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Prophage λ induction in *Escherichia coli* K12 *envA uvrB:* A highly sensitive test for potential carcinogens

(aflatoxin B1/benzo[*a*]pyrene/7,12-dimethylbenz[*a*]anthracene)

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ABSTRACT A simple, inexpensive, and sensitive test for potential carcinogens based upon the property of carcinogens to induce prophage λ is described. By using chemicals activated with microsomal enzymes and *E. coli* K12 permeable (*envA*) tester bacteria also deficient in DNA repair (*uvrB*), the range of carcinogens detected in a lysogenic induction test (inductest) has been extended. We have provided the evidence that, after activation, carcinogenic polycyclic hydrocarbons such as benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene induce prophage λ. Three variants of the test have been developed (inductests I, II, and III), which are as sensitive as the mutagenicity test of Ames *et al.* [Ames, B. N., McCann, J. & Yamasaki, E. (1975) *Mutat. Res.* 31, 347–364]. Inductests II and III provide a quantitative estimation of the inducing activity of a carcinogen. With the latter test, one can determine: (*i*) the cellular toxic effect of a carcinogen and (*ii*) the kinetics of appearance and disappearance of active metabolites. For two series of chemicals, aflatoxins and benz[*a*]anthracenes, there is a good correlation between their carcinogenic activity in rodents and their prophage inducing activity in bacteria. The fact that the majority of the cell population is induced makes it possible to test the inducing activity of carcinogens at the biochemical level, e.g., by measuring λ repressor inactivation.

More than a hundred carcinogens have recently been shown to be mutagens in *Salmonella typhimurium* (1, 2). The correlation between carcinogenicity and mutagenicity provides a useful test for potential carcinogens.

Recent work suggests that when *Escherichia coli* K12 bacteria are treated with ultraviolet light (3–6) or aflatoxin B1 (a potent carcinogen) (7, 8), persisting chromosomal DNA lesions induce a metabolic pathway that leads to mutagenesis as well as to the induction of prophage λ. One expects that carcinogens should induce prophage λ as well as they induce mutagenesis.

Until now, only a limited number of carcinogens have been shown to induce prophage λ in *E. coli* K12 (9–11). This relative failure can be ascribed to the lack of a proper technique. Most chemicals cannot penetrate bacteria (12), and, to be active, carcinogenic compounds have to be metabolized by mammalian liver enzymes (1, 2, 13).

We describe here an improved test for potential carcinogens based upon lysogenic induction. The procedure involves the use of activated chemicals and new permeable tester bacteria (*envA*) also deficient in DNA repair (*uvrB*). Three variants of this efficient, simple, and inexpensive lysogenic induction test (inductest) are reported and discussed.

MATERIALS AND METHODS

Bacterial Strains. All strains were derived from D21 env^+ and D22 *envA* (14) except GY 4015, an Amp^R derivative of C600. A pedigree of the strains used will be sent upon request.

Media and Culture. LB: Difco yeast extract, 5 g; Difco tryptone, 10 g; NaCl, 10 g; water, 1 liter. LBE: LB supplemented with 0.2% glucose and 20% medium E of Vogel and Bonner. LA-Ca: LB, 1 liter supplemented with 0.2% glucose and 2.5 mM $CaCl_2$; Biomar agar, 15 g. GT-amp: peptone, 8 g; Difco tryptone, 5 g; NaCl, 5 g; Biomar agar, 12 g; water, 1 liter; supplemented with 10 μg/ml of D-ampicillin. Soft agar: Difco agar, 7.5 g; water, 1 liter.

In all experiments, λ lysogens were grown as follows: an overnight culture in LBE medium at 37° was diluted 1:100 into fresh LBE medium and incubated with aeration at 37° up to about 4×10^7 viable cells per ml (OD_{650} = 0.3).

Chemicals. NADP and glucose-6-phosphate were from Sigma; aflatoxin B1, aflatoxin G1, and sterigmatocystin from Makor; and benzo[*a*]pyrene, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, and 3-methylcholanthrene from Fluka. Benzo[*a*]pyrene 4,5-oxide was kindly provided by D. M. Jerina.

Carcinogens were dissolved and diluted when needed in dimethylsulfoxide (Me_2SO), spectrophotometric grade (Merck), and kept in the dark.

Metabolization of Carcinogens. Except where otherwise stated, the metabolizing mixture consisted of 1 volume of postmitochondrial fraction (13) and 6 volumes of NADPH-generating system: 150 mM phosphate buffer (pH 7.5), 3 mM NADP, 10 mM glucose-6-phosphate, and 12 mM $MgCl_2$. The latter was sterilized by filtration and stored at 4°.

The postmitochondrial fraction was from rats that had received either three daily injections of 75 mg/kg of phenobarbital sodium or two daily injections of 20 and 10 mg/kg of 3-methylcholanthrene in the days preceding their being killed.

Viability of *envA* (λ) Lysogens in Liquid and on Solid Media. Starting from a saturated culture, growth of *envA* bacteria is exponential for a few generations. The exponential growth phase of *envA* (λ) lysogens cannot be maintained by repeated dilutions into fresh rich medium since bacteria begin to die. A similar situation is found when *envA* bacteria are plated because colony formation involves a long exponential growth phase (14). We found that *envA* lysogens remain alive when plated with *E. coli* bacteria killed with streptomycin. Practically, 0.1-ml dilutions of *envA* bacteria were plated with 3 ml of soft agar and 0.3 ml of strain GY 4015 at about 2×10^9 cells per ml onto LA-Ca plates containing 200 μg/ml of streptomycin. Under these conditions, *envA* bacteria have an efficiency of colony formation 5-fold less than env^+ cells taken at the same optical density. This result is consistent with the fact that *envA* bacteria have on average 5 cell units per colony former (15).

We hypothesize that the streptomycin-killed bacteria provide a factor essential for the exponential growth of *envA* bacteria; the factor seems to be accumulated in the resting cells. This factor of viability might be either an enzyme or an activator

Abbreviation: Me_2SO, dimethylsulfoxide.

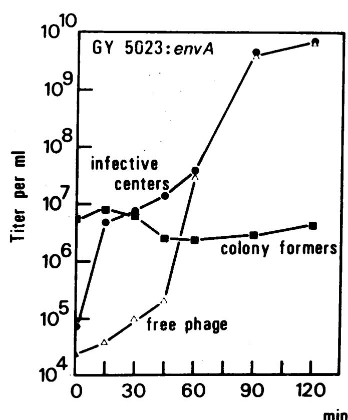

FIG. 1. One-step growth curve of phage λ induced by aflatoxin B1 metabolites. To 1.4 ml of metabolizing mixture were added a sample of 0.3 ml of *envA* (λ) bacteria at 4×10^7 cells per ml and then 40 μl of Me_2SO containing 8.7 μg of aflatoxin B1 (final concentration 5 μg/ml). The preparation was incubated with aeration in the dark at 37°. At times indicated on the abscissa, diluted samples in 10 mM $MgSO_4$ were assayed for colony formers (■), infective centers (●), and free phage (△). Microsomes were from rats treated with phenobarbital.

of the enzyme, *N*-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28) found to be reduced in *envA* bacteria (16). Our finding should help to elucidate the physiology of the *envA* defect.

Kinetics of Lysogenic Induction. A λ lysogen, when induced, gives rise to an infective center which bursts and liberates free phage. Free phage are resistant to chloroform, unlike infective centers (17); free phage and infective centers are both detected as plaques on a lawn of phage λ indicator bacteria. An example of a one-step growth curve of phage λ induced by carcinogenic metabolites, e.g., aflatoxin B1 metabolites, is shown in Fig. 1. When *envA* (λ) bacteria were mixed with aflatoxin B1 and the metabolizing mixture, prophage λ was readily induced; the lysogens began to lyse between 30 and 40 min at 37°. Before 30 min of incubation, the amount of free phage was negligible; plaques were only produced by infective centers. The number of the infective centers formed was not equal to the decrease in bacterial survivors; this can be accounted for by the fact that *envA* bacteria form chains of about 4 to 5 cell units (15). A single induced lysogen per chain is enough to give rise to an infective center, whereas the loss of a colony occurs only if all cells of a chain are killed.

Efficiency of Lysogenic Induction is calculated as the ratio of the number of infective centers to the number of original viable lysogens. To measure infective centers, the treated λ lysogens were plated onto GT-amp plates with 3 ml (or 2.5 ml in inductests I and II) of soft agar containing 0.3 ml of indicator bacteria GY 4015 at about 2×10^9 cells per ml. Ampicillin prevents the noninduced bacteria from multiplying and, therefore, from releasing phage during the overnight incubation of plates. This antibiotic eliminates spontaneous phage production but does not inhibit the induced lysogens to form infective centers (18). This technique was applied to *envA* (λ) lysogens since they are phenotypically ampicillin-sensitive (14).

In inductest III, to estimate the efficiency of lysogenic induction of any carcinogen, incubation in liquid at 37° was never carried out any longer than 25 min in order to avoid the burst of induced lysogens. However, in the case of a weak λ inducer, it is possible to magnify the number of the detected plaques by allowing the burst of the induced lysogens: then, free phage instead of infective centers are measured.

RESULTS

The Inductests. When an inductest is performed the following are used: (*a*) the tester bacteria, such as *envA uvrB* (λ) lysogens; (*b*) the λ indicator Amp^R bacteria to reveal the phage produced by induction; (*c*) the drug to be tested dissolved in Me_2SO; (*d*) if required, the metabolizing mixture that transforms the drug into active products; and (*e*) GT-amp plates.

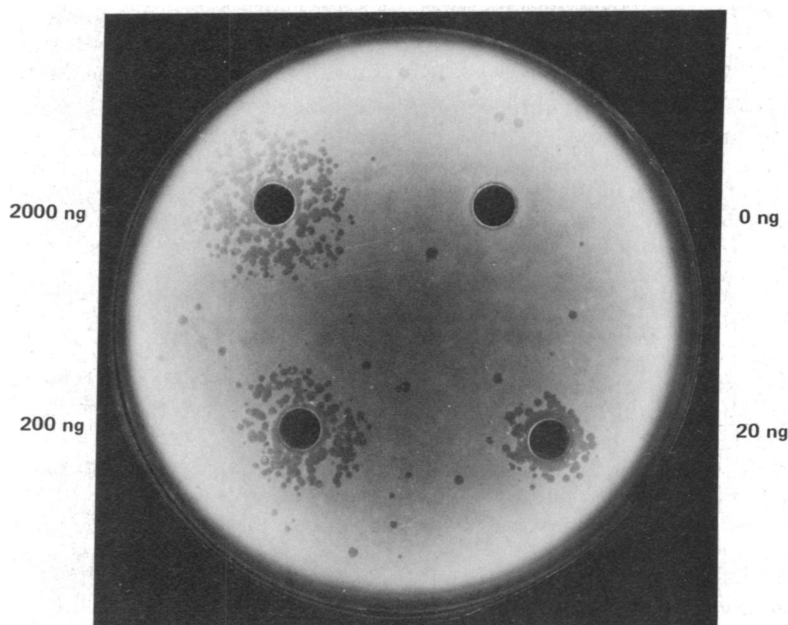

FIG. 2. Spot test (inductest I) for prophage λ induction by aflatoxin B1 metabolites. The following were poured on a GT-amp plate: 2.5 ml of soft agar with 0.3 ml of GY 4015 indicator bacteria at 2×10^9 per ml and 1 ml of *envA uvrB* (λ) bacteria at about 8×10^3 cells per ml diluted 1:8 in the metabolizing mixture. Four filter paper discs were placed on the agar when it had hardened. On each filter were deposited 20 μl of Me_2SO containing one of the four following concentrations of aflatoxin B1: 0, 20, 200, or 2000 ng. The plate was incubated overnight at 37°. Microsomes were from rats treated with phenobarbital.

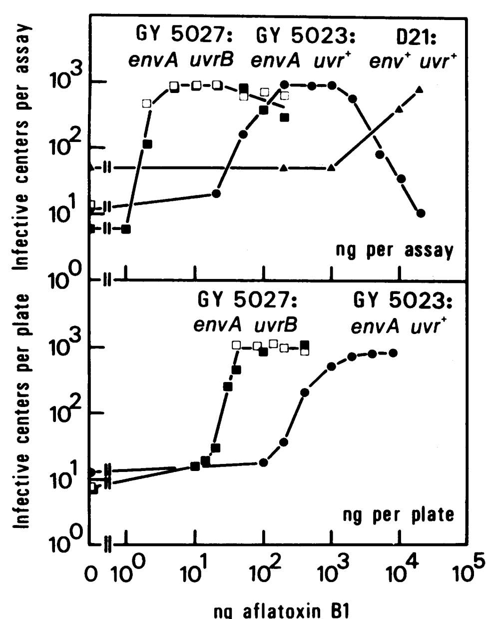

FIG. 3. Detection of aflatoxin B1: influence of *envA* and *uvrB* mutations. (Upper panel) Inductest III. One volume of bacteria of genotype indicated on the graph at about 2×10^4 cells per ml was mixed with 7 volumes of metabolizing mixture; 200-μl aliquots were distributed into test tubes containing 5 μl of Me_2SO with increasing amounts of aflatoxin B1 (indicated on the abscissa). Each preparation was gently agitated at 37° in the dark for 15 min and then poured onto GT-amp plates with 3 ml of soft agar containing 0.3 ml of GY 4015 indicator bacteria at 2×10^9 per ml. Prophage λ induction was measured as infective centers formed after overnight incubation at 37°. Microsomes were from rats treated with phenobarbital. (Lower panel): Inductest II. A sample of bacteria at about 4×10^3 cells per ml and the metabolizing mixture were mixed as described above; 1-ml aliquots were distributed into test tubes containing 20 μl of Me_2SO with increasing amounts of aflatoxin B1 (indicated on the abscissa). Preparations were then poured onto GT-amp plates with 2.5 ml of soft agar and with 0.3 ml of GY 4015 indicator bacteria at 2×10^9 per ml. Prophage λ induction was measured as infective centers formed after overnight incubation at 37°. Microsomes were from rats treated with phenobarbital.

Three variants of the test have been designed that differ with respect to how and when *a, b, c,* and *d* are mixed.

Inductest I: A qualitative spot test. The carcinogen is spotted onto plates directly or onto paper filters; the soft agar contains the λ lysogens, the phage λ indicator bacteria, and the metabolizing mixture. As soon as metabolization of the drug occurs, metabolites diffuse in the soft agar and penetrate the λ lysogens, which are induced: plaques appear around the spots after overnight incubation at 37° (Fig. 2). The drug diffusing out of a spot forms concentric rings of decreasing concentrations.

Inductest I is a simple procedure for the screening of a large number of chemicals. This test can also easily assess whether, in order to be active, a compound must be metabolized or not: aflatoxin B1 (20 ng) (Fig. 2) and 7,12-dimethylbenz[*a*]anthracene (4 μg) were found to be active only when metabolized, whereas benzo[*a*]pyrene 4,5-oxide (1 μg) and mitomycin C (0.1 μg) were active *per se*.

Inductest II: A quantitative test on plate. Five hundred to 1000 lysogenic cells are mixed with a definite amount of a carcinogen, of the metabolizing mixture, and of the phage λ indicator bacteria, and poured onto GT-amp plates.

Fig. 3 (lower panel) and Fig. 4 show that, at optimal input drug concentrations, all the treated cells were induced except when 3-methylcholanthrene was used. This compound seems

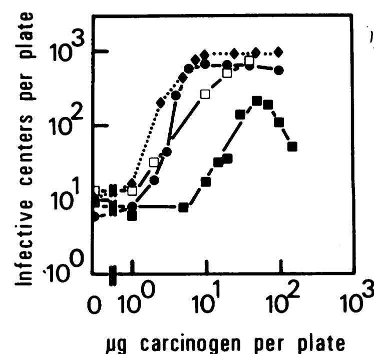

FIG. 4. Detection of polycyclic hydrocarbons in inductest II. GY 5027: *envA uvrB* (λ) bacteria were treated as in the legend of Fig. 3 (lower panel) with increasing amounts (indicated on the abscissa) of 7,12-dimethylbenz[*a*]anthracene (♦), benzo[*a*]pyrene (●), benzo[*a*]pyrene 4,5-oxide (□), or 3-methylcholanthrene (■). In the case of benzo[*a*]pyrene 4,5-oxide, the metabolizing mixture was omitted. Microsomes were from rats treated with phenobarbital, or with 3-methylcholanthrene in the case of benzo[*a*]pyrene.

highly toxic. The induction curves as a function of the drug concentration are approximately two-hit.

Inductest III: A quantitative test in liquid medium. A definite number of lysogens (500–1000) and a given amount of a carcinogen and of the metabolizing mixture are mixed and incubated at 37°. Metabolization of the drug and cell penetration of the metabolites are stopped by dilution in soft agar and plating.

The sensitivity of inductest III is generally higher than that of inductest II (Fig. 3). In the case of water-insoluble compounds, however, only about 50% of the treated lysogens were induced in liquid instead of 100% in the soft agar (Figs. 4 and 5). Diffusion of the water-insoluble metabolites and their penetration into lysogens is slow; incubation for 15 or 25 min in liquid was not enough to bring about full induction of the lysogens.

To estimate the kinetics of appearance and disappearance of the carcinogenic metabolites, we first incubated the metabolizing mixture at 37° with the carcinogen and, at various times, added the lysogens and incubated for 15 min; the frequency of infective centers produced was taken as an estimate

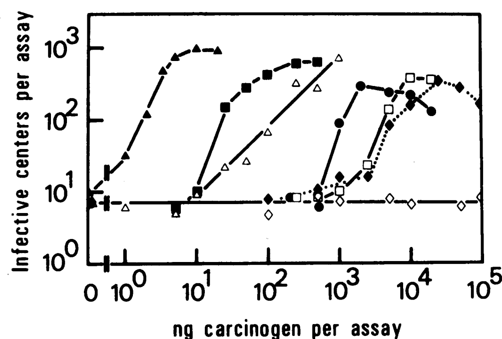

FIG. 5. Comparison of λ inducing activity of various carcinogens (inductest III). GY 5027: *envA uvrB* (λ) bacteria were treated as in the legend of Fig. 3 (upper panel) with increasing amounts (indicated on the abscissa) of aflatoxin B1 (▲), aflatoxin G1 (■), mitomycin C (△), benzo[*a*]pyrene (●), benzo[*a*]pyrene 4,5-oxide (□), 7,12-dimethylbenz[*a*]anthracene (♦), or benz[*a*]anthracene (◇). In the case of mitomycin C and benzo[*a*]pyrene 4,5-oxide, the metabolizing mixture was omitted. Treatment with 7,12-dimethylbenz[*a*]anthracene, benz[*a*]anthracene, and benzo[*a*]pyrene 4,5-oxide was for 25 min. Microsomes were from rats treated with phenobarbital, or with 3-methylcholanthrene in the case of benzo[*a*]pyrene.

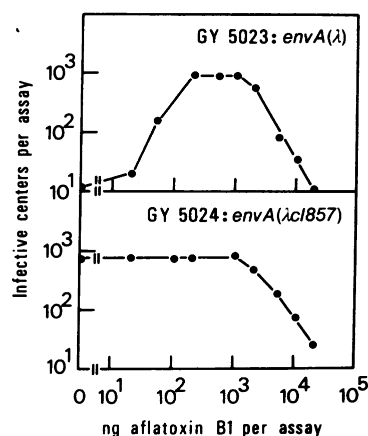

FIG. 6. Capacity of cells treated with aflatoxin B1 to reproduce prophage λ. (Lower panel) One volume of *envA* (λ*cI857*) bacteria at about 2×10^4 cells per ml was mixed at 32° with 7 volumes of metabolizing mixture; 200-μl aliquots were distributed into test tubes containing 5 μl of Me_2SO with increasing amounts of aflatoxin B1 (indicated on the abscissa). Each preparation was gently agitated at 32° in the dark for 25 min and then poured onto GT-amp plates with 3 ml of soft agar containing 0.3 ml of GY 4015 indicator bacteria at 2×10^9 per ml. The development of the induced λ prophage was measured as infective centers formed after overnight incubation at 42°. Microsomes were from rats treated with phenobarbital. (Upper panel) (Inductest III) *envA* (λ) bacteria were treated as described above except that incubation was for 15 min at 37°. Infective centers on the ordinate were measured after overnight incubation at 37°.

of the level of active metabolites. We found that the level of the active metabolites of aflatoxin B1 decreased immediately almost exponentially as a function of the time of preincubation; the half-life of aflatoxin B1 metabolites was about 10 min at 37°.

Sensitive Tester Lysogens. Fig. 3 (upper panel) shows that the combination of *envA* and *uvrB* mutations increases the detection of aflatoxin B1 metabolites more than 4000 times. The *envA* mutation augments the sensitivity of the induction test 100-fold. The fact that the *uvrB* mutation also increases the cell sensitivity to the metabolites of aflatoxin B1 and benzo[*a*]pyrene (data not shown) indicates that these metabolites produce bacterial DNA lesions which are removed by the excision repair pathway.

Drug Metabolization. We have routinely tested potential carcinogens using microsomes from rats treated with phenobarbital, except in the case of benzo[*a*]pyrene, in which microsomes from rats treated with 3-methylcholanthrene were used (19). With sterigmatocystin both types of microsomes gave identical results. With aflatoxin B_1, however, although the efficiency of induction was unchanged, the capacity to reproduce the phage decreased when the microsomes used were from rats treated with phenobarbital. This is in agreement with the previous observations that more toxic metabolites are produced by microsomes from phenobarbital-treated rats (13).

Toxicity Test for Chemical Carcinogens. All the known inducers of prophage λ have two antagonistic effects: (*i*) they trigger the multiplication of the prophage and (*ii*) they cause damage to the prophage as well as to the host bacterium so that the cell's capacity to reproduce the induced prophage is affected. At high ultraviolet doses, lysogenic induction occurs but may go unnoticed by lack of production of mature phage particles; it may be so for any chemical carcinogen when used at high concentration.

By replacing λ lysogens with λ*cI857* lysogens in inductest III, we established a means of testing the range of toxicity of a chemical compound. A culture of λcI857 lysogens is submitted at 32° to the tested drug; at this temperature the prophage is not induced (*ind*$^-$). The culture is shifted to 42°; all cells are then induced because, at this temperature, the λ*cI857* mutant repressor is inactivated. Any reduction in the number of infective centers will be due to the deleterious effect of the drug on the cell capacity to reproduce the prophage.

On Fig. 6 (lower panel) we see that at low concentrations of aflatoxin B1, 100% of the λ*cI857* treated cells are induced; at higher doses, the descending part of the curve of the λ*cI857* infective centers parallels that of the λ lysogens (Fig. 6, upper panel); this shows the toxic effect of the metabolites.

Carcinogenic Activity and Prophage λ Induction. Sterigmatocystin is, as is aflatoxin B1, a widely disseminated mycotoxin and a powerful carcinogen (20). We have found that, when metabolized, these two related compounds induced prophage λ at similar low concentrations (data not shown). Aflatoxin G1 induces tumors in the rat with a considerably reduced potency as compared to that of aflatoxin B1 (20). Similarly, the input amount of aflatoxin G1 needed to induce all the treated lysogens was higher than that of aflatoxin B1 (Fig. 5).

Polycyclic hydrocarbons with a strong carcinogenic activity, such as benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, and 3-methylcholanthrene, induced prophage λ when metabolized (Figs. 4 and 5). In contrast, benz[*a*]anthracene, which has a questionable carcinogenic activity, failed to induce prophage λ in inductest II or III. Benzo[*a*]pyrene 4,5-oxide is the most stable of the benzo[*a*]pyrene epoxide metabolites (21). We have found that, as λ inducer, it was active *per se*, but less efficient than metabolized benzo[*a*]pyrene.

DISCUSSION AND CONCLUSIONS

Until now, lysogenic induction used as a test for chemical carcinogens had been of limited value for two main reasons: bacteria are not permeable to most chemical compounds (12) and, even if they are, many of the chemicals have to be metabolized to become active carcinogens (22). We have extended the range of carcinogens detected as prophage λ inducers by using permeable mutant lysogens and rat liver homogenates which duplicate mammalian metabolism. Apparently not altered in the lipopolysaccharide carbohydrate cell wall layers, *envA* bacteria are more permeable than *lps* mutants to compounds like gentian violet. The rate of gentian violet uptake by *envA* bacteria is similar to that of spheroplasts made by lysozyme-EDTA treatment (12). It seems likely that the *envA* mutation causes as much permeation as the bacterial cell envelope can tolerate.

The three inductests we developed are as sensitive as the mutagenicity test of Ames *et al.* (1). Moreover, inductests II and III provide a quantitative estimation of the λ inducing activity of a carcinogen. Inductest III possesses unique advantages: (*i*) the cellular toxic effect of carcinogens can be easily estimated by using λ*cI857* instead of λ lysogens, (*ii*) the kinetics of appearance and disappearance of active metabolites can be determined since drug metabolization and cell penetration of metabolites can be stopped at will by dilution and plating, and (*iii*) smaller quantities of the metabolizing mixture are needed and smaller amounts of the tested drug can be used (mainly in the case of water-soluble compounds).

We have provided the evidence that, after activation, carcinogenic polycyclic hydrocarbons such as benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene induce prophage λ. Furthermore, for two series of chemicals, we found that there is a good correlation between their carcinogenic activity in rodents

and their prophage inducing activity in bacteria. Aflatoxin B1 is a potent carcinogen as well as a potent λ inducer, whereas aflatoxin G1 is weak in both cases. Benz[*a*]anthracene, a questionable carcinogen, does not induce prophage λ even though it is a potent mutagen (2); its carcinogenic derivative, 7,12-dimethylbenz[*a*]anthracene is a λ inducer as well as a mutagen.

The use of lysogenic induction to study the cellular action of carcinogens has some advantages, one of which being the fact that, since the majority of the cell population is induced, one can test the inducing activity of carcinogens at the biochemical level, e.g., by measuring λ repressor inactivation.

The results of mutagenicity and lysogenic induction tests show that there is a corroboration of carcinogenic activity in eukaryotes with the ability of the very same chemicals either to induce a prophage or to produce mutations in prokaryotes. Such a correlation leads to postulate that: (*i*) the active forms of carcinogens *in vivo* are like those generated *in vitro* by mammalian liver enzymes, and (*ii*) DNA lesions induced in eukaryotes by carcinogens are similar to those induced in prokaryotes. Recent evidence suggests that, in bacteria treated with ultraviolet light (3–6) or aflatoxin B1 metabolites (7, 8), the DNA lesions, which persist after pyrimidine dimer excision repair, induce a metabolic pathway leading to mutagenesis, lysogenic induction, and cell filamentation. We hypothesize that, in eukaryotes, there exists a similar pathway which leads to carcinogenesis (8). With the aforementioned considerations in mind, one would surmise that if a chemical responds positively to the test of mutagenesis (mutatest) and to the test of lysogenic induction (inductest), it will cause cancer.

Unpublished experiments of M. Yarmolinsky to set up an inductest type I stimulated us in developing the inductests. We thank him for his help in the preparation of the manuscript. We are thankful to Y. Moulé and A. Sarasin for providing rat liver microsomal fractions as well as instructing us in their preparation. We are also grateful to many of our colleagues for the gift of various carcinogens. P.M. received a fellowship from Ligue Nationale Française contre le Cancer. Grants from this organization and Fondation pour la Recherche Médicale are gratefully acknowledged.

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