro analogues may be easily metabolized by bacteria and that the C—F bond remains intact is provided by studies on the oxidation of fluoronicotinic acids (Hughes, 1952b) and fluorobenzoic acids (Hughes, 1953).

SUMMARY

1. The growth of *Lactobacillus arabinosus*, *Staphylococcus aureus*, *Proteus vulgaris* and *Escherichia coli* is inhibited by the five halogeno nicotinic acids in the following order of effectiveness: 5-fluoronicotinic acid > 5-chloronicotinic acid > 5-bromonicotinic acid > 2- and 6-fluoronicotinic acids.

2. The inhibition is reversed competitively by nicotinic acid, nicotinamide and cozymase.

3. The acceleration of glycolysis by nicotinic acid in washed suspensions of *Lb. arabinosus* and *Staph. aureus* is inhibited by 5-fluoronicotinic acid; acceleration by cozymase in *Lb. arabinosus* is not inhibited. 5-Fluoronicotinic acid caused similar effects on the acceleration of oxygen uptake of *Prot. vulgaris* by nicotinic acid and its derivatives.

4. Cozymase synthesis from nicotinic acid, nicotinamide, nicotinamide riboside, and to a lesser degree nicotinamide ribonucleotide, is inhibited by 5-fluoronicotinic acid in *Lb. arabinosus*. Cozymase uptake is not inhibited. A similar inhibition of synthesis of cozymase from nicotinic acid, nicotinamide and nicotinamide ribonucleotide was found in *Staph. aureus*. The inhibition is competitive when inhibitor and metabolite are added simultaneously, but non-competitive when inhibitor is added before metabolite.

5. The magnitude of the inhibition of cozymase synthesis from nicotinic acid in *Lb. arabinosus* and *Staph. aureus* was increased at least tenfold by prior incubation of the cells with the inhibitor. During the incubation the inhibitor was taken up and bound by the cells.

6. The uptake of 5-fluoronicotinic acid is dependent on the presence of a concomitant glycolytic

reaction. It is suggested that 5-fluoronicotinic acid inhibits several steps in the synthesis of cozymase possibly by being metabolized along the synthetic route.

Thanks are due to Prof. H. A. Krebs, F.R.S., for his advice and criticism, and to Mr M. Clegg and Mr N. Blumson for technical assistance. The halogen-substituted nicotinic acids were gifts from Dr K. Rohrman, Eli Lilly Corp., U.S.A. This work was aided by a grant from the Rockefeller Foundation.

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The Regulation of Respiration during the Assimilation of Nitrogen in *Torulopsis utilis*

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(Received 7 December 1953)

In micro-organisms and in higher plants there is strong evidence that cellular respiration and the assimilation of inorganic nitrogen are closely inter-related metabolic processes. Some of the mechanisms underlying their linkage have been discussed by Yemm (1949), Burris & Wilson (1952), and Syrett (1953b). In order to investigate these inter-relations more fully, experiments have recently been carried out with food yeast (*Torulopsis utilis*). This yeast has an outstanding capacity for the assimilation of nitrogen and synthesis of protein. Roine (1947) and Virtanen, Csaky & Rautanen (1949) have shown that pure cultures can be readily grown on a culture medium containing sucrose and minerals including ammonium salts, and then brought into a physiological condition in

which they will rapidly utilize either ammonia or nitrate, by aeration for a few hours in a sucrose-mineral salt solution lacking in nitrogen. Their results indicate that glutamine, asparagine and alanine are the chief primary products of assimilation when such yeast is treated with a nitrogen source in the absence of external carbohydrate, and this clearly suggests a close parallel with the data now available for roots and other tissues of higher plants (e.g. Willis, 1950, 1951).

The experiments described in this paper were designed to investigate the interrelations of cellular respiration, the assimilation of nitrogen, and the metabolism of carbohydrates. For this purpose, pure cultures of the yeast were supplied with ammonium salts under controlled conditions; the