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Nicotinic Acid Biosynthesis: Control by an Enzyme that Competes with a Spontaneous Reaction

Abstract. *Extracts of livers from diabetic rats contain normal amounts of the enzymes needed to convert 3-hydroxyanthranilic acid to nicotinic acid nucleotide. The decreased capacity of diabetic animals to synthesize nicotinic acid is therefore attributed to increased amounts of picolinic carboxylase, which competes for a common intermediate with the spontaneous reaction in which quinolinic acid is formed as a precursor of nicotinic acid. These studies were facilitated by the synthesis of 3-hydroxyanthranilic acid labeled with carbon-14 in positions 3 and 6.*

Control of biochemical processes is known to occur by many devices, including repression and derepression of gene expression, enzyme inhibition in feedback control, and substrate limitation—as in control of utilization of inorganic phosphate. An unusual opportunity for control of a biological process exists in the synthesis of nicotinic acid, since one step in the sequence of reactions that starts with the oxidation of tryptophan is the nonenzymatic formation of quinolinic acid from the oxidation product of 3-hydroxyanthranilic acid (*1*). In normal animals there is very little enzymatic reaction to interfere with the formation of quinolinic acid, but in animals with certain endocrine disturbances, in particular with diabetes, there is a great increase in the concentration of picolinic carboxylase in the liver. Diabetic animals excrete only small amounts of nicotinic acid metabolites. Therefore, it was previously suggested that the relative activity of picolinic carboxylase, which utilizes the precursor of quinolinic acid as a substrate, could control the amount of quinolinic acid formed, which would in turn determine the amount of nicotinic acid synthesized (*2*). At that time the terminal steps in nicotinic acid biosynthesis were not known. The discovery by Nishizuka and Hayaishi (*3*) of the enzymatic synthesis of nicotinic mononucleotide from quinolinic acid and PRPP (*4*) has made it possible to demonstrate that the formation of quinolinic acid is indeed limiting in systems containing large amounts of picolinic carboxylase. Our experiments show that the enzyme of Nishizuka and Hayaishi is present in livers of diabetic rats, since these livers are able to convert quinolinic acid to nicotinic acid, although they do not carry out the corresponding conversion when 3-hydroxyanthranilic acid is the substrate.

In order to follow the reactions of 3-hydroxyanthranilic acid in these studies, we have devised a synthesis for labeling this compound with carbon-14 in positions 3 and 6. This pattern of labeling permits the formation of quinolinic acid with half the radioactive carbon in the ring (which persists in the ring of nicotinic acid) and half in the carboxyl group that is converted to carbon dioxide when the nucleotide is synthesized.

Anthranilic acid and 3-hydroxyanthranilic acid labeled with C^{14} at positions 1 and 2 have been synthesized from 1,3-butadiene and C^{14}-labeled maleic anhydride (*5*). The same scheme (Fig. 2) has been used in the present investigation starting with 1,3-butadiene labeled at carbons 1 and 4 that had been prepared by vapor-phase micropyrolysis of 1,4-C^{14}-labeled 1,4-butanediol diacetate according to Hooton (*6*).

The 1,4-butanediol diacetate-1,4-C^{14} was prepared as follows. 1,4-Butanediol-1,4-C^{14} (0.05 ml) (*7*), 0.5 g of 1,4-butanediol, and 1.6 g of acetic anhydride were kept on the steam bath for 1 to 2 hours, cooled, and treated with ice.

Table 1. Nicotinic acid mononucleotide synthesis by enzymes from livers of normal and diabetic rats. The experiment in duplicate was carried out as described.

Enzyme	Count/min	
	Diabetic	Control
3-HA	69	523
	52	539
"Quin"	650	520
	643	563

After all ice had melted, ether and excess dilute potassium carbonate were added. The ether layer was washed with potassium carbonate solution, dried over sodium sulfate, and evaporated to dryness, giving 0.9 g of liquid with an index of refraction n_D^{20} 1.4250 (*8*); an infrared spectrum taken on a smear showed a maximum absorption (λ_{max}) at 5.75 μ.

The diester was pyrolyzed in a Vycor tube (20 cm in total length and 0.8-cm inside diameter) packed with pyrex helices and heated externally by a 10-cm furnace maintained at 630°C. The tube was connected directly to a 2.1-m chromatographic column containing one part of silicone grease on six parts of Chromosorb P 30/60 by weight, maintained at room temperature. Eighteen 50-μl portions (total 0.9 g) of the diacetate were injected at 60-second intervals through a serum cap at the top of the tube by means of a microsyringe while helium was passed through the tube from a side arm, attached just below the serum cap, at the rate of 1 ml per second. As indicated by the peak that appeared in the chromatogram, each portion of the diacetate produced 75 to 90 percent of 1,3-butadiene-1,4-C^{14} which was collected at the exit of the column in two test tubes (20 by 150 mm) connected in series, each containing 1.0 g of maleic anhydride in 15 ml of benzene (cooled to 5° to 10°C). At the end of the pyrolysis period the test tubes were stoppered firmly and kept at room temperature for 24 to 48 hours. The solutions were then combined and treated with excess "cold" butadiene, and the mixture was allowed to stand for an additional 24 hours. The solution was concentrated to 8 to 10 ml, treated with an equal volume of ligroin (bp 30° to 60°C), and cooled to −15°C. Filtration gave 2.3 g of 1,2,3,6-*cis*-tetrahydrophthalic anhydride-3,6-C^{14}, mp 100°-102°C; infrared absorption showed $\lambda_{max}^{CHCl_3}$ 5.4 and 5.63 μ (*9*); this was converted to 1.0 g of anthranilic acid-3,6-C^{14} (I) mp

142° to 144°C as described before (*5*).

To a stirred solution containing 1.0 g of 3,6-C^{14} anthranilic acid, 50 ml of water, and 20 ml of 2*N* potassium hydroxide (*10*) we added, drop by drop, over a period of 5 hours, 4 g of potassium persulfate (assay 97.6 percent) in 110 ml of water. After addition of 50 ml of concentrated hydrochloric acid the mixture was kept on the steam bath for 30 minutes and evaporated to dryness in a vacuum with the addition from time to time of 1-butanol to prevent excessive foaming. The dark residue was extracted five times with a total of 50 to 60 ml of absolute ethanol. The combined filtered extracts were evaporated to dryness in a vacuum. The residue (0.75 g) was dissolved in 13 ml of water and chromatographed on Dowex 1-acetate as described (*5*). The combined eluates (230 ml) containing the 3-hydroxyanthranilic acid were evaporated to dryness in a vacuum, the residue being 0.35 g. The residue was dissolved in absolute alcohol, a little hydrogen chloride gas was added, and the solution was treated with Norit. Concentration of the filtrate to 2 to 3 ml and gradual addition of 1 to 2 ml of ether gave, after 3 hours, 140 mg of 3-hydroxyanthranilic acid-3,6-C^{14} (II) hydrochloride, mp 225–227°C with decomposition. An additional 20 mg, mp 223–226°C with decomposition, was recovered from the filtrate. Its identity was further confirmed by enzymatic assay and fluorescence.

Extracts of livers from normal and diabetic rats were made essentially as described by Nishizuka and Hayaishi. After treatment with charcoal and brief dialysis, the extracts were assayed for the enzymes that metabolize 3-hydroxyanthranilic acid. Preparations from both types of livers consistently contained equivalent amounts of 3-hydroxyanthranilic oxidase (*11*). The activity of this enzyme is at least tenfold greater than the activities of the other enzymes assayed; the 2 milliliters of extract used in the experiment of Table 1 contain sufficient 3-hydroxyanthranilic oxidase to oxidize all of the 3-hydroxyanthranilic acid in less than 1 minute. The concentration of picolinic carboxylase in the dialyzed extract of normal livers was too low to measure, whereas the amount in the preparations from the diabetic livers was in the range previously found to be characteristic (*11*). In the experiment reported in the table, the extract from the diabetic animals

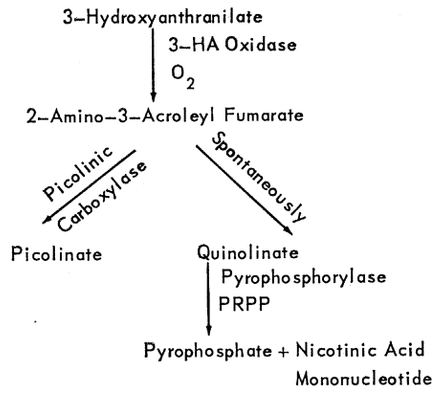

Fig. 1. Biosynthesis of nicotinic acid.

used gave a decrease in optical density of 360 mμ of 0.160 per minute when a sample of 0.2 ml was assayed for picolinic carboxylase. Thus, the amount of activity in the 2 ml of extract used in the experiment of Table 1 could quantitatively produce picolinic acid in 10 minutes from all of the 3-hydroxyanthranilic acid oxidized. This is much faster than the spontaneous formation of quinolinic acid, which has a half-time of over 40 minutes at 25°C (*1*).

In preliminary experiments the capacity of rat-liver preparations to produce $C^{14}O_2$ was tested under a variety of conditions. Very little was produced when crude extracts were used, but after the removal of interfering substances by the procedure of Nishizuka and Hayaishi (*3*) a reaction could be measured. In these experiments quinolinic acid was prepared from the labeled 3-hydroxyanthranilic acid by oxidation with an enzyme extracted from acetone-dried liver; the oxidation product was heated to accelerate cyclization. A purified preparation was obtained by chromatography on alumina, as described by Henderson and Hirsch (*12*). Small amounts of labeled CO_2 were evolved in the absence of added PRPP, and three to four times as much were produced when PRPP was added. Changing the concentrations of ATP, Mg^{++}, and PRPP or addition of ribose-5-phosphate did not increase the yield of labeled CO_2. The dependence of $C^{14}O_2$

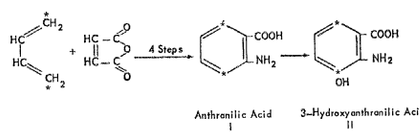

Fig. 2. Synthesis of 3-hydroxyanthranilic acid.

evolution upon PRPP is evidence that the reaction being measured is the formation of nicotinic acid mononucleotide in the reaction of Nishizuka and Hayaishi. The formation of picolinic acid from the substrate used yields only unlabeled CO_2.

Extracts from livers of normal and diabetic rats were compared with respect to their capacities to synthesize nicotinic acid mononucleotide. The reactions were carried out in Warburg vessels in which the substrate, 3-hydroxyanthranilic acid-3,6-C^{14} (0.2 mg, approximately 4500 count/min), had been incubated either in a volume of 0.8 ml with 300 mμmole of potassium phosphate, *p*H 7.0 (3-HA), or in a similar solution containing in addition 0.1 ml of an extract of acetone-dried rat liver as a source of 3-hydroxyanthranilic oxidase (Quin). After 3 hours at room temperature, when essentially all of the 3-hydroxyanthranilic acid in the "Quin" vessels had been converted to quinolinic acid, the following additions were made to all vessels: to the center well, 0.2 ml of a methanolic solution of the organic base Hyamine; to a side arm, 0.2 ml of 5*N* H_2SO_4; to the substrate solution in the main compartment, 0.1 ml of PRPP (5 mg/ml), 0.05 ml of 0.2*M* ATP, 0.1 ml of 0.1*M* $MgCl_2$, and 2.0 ml of liver extract. The vessels were immediately sealed with rubber stoppers and incubated with shaking in a 35°C bath for 40 minutes. The acid was then tipped into the main compartment and the shaking was continued for 15 minutes. The Hyamine was then transferred to counting vials together with three rinses totaling 1.8 ml of ethanol. To each vial was added 12 ml of a solution of phosphors (*13*) in toluene, and the radioactivity was determined in a Tricarb scintillation counter.

The data of Table 1 show that the prior conversion of 3-hydroxyanthranilic acid to quinolinic acid enables the diabetic-liver preparation to synthesize the nucleotide of nicotinic acid, as measured by the production of labeled CO_2. The activity of this preparation is as great as that of the normal control. However, only a trace of labeled CO_2 is released when 3-hydroxyanthranilic acid is used as the substrate.

This experiment shows that the capacity of diabetic livers to form nicotinic acid from quinolinic acid is equivalent to that of normal livers. Since there is no decrease in 3-hydroxyan-

thranilic oxidase in these livers, it may be concluded that the failure of diabetic animals to synthesize nicotinic acid is not caused by a decrease in any essential enzyme, but must be attributed to some other type of control mechanism. The striking increase in picolinic carboxylase, from immeasurably low concentration to appreciable concentration, has previously been correlated with the decreased production of nicotinic acid. The decrease in quinolinic acid formation caused by an increase in picolinic carboxylase activity provides an adequate explanation for the control of nicotinic acid biosynthesis.

The physiological significance of this phenomenon remains obscure, since it is not clear what advantage is gained by the diabetic organism through diverting an intermediate from nicotinic acid synthesis. The mechanism by which hormonal regulation of picolinic carboxylase operates is also not known. It does seem clear, however, that the more general phenomenon of competition for available substrate by different enzymes is exhibited in an extreme form in this system, in which the substrate for a spontaneous reaction is utilized by an enzyme whose activity changes from very low to higher values.

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Nucleic Acid Metabolism in L Cells Infected with a Member of the Psittacosis Group

Abstract. *Multiplication of the meningopneumonitis agent in the cytoplasm of L cells in suspension culture is accompanied by parallel increases in cytoplasmic DNA, RNA, and phospholipid. Synthesis of RNA and phospholipid in infected cells is inhibited at the height of meningopneumonitis multiplication, but nuclear DNA synthesis is inhibited earlier and to a greater degree.*

Members of the psittacosis group are obligate intracellular parasites that grow in the cytoplasm of their host cells. Purified suspensions of these infectious agents contain approximately equal amounts of DNA and RNA (*1*), but information about the kinetics of DNA and RNA synthesis in the agent, and the effects of multiplication of the parasite on the nucleic acid metabolism of the host cell, is meager and contradictory. The total DNA and RNA content of infected cell cultures increases as the infectious titer rises (*2, 3*). It was concluded from studies with the agent of psittacosis that DNA synthesis begins early (*4*) but that there is a 10-hour lag between synthesis of DNA and its incorporation into particles (*5*), and another investigation with the trachoma agent indicated that DNA synthesis is most active late in the infection while RNA synthesis occurs at an earlier stage (*3*).

To gain a clearer picture of the nucleic acid metabolism of cells infected with the agent of meningopneumonitis (Cal 10 strain), a well-characterized member of the psittacosis group (*1, 2*), we determined the rate of DNA and RNA synthesis by measuring the uptake of orthophosphate labeled with P^{32}. The cells were exposed to the isotope for brief periods at various times after infection. Labeled cells were separated into nuclear and cytoplasmic fractions, and the cytoplasm was further separated into supernatant and sediment. In this way, synthesis of agent nucleic acid in the cytoplasm could be differentiated from synthesis of host nucleic acid in the nucleus. Synthesis of agent DNA and RNA was clearly evident after 10 to 15 hours of infection and was at a maximum after 20 to 25 hours, the period of highest multiplication rate. The rate of host RNA synthesis was significantly reduced only at the time of maximum agent multiplication, but the rate of host DNA synthesis was profoundly inhibited as early as 10 to 15 hours, when agent multiplication had only just begun.

Mouse fibroblasts (strain L cells) exponentially multiplying in suspension cultures were infected with a strain of meningopneumonitis agent well adapted to L cells. As measured by microscopic count of inclusions, 40 to 70 percent of the cells were infected. The growth medium was M-199 containing 16 percent fetal calf serum, and streptomycin. Uninfected suspensions of L cells were included in each experiment. At 5-hour intervals after infection, 0.67 mc P^{32} as carrier-free orthophosphate was added for each 3 to 5 $\times$ 10^7 cells. Exactly 5 hours after the addition of P^{32}, the cells were collected by centrifugation, washed three times in Hanks balanced salt solution (0.01*M* in phosphate) containing 0.15 percent Methocel to preserve cell integrity, and separated into nuclear and cytoplasmic fractions by shaking at 4°C for 1 hour with 20 percent citric acid and then for one-half hour with 30 percent citric acid. Less than 2 percent of the P^{32}-labeled DNA in uninfected cells appeared in the cytoplasmic fraction. The cytoplasmic fraction was further separated into supernatant and sediment by centrifuging at 5000*g* for 30 minutes, just sufficient to sediment the meningopneumonitis agent. The cytoplasmic sediment was washed twice with balanced salt solution containing Methocel. The phosphorus of each fraction was partitioned by the Schneider-Schmidt-Thannhauser technique (*6*) into acid-soluble P, lipid P, RNA P, DNA P, and residue P, and the P^{32} content of each part was determined in a thin-window counter. On the assumption that the uninfected cells in an infected culture were metabolizing at a normal rate, the data were corrected for the contribution of uninfected cells