8. W. R. Stinebring and J. Youngner, *Nature* **204**, 712 (1964); M. Ho and Y. Kono, *Proc. Nat. Acad. Sci. U.S.* **53**, 220 (1965); J. Youngner, W. R. Stinebring, S. Taube, *Virology* **27**, 541 (1965).
9. T. C. Merigan, C. A. Winget, C. B. Dixon, *J. Mol. Biol.* **13**, 679 (1965).
10. R. Z. Lockart, Jr., and T. Sreevalson, in *Viruses, Nucleic Acids, and Cancer* (Williams and Wilkins, Baltimore, 1963), p. 447.
11. J. Taylor, *Biochem. Biophys. Res. Commun.* **14**, 447 (1964); R. Z. Lockart, Jr., *ibid.* **15**, 513 (1964); R. M. Friedman and J. A. Sonnabend, *Nature* **203**, 366 (1964); S. Levine, *Virology* **24**, 586 (1964).
12. O. Kempthorne, in *The Design and Analysis of Experiments* (Wiley, New York, 1952), p. 47.
13. Statistical analysis and interpretation by Dr. D. W. Alling of the NIAID are gratefully acknowledged.

15 February 1966

Morphogenetic Substance in Legume Nodule Formation

Abstract. *Addition of extracts of bean cotyledon or hypocotyl or of a coconut-water preparation to roots grown from excised bean hypocotyls markedly increased the number of nodules, while removal of part of the hypocotyl tissue reduced the number. The active morphogenetic substance in the coconut water has been partially purified.*

Ability of plants to nodulate and to enter into a N_2-fixing symbiosis with species of *Rhizobium* is restricted to members of the Leguminosae. This fact suggests existence in the host of substances that govern the limited range of plants that the bacteria can invade and with which the microsymbionts can develop a functional relation. Moreover, the specificity of bacterium for plant, the morphological changes preceding infection, the alterations in root-hair structure during development of the infection thread, and the morphological changes associated with nodule genesis undoubtedly involve substances excreted by or contained within cells of one or both symbionts. There is also evidence of host control of nodule abundance, a control possibly implicating a morphogenetic substance synthesized in the green part of the plant and translocated to the root (*1*).

This report concerns the existence of morphogenetic substances implicated in nodule formation, and the development of a reliable bioassay for these substances. Our technique is based on a procedure developed by Raggio, Raggio, and Torrey (*2*), who demonstrated that roots excised from legumes will nodulate adequately in culture. This excised-root technique has produced direct evidence that a host factor is concerned in nodule genesis (*3*); and it was observed that the frequency of nodules is increased if the excised roots retain a portion of the hypocotyl (*4*).

Seeds of *Phaseolus vulgaris* L. were surface sterilized and soaked in water for 24 hours, and the axes below the cotyledons were excised—each piece about 6 mm long. The segments of excised tissue were incubated for 9 days at 22° to 23°C on White's medium (*5*), by which time they consisted of about 2 cm of hypocotyl tissue, primary root, and a few short adventitious roots. These structures were transferred to the split medium of Raggio *et al.* (*2*) containing 10 percent sucrose. The cut end of the plant material was inserted into a small glass vial containing nitrate, organic components of the autoclaved medium, and substances whose activity was to be tested. After 10 more days of incubation, when vigorous growth of the adventitious roots had occurred on the inorganic portion of the solidified medium, the roots were inoculated with a dilute suspension of *Rhizobium phaseoli*. Nodule counts and root measurements were made 3 or 4 weeks later; usually, a randomized complete-block design was employed; nodule numbers were statistically analyzed by use of a square-root $(x + \frac{1}{2})$ transformation. All attempts to culture the roots of this bean variety, detached from the hypocotyl, failed.

Table 1. Effect of coconut-water dialyzate on nodulation and root development by axes initially 6 mm long. In the analysis for statistical significance, the means from the 12 experiments were paired.

Nodules (mean No.)	Nodulation frequency (%)	Major roots, length (cm)	Lateral roots (No.)
	Untreated tissue		
10.5	90.5	45.9	29.1
	Treated tissue		
17.5	90.6	38.8	39.5
	Least significant difference (P=*0.1*)		
6.7	13.5	6.2	6.3

Table 2. Nodulation and growth of roots borne on segments of hypocotyls. Each treatment was replicated eight or nine times. Statistical significance between the untreated control and treated segments is shown by letters in parentheses: *N*, no significant difference at $P = .1$; *A*, *B*, *C*, significant differences at $P = .1$, .05, and .01, respectively. In each instance the frequency of nodulation was 100 percent. Treatment was with dialyzate of coconut water (CW) or of cotyledon extract (CE).

Treatment	Nodules (mean No.)	Major roots, length (cm)	Lateral roots (No.)
	Segments 10 mm long		
None	3.9	35	28
CW	13.9 (*C*)	37 (*N*)	29 (*N*)
CE	12.1 (*C*)	26 (*A*)	12 (*B*)
	Segments 15 mm long		
None	7.1	36	10
CW	13.7 (*B*)	26 (*A*)	18 (*N*)
CE	11.0 (*N*)	27 (*A*)	16 (*N*)

Table 1 summarizes the results of 12 experiments designed to determine the effect on nodulation of the dialyzate of autoclaved coconut water (30 percent by volume). Each tabulated value of nodule number and frequency and of root length and number represents the average of the means of observations from the 12 separate experiments, each containing seven to ten replicates. Although stimulation by the coconut-water preparation is unmistakable and statistically significant when all experiments are considered together, many nodules appeared even in the absence of the coconut water, making the enhancement of nodulation by the addition more difficult to evaluate. Consequently, possible modifications of the bioassay were studied that would permit more ready identification of substances implicated in nodule genesis.

The influence of the hypocotyl tissue was assessed by removing 10- or 15-mm segments of the hypocotyl from the structures developed from 6-mm axes. Roots developing from the remaining portions bore fewer nodules than the complete structures, yet no adverse effect on root growth was observed. The dialyzate from the autoclaved coconut water (provided in the organic medium at a concentration 30 percent of that in the original endosperm) trebled the numbers of nodules on the roots growing from the shorter structures, but the addition did not significantly influence root extension.

The coconut water had a similar effect when the 10- and 15-mm segments of hypocotyl tissue, cut from the complete structures, were employed; in addition, the dialyzate derived from the cotyledon extract (provided in the organic medium at a concentration equivalent to one cotyledon per milliliter) highly significantly increased the abundance of nodules ap-

pearing on adventitious roots growing from the 10-mm segments (Table 2). An aqueous extract of hypocotyl tissue from bean seedlings 5 to 8 days old also stimulated nodulation when roots borne by 10-mm hypocotyl segments were used in the assay.

Although there is considerable variation in nodule number and root development between experiments with these procedures, the method employing 10-mm hypocotyl segments appears to be a suitable assay for identifying the unknown principle, because the effect of the unknown manifests itself by at least doubling nodule abundance, the difference being significant at the .05 level of probability; and the roots grow vigorously without appearing to be influenced by the medium supplements. The major advantage of this method over methods that employ only root tissue (*2, 3*) is that the frequency of nodulation is high, usually 100 percent, thus enabling one to detect readily fractions containing the active substance.

Yeast extract, casamino acids, indole-3-acetic acid, kinetin, *p*-chlorophenoxyisobutyric acid, or inositol, or increase in the concentration in the test medium of pyridoxine, niacin, and thiamin, did not significantly influence nodulation.

The active factor in the coconut water was partially purified by discarding the precipitate formed during autoclaving; the diffusible fraction obtained after dialysis was collected and subjected to ion-exchange chromatography. The unknown passes through a cation-exchange resin but is retained by an anion-exchange resin, from which it can be eluted with acetic or formic acid. The morphogenetic substance was purified ninefold relative to the coconut-water dialyzate on a dry-weight basis.

Our results suggest that a soluble morphogenetic substance, controlling nodulation, is produced by the host plant. The chemical, or a substance that exerts the same effect, is not peculiar to legumes; it is also found in the coconut endosperm, and the substance (or substances) present in the autoclaved coconut water appears to replace that found in the bean hypocotyl tissue. Thus removal of part of this tissue makes it possible to use the remainder of the hypocotyl in the bioassay. The question of whether the hypocotyl factor and the substances found in coconut water and bean cotyledons are identical awaits investigation.

A. G. SCHAFFER
M. ALEXANDER

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York

References and Notes

1. H. G. Thornton, *Proc. Roy. Soc. London* **B106**, 110 (1930); M. Raggio and N. Raggio, *Phyton* **7**, 103 (1956).
2. M. Raggio, N. Raggio, J. G. Torrey, *Amer. J. Botany* **44**, 325 (1957).
3. C. L. Valera and M. Alexander, *J. Bacteriol.* **89**, 1134 (1965).
4. A. H. Bunting and J. Horrocks, *Ann. Botany* **28**, 229 (1964).
5. J. Paul, *Cell and Tissue Culture* (Williams and Wilkins, Baltimore, 1960), p. 107.
6. Supported by NSF grant GB3687.

27 December 1965

Liver Carcinogenesis by Diethylnitrosamine in the Rat

Abstract. *Diethylnitrosamine was continuously administered to rats at a dose rate of low toxicity. Ninety-two percent of the animals died with multicentrical hepatocellular carcinomata within a narrow and highly reproducible time interval. Discontinuing the carcinogen during the experiment resulted in a prolonged median time until death, a reduced tumor yield, and a lessened slope of the dose-response curve. Partial hepatectomy after discontinuation of the drug did not change either tumor yield or time of death. The obtained dose-response relationships support the concept that carcinogenic effects of single doses are irreversible and cumulative. Daily, low-dose, total-body x-irradiation had no significant effect on the response of rat liver to the carcinogen.*

One of the most powerful chemical liver carcinogens is the alkylating agent diethylnitrosamine (DEN) (*1*). When continuously administered to rats at a dose rate of low toxicity it induces multicentrical hepatocellular carcinomata in nearly all the animals. Under standardized conditions highly characteristic dose-response curves are obtained (Fig. 1). It is believed that DEN is transformed in vivo into the effectively alkylating diazoethane after enzymatic oxidative dealkylation. Besides other cell components, the guanine molecules of RNA and DNA seem to be an important site of alkylation (formation of 7-ethylguanine) (*2*). We have found (*3*) that 10 days after a single oral dose of ^{3}H-DEN (*4*) to rats the levels of bound-^{3}H activity in liver, kidney, spleen, and intestine follow the ratio 100 : 74 : 40 : 17. These figures are possibly correlated with the amount of alkylation in the different organs and may help to explain the organ specificity of the carcinogenic effect of DEN.

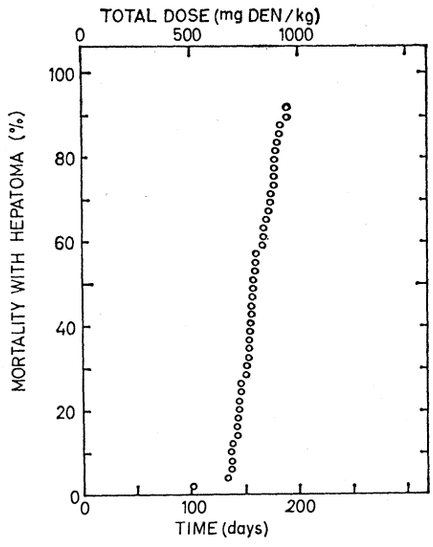

Fig. 1. Liver carcinogenesis in rats that had been fed DEN. 50 adult, male Sprague-Dawley rats received daily doses of 5 mg of DEN per kilogram of body weight in their drinking water. Each point represents one animal. Median time (T) until death with multicentrical liver carcinomata is 158 days ± 16 (S.D.).

We now report the influence of two different factors on liver carcinogenesis by DEN in the rat: (i) discontinuing the administration of DEN after half of the median time (T) until death from hepatomata and subsequent removal of two-thirds of the liver, and (ii) combination with daily low-dose whole-body irradiation.

Results from continuous administration of a variety of carcinogens have led to the conclusion that within a dose range of low toxicity the carcinogenic effect is independent of the size of individual doses and is essentially a function of the total dose ("irreversible summation") (see *5*). There is, however, evidence that time is an important factor in the process of carcinogenesis.

From studies with 4-dimethylaminoazobenzene, 4-dimethylaminostilbene, and DEN (*5*) and with ultraviolet light