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.

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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.

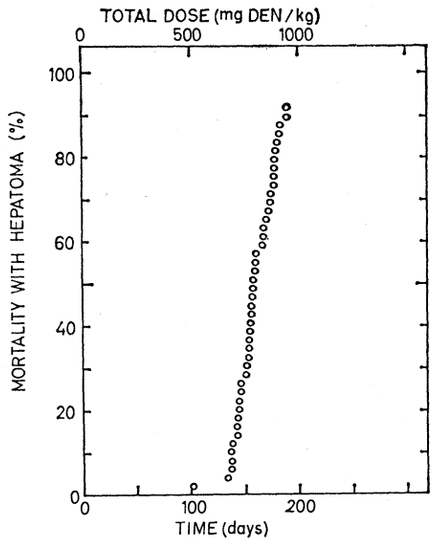

Fig. 1. Liver carcinogenesis in rats that had been fed DEN. 50 adult, male Sprague-Dawley rats received daily doses of 5 mg of DEN per kilogram of body weight in their drinking water. Each point represents one animal. Median time (T) until death with multicentrical liver carcinomata is 158 days ± 16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

(6), it has been concluded that in these systems the daily dose (d) and T are linked through the relationship

$$d = c\frac{1}{T^n}, \qquad (1)$$

where c is a constant of proportionality and the exponent n has always been found to be greater than 1. It has, for instance, been shown that n is 2.3 for liver carcinogenesis by DEN in rats (5). Consequently, in these systems carcinogenesis is an accelerated process. If doses are discontinued during an experiment, the time of tumor appearance is delayed and the slope of the dose-response curve is lessened; also, tumor incidence may be lower (5, 6).

We performed experiments to find out whether discontinuing administration of DEN and renewal of two-thirds of the liver cells after 68-percent hepatectomy, at a stage of the carcinogenic period where microcarcinomata are still not detectable, would change T according to Eq. 1, or whether the value of 2.3 for the exponent n would be influenced. Three groups of adult, male, Sprague-Dawley rats (mean body weight 290 g ± 10 percent S.D.) were given daily doses of 5 mg of DEN per kilogram of body weight. In group I (50 animals), DEN was fed continuously until the animals died from hepatomata. For this group, T was 158 days ± 16 (S.D.). Groups II and III (25 animals each) received DEN at the same dose rate up to the 82nd day (total dose D_{82}, 410 mg DEN/kg). On the 84th day animals of group III underwent 68-percent hepatectomy (7). For "discontinued" groups II and III, T was indeed prolonged by a factor of 1.7, and the slope of the curve was less steep than that of "continuous" group I (Fig. 2). In addition, the tumor yield of groups II and III was reduced to about 55 percent as compared with 92 percent in group I.

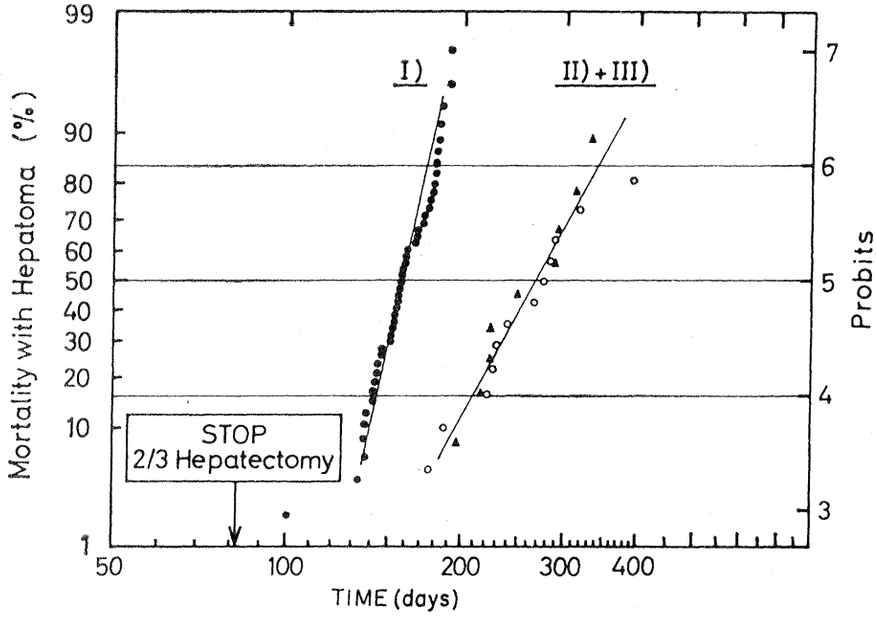

Fig. 2. Mortality with liver carcinomata plotted against time in a probability grid [experimental points corrected after the method of Miescher *et al.* (14)]. Group I (●), continuous administration of DEN (daily dose, 5 mg/kg). Group II (○), administration of DEN discontinued after 82 days. Group III (▲), administration of DEN discontinued after 82 days; two-thirds hepatectomy performed on 84th day. Each point represents one animal.

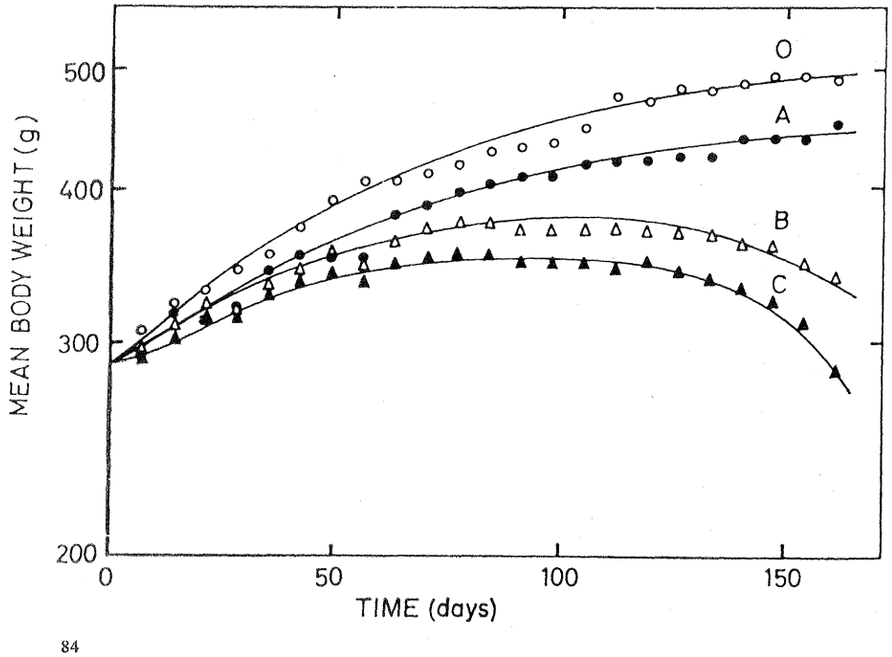

Fig. 3 (left). Combination of continuous administration of DEN with daily low-dose x-irradiation. Four experimental groups of 50 male Sprague-Dawley rats each. Group O, control; the others received the following treatments: group A, 5 r per day; group B, 5 mg of DEN per kilogram per day; and group C, 5 mg of DEN per kilogram per day plus 5 r per day. The graph shows the mean body weights as a function of time.

The second result of the experiment is that there seems to be no significant difference in T between the animals of groups II and III. This finding agrees, at least in part, with results of earlier studies on effects of liver regeneration on hepatocarcinogenesis after administration of 4-dimenthylaminoazobenzene to rats (8, 9). One might conclude that the "carcinogenic information" accumulated in the cells of the remaining third of the liver after hepatectomy is more or less completely passed over to the progeny of these cells which by

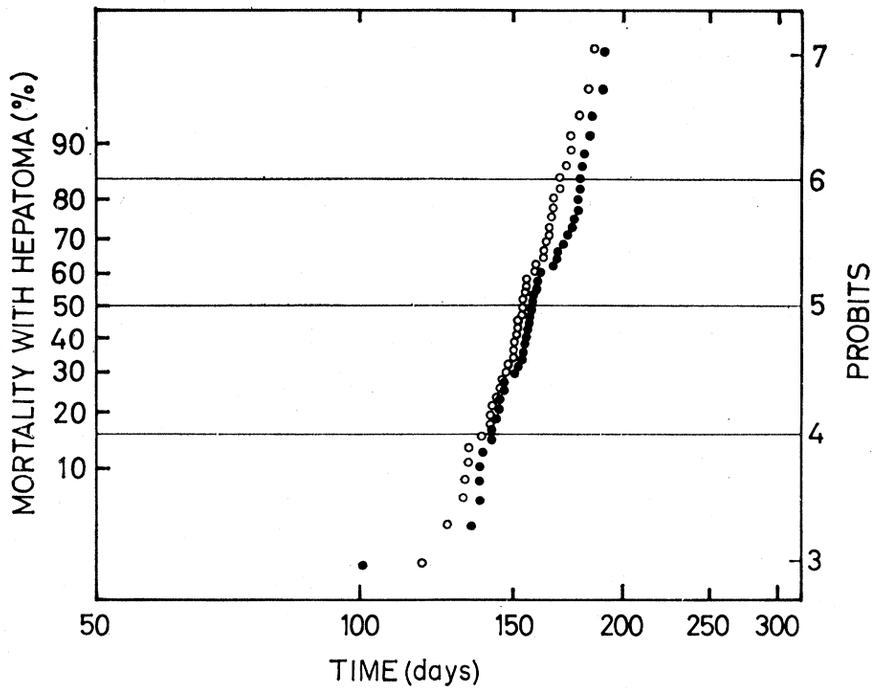

Fig. 4. Effect of daily low-dose x-irradiation on tumor development in the DEN–rat liver system. Mortality with liver carcinomata is plotted against time in a probability grid [experimental points corrected after the method of Miescher *et al.* (*14*)]. Treatment: (●) 5 mg of DEN per kilogram per day; and (○) 5 mg of DEN per kilogram per day plus 5 r per day. Each point represents one animal.

rapid proliferation restore the liver mass to its original size within a short time after operation (*10*).

The effect of daily low doses of irradiation on response of the liver to DEN carcinogenesis was studied in another series of experiments. In Wistar rats treated with daily doses of 3 mg of DEN per kilogram of body weight and irradiated with a single dose of 320 roentgens before starting the experiment, there was no statistically significant effect on T, although T for irradiated animals was slightly shorter than for animals treated with DEN only (*11*). We administered daily doses of 5 mg of DEN per kilogram combined with daily doses of 5 r (±3 percent S.D.) (*12*) whole-body irradiation.

Four groups of 50 adult, male Sprague-Dawley rats each (mean body weight, 290 g ± 10 percent S.D.) were used: group O was the untreated control, group A received irradiation only, group B was given DEN only, and group C was treated with DEN plus irradiation. According to the generally accepted observation that body weight of rats is a very sensitive indicator of radiation (*13*), reduction of mean body weight by additional low-dose irradiation is reflected in Fig. 3. The reduction factor is very nearly the same for the untreated control group O and for group B which was treated with DEN only. Hence, concerning reduction of body weight, there is summation of the effects of DEN and x-rays. Figure 4, however, shows that there is no significant difference in T between the combined group C and group B, although, as in an earlier experiment (*11*), there is a slight shift to the left for group C. There may be a better chance to demonstrate radiation effects on this system of experimental carcinogenesis by lowering the daily dose of DEN in order to increase T in the unirradiated group.

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Lithium's Failure to Replace Sodium in Mammalian Sympathetic Ganglia

Abstract. *Ganglionic responses to electrical stimulation, acetylcholine, and potassium ions were studied in superior cervical ganglia of cats perfused with media containing lithium chloride instead of sodium chloride. In lithium-Locke, ganglionic transmission and depolarization evoked by acetylcholine were blocked completely but reversibly, while the depolarization produced by potassium ions was unaltered.*

There is much evidence that acetylcholine depolarizes neuronal and junctional tissues primarily by causing a marked increase in sodium permeability at those sites and that it also causes an increase in permeability of these tissues to other cations (*1*). In peripheral nerve and sympathetic ganglia, complete replacement of sodium ions in the bathing medium with iso-osmotic equivalents of calcium ions maintains acetylcholine-induced depolarization until such time as stabilizing action of the divalent cation exerts itself. Moreover, the tissues retain considerable sensitivity to acetylcholine when sodium in the medium is replaced by nonelectrolytes. Under these conditions, removal of calcium from the bathing medium results in loss of sensitivity to acetylcholine. When acetylcholine is applied to these tissues in the absence of sodium ions, it is apparently the movement of calcium ions into the cells that provides current necessary for discharge