whether the observed activity has a functional significance in the alimentary tracts of these insects. A series of tests with homogenates made from larvae from which the alimentary tracts had been removed were negative for pepsin-like activity, indicating that the gut tissue is the principal source of the enzyme.

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Biogenesis of Nicotine

Abstract. Radioactive nicotine was synthesized by *Nicotiana rustica L.* which was fed sodium acetate-1-C^{14}, sodium acetate-2-C^{14}, sodium pyruvate-1-C^{14}, or sodium pyruvate-3-C^{14}. Acetate-2-C^{14} and pyruvate-3-C^{14} were converted to nicotine with the least dilution of radioisotope, whereas pyruvate-1-C^{14} was incorporated with a relatively large dilution. When acetate-1-C^{14} was administered to the plants the nicotine contained C^{14} only in the pyrrolidine ring. After acetate-2-C^{14} was fed, C^{14} was located in both the pyrrolidine and pyridine rings.

Biogenetic pathways for the synthesis of the pyrrolidine and N-methyl groups of nicotine in tobacco plants have been firmly established (*1, 2*). Although Dawson *et al.* (*3*) have demonstrated the incorporation of the pyridine ring of nicotinic acid into nicotine, the *de novo* biogenesis of the pyridine ring of nicotine or other pyridine compounds in higher plants has not been elucidated. It has been shown that nicotinic acid is synthesized from tryptophan in several microorganisms and animals (*4*). However, higher plants do not appear to be capable of synthesizing the pyridine ring system of nicotine, trigonelline, or niacin from tryptophan (*5, 6*).

Recently, Il'in showed that acetate-C^{14} is incorporated to a large extent into nicotine in tobacco plants (*7*) but did not ascertain the location of the radioactivity in the nicotine molecule. Leete *et al.* (*8*) also demonstrated incorporation of acetate-2-C^{14} into nicotine and indicated that the radioactivity was randomly distributed in the alkaloid.

This report is a study of the incorporation of acetate-1-C^{14}, acetate-2-C^{14}, pyruvate-1-C^{14}, and pyruvate-3-C^{14} into nicotine in tobacco plants and presents data of a preliminary degradation to discover the location of radioactivity in nicotine following the feeding of radioactive acetate.

Intact *Nicotiana rustica L.* were prepared for hydroponic administration of the C^{14}-labeled substrates as described in an earlier report (*5*). Each plant was fed 2.44×10^{-5} mole of a given compound which had a specific activity of 4.10×10^{8} count/min per millimole. The plants were harvested 7 days after administration of a labeled compound, and the nicotine was purified and isolated as the dipicrate (*9*).

Nicotine was degraded by oxidation with neutral potassium permanganate to yield nicotinic acid, potassium bicarbonate, and methylamine (*10*). In this procedure 3 moles of potassium bicarbonate were formed from the 3-, 4-, and 5-carbons of the pyrrolidine ring. The nicotinic acid was then decarboxylated, and the carboxyl carbon, which originally was the 2-carbon of the pyrrolidine ring of nicotine, was obtained as barium carbonate (*11*). The pyridine resulting from the decarboxylation was recovered as the picrate. The N-methyl group of nicotine was obtained by treatment of the alkaloid with hydriodic acid and conversion of the resulting methyl iodide to methyltriethylammonium iodide (*9*).

The radioactivity of each compound was determined with a windowless flow counter, and all counts were corrected for self-absorption.

The extent of incorporation of labeled substrates into nicotine is shown in Table 1. Acetate-2-C^{14} and pyruvate-3-C^{14} were converted to nicotine with the least dilution, whereas pyruvate-1-C^{14} was incorporated only to a relatively small extent. These results indicated that pyruvate was metabolized to acetate for the most part before it was incorporated into nicotine. However, the extent of conversion of pyruvate-3-C^{14} and the small but significant incorporation of C^{14} from pyruvate-1-C^{14} are an indication that not all pyruvate was converted to acetate before it was utilized for nicotine synthesis.

Nicotine which was synthesized in plants fed acetate-1-C^{14} and acetate-2-C^{14} was partially degraded to determine the location of C^{14} in the molecule in each case. The results of these degradations are presented in Table 2. When acetate-1-C^{14} was the labeled precursor, about one-half the activity was located in the 2-carbon of the pyrrolidine ring. The remaining C^{14} was located in the barium carbonate obtained from the 3-, 4-, and 5-carbons of the pyrrolidine ring. Previous studies, in which nicotine was degraded following administration of ornithine-2-C^{14} (*12*) and glutamate-2-C^{14} (*1*), have shown C^{14} in the alkaloid to be equally distributed between positions 2 and 5 of the pyrrolidine ring. The precursors of the pyrrolidine ring therefore appear to pass through a symmetrical intermediate during the synthesis of nicotine. Since acetate was probably incorporated first into glutamate by way of the tricarboxylic acid cycle, the quantity of the C^{14} in the 2- and 5-carbons of the pyrrolidine ring should be equal. In the present study one-half the C^{14} in nicotine obtained from plants fed acetate-1-C^{14} was found in position 2 of the pyrrolidine ring. It is postulated, therefore, that the remaining C^{14} resides in position 5 of the pyrrolidine ring. Essentially no C^{14} was incorporated into the pyridine ring when plants were fed acetate-1-C^{14}. In contrast, radioactive carbon in the nicotine from plants fed acetate-2-C^{14} was located in both rings. The pyrrolidine ring contained about 60 percent of the total C^{14} in the molecule. If one again assumes an equal distribution of radioactivity between carbons 2 and 5, these positions contained about 20 percent of

Table 1. Incorporation of several labeled precursors into nicotine in tobacco plants.

Substrate	Nicotine dipicrate (10^5 count/min mmole)	Dilution
Acetate-1-C^{14}	4.50	911
Acetate-1-C^{14}	4.18	981
Acetate-2-C^{14}	7.47	550
Acetate-2-C^{14}	12.70	323
Pyruvate-1-C^{14}	0.49	8370
Pyruvate-3-C^{14}	16.90	243

Table 2. Distribution of C^{14} in nicotine from plants fed sodium acetate-1-C^{14} and sodium acetate-2-C^{14}.

Compound	Specific activity (10^3 count/min mmole)	
	Acetate-1-C^{14}	Acetate-2-C^{14}
Nicotine dipicrate	9.3	21.0
Barium carbonate*	3.7	8.6
Nicotinic acid	4.5	9.3
Methyltriethylammonium iodide	0.1	0.6
Barium carbonate†	4.4	1.8
Pyridine picrate	0.4	8.6

* Barium carbonate from the 2-, 3-, and 4-carbons of the pyrrolidine ring. The specific activity was multiplied by 3.
† Barium carbonate resulting from the decarboxylation of nicotinic acid.

the radioactivity of the original nicotine molecule, and about 40 percent was located in carbons 3 and 4 of the pyrrolidine ring. Approximately 40 percent of the C^{14} in the nicotine was located in the pyridine ring.

Even though a 7-day period of metabolism was used with the radioactive acetate, only a small amount of randomization of the C^{14} in the synthesized nicotine occurred. These data also show for the first time the utilization of a specific precursor for the *de novo* synthesis of the pyridine ring in a higher plant (*13*).

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Nucleoprotein Constituents Stimulating Growth in Tissue Culture: Active Protein Fraction

Abstract. A new method has been developed for removing the nucleic acid portion of the nucleoprotein fraction which stimulates growth in tissue culture. Biological activity resides in the purified protein fraction, while the high-polymer nucleic acid fraction is inert. The active protein fraction contains extractable lipids which have no effect on the biological activity.

As was previously reported, the factors of high molecular weight in embryo extract which are needed for stimulation of certain types of tissue culture can be isolated with streptomycin as a protein precipitate both from embryo extract (*1*) and from adult tissues (*2*). These growth factors, which are active in both plasma and glass substrate tissue cultures (*3*), are obtained from the streptomycin precipitate in the form of a nucleoprotein fraction (NPF) (*1*). Since the nucleoprotein fraction is a mixture containing four electrophoretic components (*4*), further fractionation was attempted to identify the individual active growth principles.

In view of recent work on viruses (*5*) demonstrating biological activity in a specially prepared nucleic acid fraction of very high molecular weight, attempts were made to obtain similar nucleic acid fractions from the nucleoprotein fraction, although earlier, lower polymer nucleic acid preparations were inactive (*1*). Various new methods for isolating these high polymers (*6*) were tried with the nucleoprotein fraction and chick embryo homogenates; these resulted in nucleic acid preparations of varying polymer size, but all nucleic acid preparations were inactive in culture (*7*). Although most of the protein residues from these experiments were insoluble in salt solutions and inactive in cultures, some protein residues from these experiments partially redissolved and possessed growth-promoting activity when tested in tissue culture. This suggested the possibility of an active soluble protein fraction. Separation of soluble proteins from nucleic acids in the nucleoprotein fraction was attempted on the basis of an earlier observation (*7*) that a high concentration of phosphate buffer caused the nucleoprotein fraction to separate in this dense medium and to form a floating scum. Since high salt concentrations are frequently used to split off nucleic acids from proteins, the nucleic acid was presumably split off in this instance. More important, the floating material redissolved easily in buffers of low ionic strength (*7*).

On reinvestigation the flotate was indeed found to exhibit a typical protein absorption spectrum with a maximum of 278 mμ, in contrast to the spectrum of the nucleoprotein fraction, with a maximum of 260 mμ (*1*). This change suggested a loss of nucleic acid from the nucleoprotein fraction to form the protein fraction (PF); this was confirmed by chemical analysis for nucleic acid (*8*): NPF, 6.5 percent; PF, 0.8 percent (on a dry weight basis).

The growth-promoting activity of the protein fraction was tested on chick embryo heart fibroblasts in Carrel flask cultures, as previously described (*1*). The potency of the protein fraction was found to be unchanged from that of the nucleoprotein fraction, while the nucleic acid moiety was inactive (Table 1). The dose-response curves were alike, and similar to those published for the nucleoprotein fraction (*2*). Likewise, as already reported (*3*), no essential loss of biological activity was observed when a protein fraction was isolated from the nucleoprotein fraction of adult chicken spleen and tested on chick myoblasts in glass substrate cultures. The protein fraction from adult spleen appeared to be more active than the protein fraction from the embryo in this test system.

Table 1. Biological activity of protein fraction in plasma clot cultures of chick embryo heart fibroblasts.

Component	Amount (mg/ml)	Areal outgrowth at 7 days* (mm^2)
Serum control		8.0 ± 1.5
Embryo NPF	0.4	36.3 ± 6.1
Embryo PF	0.4	42.4 ± 5.4
Embryo nucleic acid	0.4	10.3 ± 3.3
Embryo delipidized PF	0.4	33.2 ± 4.7

* Averages, with standard error, for six flasks.

A routine procedure for isolation of protein fraction is as follows (all steps at 0°C): Clear fresh nucleoprotein fraction in Gey's solution is treated with 2 volumes of 3.3*M* potassium phosphate buffer at *p*H 7.8. The white turbid solution is allowed to stand for 1 hour, then is centrifuged for 20 minutes at 28,000*g* in the Servall centrifuge to concentrate the floating insoluble proteins as a flotate. The subnatant, containing nucleic acids and some protein, is discarded, and the flotate is further compacted by spinning for 30 minutes at 144,000*g* in the Spinco ultracentrifuge. The solid flotate is dialyzed for 48 hours against 2000 volumes of 0.1*N* sodium bicarbonate in which the protein fraction is stable. This step removes phosphate and dissolves much of the solid flotate. The contents of the dialysis sac are then spun for 1 hour at 144,000*g*, the floating lipid is removed with a capillary pipette, and the clear midfraction (in concentrations up to 20 mg/ml) is saved as the protein fraction.

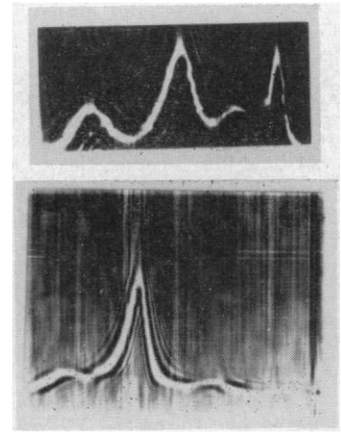

Fig. 1. Comparison of electrophoretic patterns of (top) nucleoprotein fraction (top) and protein fraction (bottom).