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N-hydroxyurea, mitomycin C and actinomycin D activity in the process of tumour formation on the primary leaves of the 'Pinto' bean

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Abstract

Mitomycin C (MC), N-hydroxyurea (HU) and actinomycin D (AD) inhibit tumour formation on the primary leaves of Pinto beans. *Agrobacterium tumefaciens* was inoculated into bean leaves with application of the above named inhibitors at various times. It was found that MC action is strongest during inoculation and immediately after it, the maximal effect of HU take place within 12 h after inoculation, whereas the antitumour action of AD starts as late as 12 h after leaf inoculation. In view of the different degree of susceptibility of bacteria and plant cells to the inhibitors applied, the above described results allowed to distinguish three critical periods in the process of tumour formation in the tested host-pathogen system.

INTRODUCTION

One of the methods of investigation of the course of tumorous transformation under the action of *Agrobacterium tumefaciens* is the treatment of the infected plant parts with various agents. A particular role is played by inhibitors and other agents disturbing metabolism and DNA functions. The specific action of these inhibitors allows to recognize the role of macromolecular processes in the successive steps of the transformation. From among the typical DNA synthesis inhibitors mitomycin C (MC) and fluorodeoxyuridine (FUdR) inhibited tumour formation. MC inactivates the pathogenic bacterium (Heberlein, Lippincott, 1967), whereas the antitumour effect of FUdR (Bopp, 1965a) consists in interference at a late stage of the transformation process, corresponding according to Klein (1957) to the promotion phase. Bopp (1965b) refered this effect to the action of FUdR on plant cells.

It has been earlier demonstrated that hydroxyurea (HU) inhibits tumour formation on the stems of sunflower plants (Rennert, 1978). The period of maximal activity of the inhibitor falls to the induction phase and starts at the time when a wave of DNA synthesis, evoked by the stimulus of wounding in the plant cells, is to be expected (Kupila-Ahvenniemi, Therman, 1971; Kupila, Stern, 1961).

The present studies were undertaken to compare the effects of two DNA synthesis inhibitors, mitomycin C and hydroxyurea as well as that of actinomycin D, an inhibitor of DNA-dependent RNA synthesis, on the process of tumour initiation on primary leaves of the bean 'Pinto'.

METHODS

Bacterial culture and counting of bacteria

Agrobacterium tumefaciens (Smith and Town) Conn., the virulent strain CCM 1037 from the Czechoslovak Collection of Microorganisms in Brno was cultured on a liquid medium containing 0.8 per cent nutrient broth, 0.1 per cent yeast extract and 0.5 per cent sucrose in darkness at 27°C with continuous shaking (Lippincott, Heberlein, 1965b). Cultures in stationary phase (48 h) were used for the experiments. The number of cells in the inoculum and of viable cells in the cultures treated with inhibitors was determined by the method of series dilutions and plating on agar (Lippincott, Lippincott, 1970).

Test plants and biotest conditions

Phaseolus vulgaris L. cv Pinto, 7-8 day-old, selected for uniform size of primary leaves (Lippincott, Heberlein, 1965a) were infected, as described by Lippincott and Heberlein (1965b), with the use of carborundum No 400 and by placement of 0.05 cm^3 of bacterial suspension on each leaf. The number of tumours formed was determined 9 days after inoculation at a × 8 magnification. Each series of determinations comprised 14-18 leaves and was replicated three times.

Determination of inoculum concentration

Before starting the experiments with the inhibitors, the infectivity of the *A. tumefaciens* strain (CCM 1037) was tested on beans. The range of inoculum concentrations effective in tumour induction is shown in Fig. 1. On the basis of the data obtained, most experiments were carried out with the use for leaf infection of a suspension of about 10^9 cells/cm^3. The average number of tumours elicited in this way on

one bean leaf varied within the limits of 60-80 (control variants). These values do not differ much from those of the quantitative biotest procedure described for this host and *A. tumefaciens* strain B_6 (Lippincott, Heberlein, 1965b). However, the infectivity of strain CCM 1037 is lower as compared with that of strain B_6.

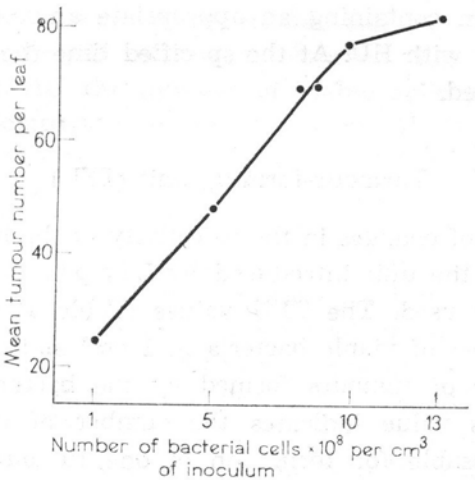

Fig. 1. Relation between *A. tumefaciens* CCM 1037 concentration of the inoculum and the number of tumours initiated on primary 'Pinto' bean leaves

Treatment of leaves with inhibitors

N-hydroxyurea (Schuchardt), mitomycin C and actinomycin D (Calbiochem) were used in the experiments in the form of aqueous solutions containing in 1 ml: HU — 3.8 mg, MC — 10 μg, AD — 40 μg. Drops of these solution were placed by means of a synringe on bean leaves in amounts of: HU — 190, MC — 0.5, AD — 2 μg per leaf and spread with a glass rod on the leaf surface. In order to establish the time of maximal activity of the particular inhibitors in the course of tumour formation, the leaves were moistened with the tested solutions once, but at various times, before or after inoculation with the virulent bacteria.

Treatment of bacteria with the inhibitors

To a 48-h culture of *A. tumefaciens* HU was added in such an amount as to obtain a 3.8 mg/dm³ solution. The cultures were shaken in darkness at 27°C. At definite time intervals test samples (5 ml) were collected, and for removing the inhibitor they were washed with

phosphate buffer pH 7 three times with centrifugation. Then the bacteria were mixed with the buffer (5 ml) until a homogeneous suspension was obtained and stored in a vessel with ice for the experiments. These samples served for determination of the number of viable bacteria and for leaf infection. In the case of actinomycin D, samples of 40-h culture of *A. tumefaciens* of concentration about 10^8 cells/cm^3 were twice diluted with fresh medium containing an appropriate amount of AD or none and incubated like with HU. At the specified time the number of viable cells was determined.

Turmour-forming unit (TFU)

For evaluation of changes in the infectivity of the bacteria in samples treated with HU, the unit introduced by Lippincott and Heberlein (1965b) was used. The TFU values (Table 1) were obtained by dividing the number of viable bacteria in 1 cm^3 of the given sample by the mean number of tumours formed by the bacteria of this sample on one leaf. This value indicates the number of cells in 1 cm^3 of inoculum indispensable for formation of one tumour. The higher the TFU values the less infectious is the preparation. TFU eliminates the decrease of the number of tumours connected with the diminution of the number of viable bacteria in the treated samples.

Table 1

Viability and infectivity of *A. tumefaciens* treated with hydroxyurea (HU)

Time of exposure min.	Viable bacterial cells/cm^3 $\times 10^8$		Tumours/leaf		TFU $\times 10^6$
	Number	%	Number	%	
0	8.7	100	72.0	100	12.0
20	8.0	92	56.8	79	14.0
40	7.6	87	43.2	60	17.6
60	7.1	81	36.7	51	19.3
120	5.3	62	21.0	29	25.2
180	3.9	45	11.0	15	35.4
180 + one passage without HU	9.0		77.0		11.7

48-hrs *A. tumefaciens* culture was supplemented with HU to a final concentration of 3.8 mg/cm^3 and incubated under standard conditions (see Methods). Test samples of this culture were removed at suitable time intervals, centrifuged and washed with phosphate buffer (pH 7). The sedimented cells were resuspended in phosphate buffer and used for viability and infectivity determinations. TFU—number of viable *A. tumefaciens* cells in 1 cm^3 of inoculum per one initiated tumour (Lippincott, Heberlein, 1965b).

RESULTS

Influence of HU on the infectivity and viability of *A. tumefaciens*

Virulent bacterial cells incubated for 3 h with HU of 3.8 mg/cm³ concentration (5×10^{-2}M) show with lapse of time a decrease of their ability of initiating tumours on 'Pinto' bean leaves. This effect, however, is dependent on the density of the treated cell population. The higher cell density the lower HU effect was observed (Fig. 2). In the course of incubation with HU the number of viable cells of *A. tumefaciens* also diminishes. Comparison of the action of HU on the viability of the bacterial cells and on tumour formation (Table 1) indicates a weak correlation of these effects. After 1-h incubation of the bacteria with HU the number of initiated tumours diminishes to about 50 per cent. Under the same conditions a 50 per cent fall of viability is observed after about 3 h. A similarly low depressing effect on the viability of *A. tumefaciens* was noted after AD application (40 μg/dm³, Table 2). A more effective inhibition of infectivity than of viability of *A. tumefaciens* by HU is indicated by the TFU value (Table 1). The number of viable bacteria per 1 cm³ of the inoculum, indispensable for initiating one tumour per 1 leaf increases with the time of incubation with HU. It would seem, therefore, that the HU-induced fall of infectivity of *A. tumefaciens* cells is not the result of depression of their viability. This decrease of infectivity, however, is transient. The cells incubated

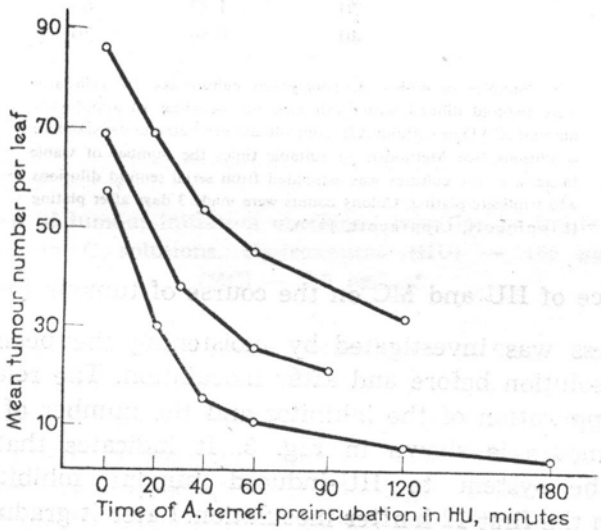

Fig. 2. Effect of *A. tumefaciens* cell density in hydroxyurea (HU)-treated samples on the degree of infectivity inhibition. Densities of bacterial suspensions treated with HU for successive descending curves were respectively: 13; 8.1 and 5.7×10^{9} cells/cm³

with HU for 3 h recovered their tumour-initiating ability after one passege on medium free of inhibitor (Table 1). All these data show that the sensitivity of *A. tumefaciens* CCM 1037 to HU is limited to the concentration interval of this inhibitor lying between 10^{-2} and 10^{-1} M, typical for prokaryots (Rosenkranz, Levy, 1965). If we compare the effects of HU and MC concentrations tested on *A. tumefaciens*, it is seen that the action of HU is not strong, MC in 1 μg/cm³ concentration abolished within 15 min completely the ability of tumour initiation on 'Pinto' bean leaves by *A. tumefaciens*. This effect was correlated with a decrease o viability (Heberlein, Lippincott, 1967). It results from other data that the MC concentration applied in the present experiments (10 μg/m³) destroys completely and irreversibly the ability of DNA synthesis by the bacteria (Matsumoto, Lark, 1963).

Table 2

Viability of *A. tumefaciens* treated with actinomycin D (AD)

Time of exposure, minutes	AD concentrations, μg/cm³	Viable bacterial cells per cm³ × 10⁸	
		Number	%
210	0	1.20	100
	20	1.22	102
	40	0.48	40
390	0	2.80	100
	20	1.83	64
	40	0.56	20

Samples of 40-hrs *A. tumefaciens* culture (ca 10^8 cells/cm³) were twofold diluted with fresh medium including an appropriate amount of AD or without AD (control) and incubated under standard conditions (see Methods). At suitable times the number of viable bacteria in the cultures was estimated from serial tenfold dilutions and triplicate plating. Colony counts were made 3 days after plating (Lippincott, Lippincott, 1970).

Influence of HU and MC on the course of tumour formation

This process was investigated by moistening the bean leaves with HU and MC solution before and after inoculation. The relation between the time of application of the inhibitor and the number of subsequently appearing tumours is shown in Fig. 3. It indicates that the susceptibility of the system to HU-induced tumour inhibition is high, particularly in the first 12 h after inoculation. Later it gradually decreases and ends after about 70 h. It results from experiments conducted parallelly with MC that its effect is similar to that o HU. This could be expected since both these substances inhibit DNA synthesis, although the mechanisms of their action are different. The agreement of the

general course of the curves for HU and MC in Fig. 3 may be evidence of a good adjustment of the concentrations of both inhibitors. MC applied during inoculation almost completely abolishes tumour formation, whereas HU under the same conditions enables the formation of a certain number of them. The stronger influence of MC than of HU on tumour formation is also observed in the period preceding leaf infection. After inoculation the affect of MC drastically decreases. In the same period HU inhibits stronger tumour formation than does MC.

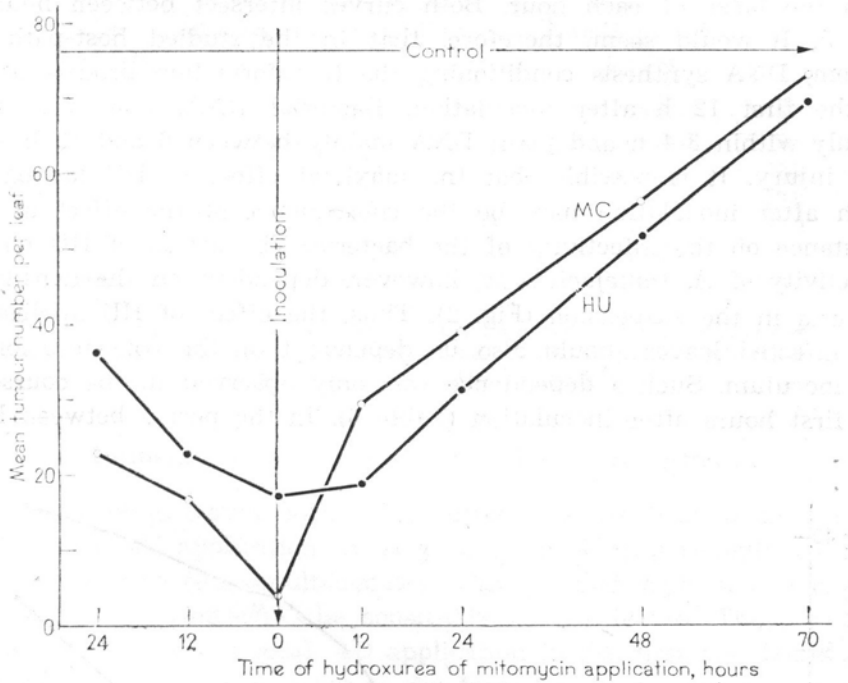

Fig. 3. Inhibition of tumour initiation on 'Pinto' bean leaves treated with hydroxyurea or mitomycin C solutions. Hydroxyurea (HU) — 180 µg, mitomycin C (MC) — 0.5 µg/leaf

Although bacterial and plant cells react to both inhibitors, their susceptibility is different. On the basis of the inactivation rate of cells treated with MC, Heberlein and Lippincott (1967) demonstrated that *A. tumefaciens* belongs to a species particularly susceptible to MC, this being possibly connected with the high content of G-C pairs in the DNA of this bacterium. It is known, on the other hand, that the cells of eukaryota are much more sensitive to HU than those of prokaryota. This relation has been discussed earlier (Rennert, 1977b). If we assume that MC inhibits tumorous transformation by acting mainly on bacteria, while HU acts mainly on plant cells, the time of DNA

synthesis by the bacteria and by the host, indispensable for the occurrence of transformation can be approximately established.

Since the differences in the effects of HU and MC appeared in the first several hours after inoculation, this period was studied for the second time while moistening the leaves with inhibitors' solutions at shorter time intervals. As seen in Fig. 4, maximal MC activity lies within the limits of the 3-4 and that of HU within 9-12 h after inoculation. It is significant that during this time HU activity remains at a constant level forming a plateau, whereas the MC action declines with the lapse of each hour. Both curves intersect between hours 4 and 5. It would seem, therefore, that in the studied host-pathogen system, DNA synthesis conditioning the transformation process occurs in the first 12 h after inoculation. Bacterial DNA, however, forms mainly within 3-4 h and plant DNA mainly between 6 and 12 h after leaf injury. It is possible that the maximal effect of HU lasting for 12 h after inoculation may be the consequence of the effect of this substance on the infectivity of the bacteria. The action of HU on the infectivity of *A. tumefaciens* is, however, dependent on the density of bacteria in the suspension (Fig. 2). Thus, the effect of HU applied on the infected leaves should also be dependent on the concentration of the inoculum. Such a dependence was only observed in the course of the first hours after inoculation (Table 3). In the period between hour

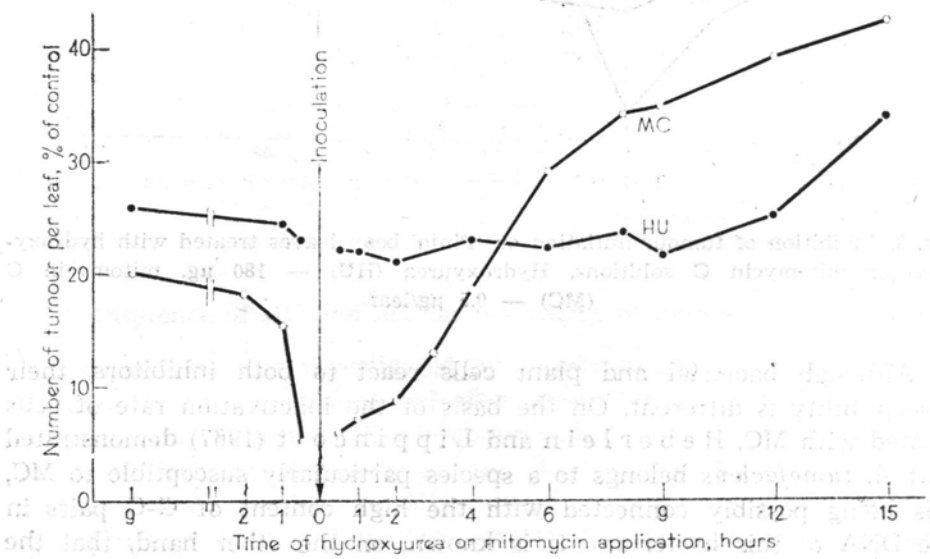

Fig. 4. Comparison of the effects of hydroxyurea (HU) and mitomycin C (MC) in the early period of tumour formation. The mean number of control tumours in experiments with HU and MC was respectively 69 and 80 per leaf. Amounts of inhibitors per leaf were as in Fig. 3

6 and 12 the susceptibility of leaves to HU is not related to the inoculum concentration. It results there from that at that time HU may mainly act on the host's DNA synthesis.

Table 3

Partial relation between effect of hydroxyurea (HU) on tumour formation and *A. tumefaciens* cell density of the inoculum

Time of HU application, hours		Concentration of the inoculum, cells/cm³			
		5.3×10^9		4.8×10^8	
Before inoculation	After inoculation	Tumour number per leaf	%	Tumour number per leaf	%
1	—	42.1	40.0	8.5	16.3
—	2	37.8	36.0	9.9	19.0
—	6	22.0	21.0	11.4	22.0
—	9	23.1	22.0	10.3	20.0
—	12	25.2	24.0	14.0	27.0
—	24	53.1	50.6	24.8	47.7
Control		105	100	52	100

Primary 'Pinto' bean leaves were inoculated with suspensions of two different concentrations of *A. tumefaciens* cells. At various times after inoculation each leaf was treated with HU-solution in a single dose of 190 μg. Control leaves received similar treatment with water.

Influence of AD on the course of tumour formation

Treatment of leaves with AD solution was identical as in the case of HU and MC application. In several series of experiments with AD two series were run simultaneously with HU and with MC to exclude any eventual changes in the sensitivity of the leaves. The results in all series were unequivocal. AD application in the first few hours after inoculation of the leaves leads to the formation of an enhanced number of tumours. This rather unexpected stimulation is strongest immediately after inoculation (180% of tumours) and comprises exactly the time period when the sensitivity of the system to inhibition of DNA synthesis is highest (Fig. 5). The inhibitory action of AD on the number of tumours formed appears as late as between hour 9 and 12 after inoculation, that is at the time when the plateau of HU activity comes to an end. Inhibition reaches its peak around 24 h and is manifested in a 50 per cent reduction of the number of tumours. This state persists rather long (Fig. 5). The time when maximum tumour inhibition by AD starts and lasts suggests that this substance affects the synthesis of RNA indispensable for the development of already initiated tumours. It remains to be elucidated whether the observed increase in the number of appearing tumours is the result of increased infectivity of the bacteria under the action of AD or of its action on plant cells.

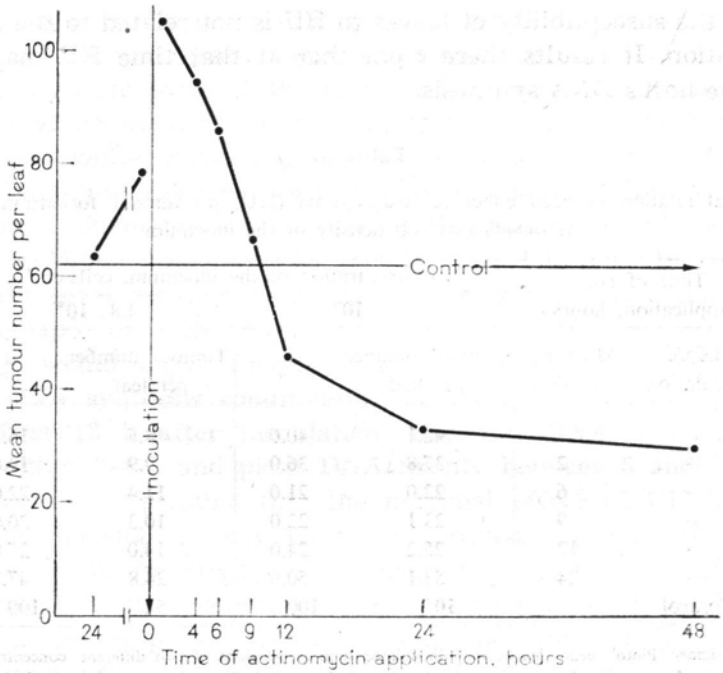

Fig. 5. Promotion and reduction of tumour number on bean leaves treated with actinomycin D (AD) solution (2 µg/leaf)

DISCUSSION

Sunflower stems infected with *A. tumefaciens* react to the antitumour action of HU (760 µg/lesion) applied within 5 days after inoculation (Rennert, 1978). The same amount of time is required for the full cycle of crown-gall transformation in classical host-bacterium systems where the reaction involves a single large wound on the stem or root of numerous plants. 'Pinto' bean leaves are susceptible to the antitumour action of HU (190 µg/leaf) within a time limited to about 70 h after inoculation with virulent bacteria. This seems also to be the maximal time period after injury to leaves when the crown-gall transformation can occur. It is in agreement with the results of Lippincott and Lippincott (1967) since the placement of the *A. tumefaciens* inoculated plants at the temperature of 32°C, preventing tumour formation gives a full effect as late as after 72 h of exposure.

The tendency to tumourous transformation appears in the period between the injury to the plant and the formation of wound cambium. This tendency is exhibited by plant cells before wound-stimulated divisions (Lipetz, 1965, 1966). This state is connected with DNA synthesis (Kupila, Stern, 1961; Kupila-Ahvenniemi, Therman, 1971) and gradually disappears along with the progressing in cambium formation. One can demonstrate that the time when HU

inhibits strongest tumour formation of sunflower stems (2nd and 3rd days after injury and inoculation) corresponds to the period of DNA synthesis in the plant cells (Kupila-Ahenniemi, Therman, 1971; Broekaert, Van Parijs, 1973). No data are available on the transformation cycle in the leaves of the 'Pinto' bean, concerning the course of DNA synthesis. It may be concluded on the basis of HU activity in this system that DNA synthesis starts shortly after inoculation and hardly lasts a dozen hours or so. This conclusion seems reasonable in the light of data that DNA formation atfer wounding is the condition for tumour initiation (Broekaert, Van Parijs, 1973; Guille, Quetier, 1970) and that tumour initiation on 'Pinto' bean leaves starts shortly after inoculation, continuing for 19 h (Lippincott, Lippincott, 1967).

Tumour initiation must be preceded by a period of normal metabolic activity of the bacterium within the wound (Lippincott, Lippincott, 1966). A certain per cent of tumours are initiated on 'Pinto' bean leaves in the course of 2 h after inoculation, the effectiveness of their formation is however, low for the first few hours (Lippincott, Lippincott, 1965). Thus, it is probably a period of adaptation of the bacteria to the environment, when their metabolic activity and DNA synthesis are very intensive. This is distinctly indicated by the results obtained with MC. In this short period (ca. 6 h) after inoculation the antitumour effect of HU depends on the concentration of the bacteria in the inoculum, this pointing to the action of the inhibitor on the bacteria. The absence of this relation at a later time points to the action of the inhibitor on processes occurring within the host cell. Notwithstanding the distinction of the effects o HU on bacteria and on plant cells, it seems doubtless that, under the tested conditions, HU inhibits the synthesis of DNA indispensable for the occurrence of tumorous transformation and that this synthesis take place mainly in the course of 12 h after inoculation. The gradual depression of HU activity after this time may indicate a decrease in the number of cells synthesising DNA since about 50 per cent of the tumours are initiated within 12 h after leaf infection (Lippincott, Lippincott, 1967). It may also be supposed, on the basis of the decrease of HU activity that in the process of tumorous transformation there occurs a decline of plant cell susceptibility to the action of the inhibitor. Actually tumour tissues in cultures *in vitro* react to higher HU concentrations than do homologous normal tissues (Rennert, 1977a and b). It seems logical to refer this phenomenon to the presence of bacterial DNA in the tumour cells since the mechanism of bacterial DNA synthesis is less sensitive to HU than that in eukaryotic cells.

Some results suggest that RNA is involved in the mechanism of tumour transformation. On the leaves of the 'Pinto' bean the step of tumour formation dependent on AD occurs after the period of DNA synthesis. AD applied during inoculation or, in the period of highest susceptibility of the system to the DNA inhibitor, enhances tumour formation. This is in contrast with the effect of rifampicin which inhibits RNA synthesis in bacteria by binding the RNA polymerase. The antitumour influence of rifampicin was strongest when the antibiotic was applied at the moment of infection or within a 24-h period preceding inoculation (Beiderbeck, 1970). This points to the indispensability of bacterial RNA synthesis in the early phase of tumour induction. Rifampicin does not affect the host cell, AD, however, seems to inhibit RNA synthesis in plant cells after tumour initiation. This is in agreement with earlier observations that tumour initiation leads to an early activation of RNA and protein synthesis systems (Rasch et al., 1959). The action of AD in this period may, therefore, lead to inhibition of divisions of the transformed cells. The results of Kurkdjian et al. (1975) actually suggest that AD does not act in the stage of induction, but inhibits tumour proliferation. This effect of AD is, however, attributed by these authors to the action on the bacterium and not on the host cells. If the antitumour influence of AD is connected with bacterial cells, the discrepancy in the results obtained with rifampicin and with AD should be ascribed to the different mechanisms of action of these two antibiotics.

The stimulation of tumour formation by AD, observed in the present experiments, is, however, unclear. In various cell and organism types AD preferentially inhibits RNA synthesis without interfering with DNA replication. There exist examples, both among prokaryotes and eukaryotes, that when there is no RNA synthesis, the DNA content may increase about twice above the normal level under the influence of AD (Kersten, Kersten, 1962; Cleffmann, 1966). Since DNA synthesis occuring in a relatively short time after inoculation is a condition for tumour formation of 'Pinto' bean leaves, it is possible that in the presence of AD the amount of DNA synthesised in this period increases. The period of appearance of the stimulation may be an indication of its relation with the bacteria.

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Aktywność N-hydroksymocznika, mitomycyny C i aktynomycyny D w procesie tworzenia tumorów na pierwszych liściach fasoli 'Pinto'

Streszczenie

Pierwsze liście fasoli 'Pinto' zwilżano roztworami hydroksymocznika (HU), mitomycyny C (MC) i aktynomycyny D (AD), w różnym czasie przed lub po ich zakażeniu wirulentnym szczepem *A. tumefaciens*. Traktowanie to prowadziło do redukcji liczby powstających tumorów. Wrażliwość systemu na hamujące działanie HU i MC (180 i 0.5 µg/liść) ograniczała się do około 70 godzin po infekcji, jednakże stopień zahamowania był zależny od czasu zastosowania inhibitorów. Podczas gdy maksymalna aktywność HU występowała w okresie pierwszych 12 godzin po inokulacji, utrzymując się przez ten czas na równym poziomie, najsilniejsze działanie MC obserwowano jedynie w czasie infekcji i tuż po niej. AD (2 µg/liść) stosowana krótko po inokulacji stymulowała proces infekcji; jej antytumorowy wpływ rozpoczynał się dopiero 12 godzin po zakażeniu liści.

Biorąc pod uwagę różny stopień wrażliwości bakterii i komórek roślinnych na zastosowane inhibitory oraz skuteczność ich działania, w procesie tworzenia tumorów na pierwszych liściach fasoli 'Pinto' można wyróżnić trzy krytyczne okresy:

1. Okres aktywności metabolicznej i syntezy DNA *A. tumefaciens*, trwający kilka pierwszych godzin po infekcji;
2. Okres syntezy DNA gospodarza, która dominuje w czasie między 6 a 12 godziną po inokulacji;
3. Okres, w którym pojawienie się tumorów zależy od syntezy RNA; ten okres zaczyna się po zainicjowaniu tumorów.