CCl_3COOH were added. After centrifugation, to 0.5 ml of the supernatant 0.5 ml of 0.3 *M* trisodium citrate was added. This mixture was heated with 2 ml of ninhydrin reagent according to the modified method of MOORE and STEIN[13] in which, on molar basis, ammonia, aspartic acid and asparagine give practically the same color intensity.

Results. A strong inhibition of rat liver asparaginase by carbobenzoxy derivatives of amino acid with more than one aromatic group was described previously[7]. As can be seen from the Table, this enzyme is also markedly inhibited by phenylacetyl derivatives of amino acids with more than one aromatic group. We found, however, that the following carbobenzoxy (CBZ), 3-phenylpropionyl (PP) and phenylacetyl (PA) derivatives of amino acids did not inhibit, at a molar ratio of 10:1 of derivative to substrate, the *E. coli* asparaginase (as tested by the above methods): CBZ-L-phenylalanine; N-CBZ-S-benzyl-L-cysteine; di-CBZ-L-lysine; N-CBZ-*O*-benzyl-L-tyrosine; CBZ-L-glutamic acid-γ-benzyl ester; PP-L-phenylalanine; N-PP-S-benzyl-L-cysteine; di-PP-L-lysine; PA-L-phenylalanine; PA-D-phenylalanine; N-PA-S-benzyl-L-cysteine; di-PA-L-lysine; N-PA-*O*-benzyl-L-tyrosine; di-PA-L-cystine; PA-L-phenylglycine; PA-L-asparagine; PA-L-glutamine.

[13] S. MOORE and W. H. STEIN, J. biol. Chem. *192*, 663 (1951).

Since the experiments with mammalian and *E. coli* asparaginase were carried out in different buffers, some of the experiments with the latter enzyme were repeated using phosphate buffer instead of *tris*, with the same results. We also tested, at the same molar ratio, the effect of most of the above derivatives on asparaginase from *Erwinia carotovora*. With this enzyme, too, no inhibition was observed.

It follows from these findings that whenever one of the above, or similar, amino acid derivatives inhibits an asparaginase-sensitive tumor, a test for the combined action of this compounds and *E. coli* (or *Erwinia*) asparaginase appears desirable.

Zusammenfassung. Während Acylderivate von Aminosäuren mit mehr als einer aromatischen Gruppe (wie z.B. N-Phenylacetyl-S-benzyl-L-cystein) auf Rattenleberasparaginase eine stark hemmende Wirkung ausüben, werden Asparaginase aus *Escherichia coli* und *Erwinia cartovora* durch solche Verbindungen nicht gehemmt.

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In vitro Binding of ^3H-Acrolein to Regenerating Rat Liver DNA Polymerase

Our previous studies on DNA synthesis in vitro[1] suggested that the inhibitory action of acrolein on regenerating rat liver DNA polymerase was located in the sulfhydryl groups essential for the enzyme activity. The present investigation was designed in order to test this hypothesis. For this purpose (^3H) acrolein has been used. The binding of the labelled aldehyde to regenerating rat liver DNA polymerase and to *Escherichia coli* DNA polymerase I was studied. As previously pointed out[1], the enzymatic activity of the first is associated with functional thiol group(s), whereas the single cysteine residue of the second is not included in the active centre of the molecule. The competition between (^3H) acrolein and 2-mercaptoethanol for the enzymes as well as the action of iodoacetamide were investigated. The fixation of (^3H) acrolein to the templates, DNA or synthetic polymers, was also measured.

Table I. Labelling of DNA polymerase templates by (^3H) acrolein

(^3H) acrolein concentration (*M*)	6×10^{-6}	6×10^{-5}	6×10^{-4}
Native DNA	0	0	65
Denatured DNA	0	0	35
Poly [d(A–T) · (T–A)]	0	0	23
Poly (dC)	0	0	12

The various templates were incubated with increasing concentrations of (^3H) acrolein (10 mCi/m*M*) for 60 min at 38 °C. The reaction mixtures (0.5 ml) contained native or denatured DNA (100 μM), poly [d(A–T) · d(T–A)] or poly dC (23 μM). The results are expressed in pmoles of (^3H) acrolein incorporated per ml of reaction mixture in 1 h.

Material and methods. We used freshly prepared (^3H) acrolein (CEA, Saclay, France) and unlabelled acrolein (Prolabo) distilled just before use, 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y., USA), iodoacetamide (Sigma Chemical Co.). The templates were native or heat denatured calf-thymus DNA (Choay, France), double stranded alternating copolymer poly [d(A-T)·d(T-A)] and single stranded homopolymer poly (dC) (Biopolymers Inc.). DNA dependent DNA polymerase of regenerating rat liver was prepared according to the method published elsewhere[2]. Fraction IV obtained after hydroxylapatite chromatography was used. The specific activity of this fraction in the presence of poly [d(A-T) · d(T-A)] was equal to 100 U/ml. *E. coli* DNA polymerase I (Fraction VII) (Biopolymers Inc.) was diluted to obtain a specific activity of 300 U/ml in the presence of poly [d(A-T) · d(T-A)].

Two types of assays were performed: 1. (^3H) acrolein was incubated 1 h at 38 °C with templates, enzymes, reagents or alone as a control. 2. Regenerating rat liver DNA polymerase or *E. coli* enzyme was preincubated 30 min at 38 °C with templates or other products before (^3H) acrolein was added and incubated 30 min at 38 °C. The incubations were performed as previously described[1]. The results, obtained by determining the difference between assays and controls, were expressed in pmoles of (^3H) acrolein incorporated per ml or reaction mixture.

Results. Binding to DNA or synthetic templates. The data in Table I show that only with 6×10^{-4} *M* (^3H) acrolein can an incorporation be measured in native or denatured DNA, poly [d(A-T) · d(T-A)] or poly (dC). Calculations

[1] N. MUNSCH, A. M. DE RECONDO and C. FRAYSSINET, FEBS Lett. *30*, 286 (1973).

[2] A. M. DE RECONDO, J. A. LEPESANT, O. FICHOT, L. GRASSET, J. M. ROSSIGNOL and M. CAZILLIS, J. biol. Chem. *248*, 131 (1973).

Table II. Binding of regenerating rat liver DNA polymerase or *E. coli* DNA polymerase I to (^{3}H) acrolein of constant or decreasing specific activity

Incubation (60 min at 38 °C) of		Binding of (^{3}H) acrolein
A) Rat liver DNA polymerase with		
Experiment I		
(^{3}H) acrolein (4.65) [a]	6×10^{-6} *M*	24
(^{3}H) acrolein (4.65)	6×10^{-5} *M*	252
(^{3}H) acrolein (4.65)	6×10^{-4} *M*	2468
Experiment II		
(^{3}H) acrolein (465)	6×10^{-6} *M*	40
(^{3}H) acrolein (46.5)	6×10^{-5} *M*	214
(^{3}H) acrolein (4.65)	6×10^{-4} *M*	2002
B) *E. coli* DNA polymerase I with		
(^{3}H) acrolein (465)	6×10^{-6} *M*	0.5
(^{3}H) acrolein (46.5)	6×10^{-5} *M*	11
(^{3}H) acrolein (4.65)	6×10^{-4} *M*	151

All reaction mixtures (0.5 ml) contained 100 µl of enzyme corresponding either to 10 units of regenerating rat liver DNA polymerase or to 30 units of *E. coli* DNA polymerase I. The results are expressed in pmoles of (^{3}H) acrolein incorporated per ml of reaction mixture in 1 h.
[a] (^{3}H) acrolein specific activities expressed in mCi/m*M*.

based on these data show that native bihelical DNA or poly [d(A-T) · d(T-A)] binds 1 molecule of (^{3}H) acrolein per 10^3 nucleotide units, whereas denatured DNA or poly (dC) binds 2 molecules of (^{3}H) acrolein per 10^3 nucleotide units.

In vivo experiments[3] revealed that the binding of regenerating rat liver DNA to (^{3}H) acrolein was constant throughout the experiment (24 h) and corresponded to 1 molecule of (^{3}H) acrolein per 4×10^4 nucleotides. This result is in good agreement with those obtained in vitro considering the fact that the concentration of (^{3}H) acrolein measured in rat liver was 10 times lower than in vitro.

Binding to regenerating rat liver DNA polymerase. 1. When DNA polymerase was incubated with increasing amounts of (^{3}H) acrolein, the incorporation increased linearly (Table II). The fixation of the aldehyde to the regenerating rat liver DNA polymerase explains the progressive inhibition of in vitro DNA synthesis observed in a previous work[1].

It must be pointed out that in the first experiment the specific activities of the different (^{3}H) acrolein batches remained constant (4.65 mCi/m*M*). In the second experiment, they varied from 4.65 to 465 mCi/m*M*. In the latter case the radioactivity of each assay was constant, whereas the quantities of acrolein varied from 6×10^{-4} *M* to 6×10^{-6} *M*. Since the labelling of the enzyme samples increased identically in the 2 experiments, we can assume that there was no ^{3}H exchange but a real fixation of (^{3}H) acrolein.

2. The preincubation of enzyme with iodoacetamide partially inhibited the incorporation of (^{3}H) acrolein by blocking SH groups of the enzyme (Table III).

3. Because of the antagonistic action of 2-mercaptoethanol and acrolein, it has been previously emphasized that acrolein inhibited DNA polymerase by oxidizing the thiol groups of the enzyme[1]. The results of Table III, confirm this hypothesis as the fixation of (^{3}H) acrolein was almost nil after preincubation of enzyme with 2-mercaptoethanol. The data obtained when the preincubation of enzyme with (^{3}H) acrolein was followed by addition of 2-mercaptoethanol demonstrate the irreversibility of the fixation of acrolein to the enzyme and thus its inhibition.

Binding to E. coli DNA polymerase I. 1. When *E. coli* DNA polymerase I was incubated with increasing concentrations of (^{3}H) acrolein, the fixation of (^{3}H) acrolein increased (Table II) and was uniformly 10 to 20 times lower than the fixation of the aldehyde to the regenerating rat liver DNA polymerase.

2. The fixation of (^{3}H) acrolein was poorly affected by the preincubation of *E. coli* DNA polymerase I with iodoacetamide (Table III).

The lack of incorporation of (^{3}H) acrolein after preincubation of the enzyme with 2-mercaptoethanol revealed a competition of acrolein and 2-mercaptoethanol for the SH group of *E. coli* DNA polymerase. As for regenerating rat liver DNA polymerase, the fixation of acrolein on the enzyme was irreversible.

Discussion. The binding of (^{3}H) acrolein to the DNA or synthetic templates was observed for the highest molarity of (^{3}H) acrolein tested (6×10^{-4} *M*). This incorporation corresponds roughly to that measured in vivo in regenerating rat liver DNA (1 mole ^{3}H-acrolein per 4×10^4 nucleotide units).

In a previous paper[1] it was shown that acrolein had an inhibitory effect on regenerating rat liver DNA polymerase during in vitro DNA synthesis. Owing to the

Table III. Opposite action of 2-mercaptoethanol and iodoacetamide to (^{3}H) acrolein on regenerating rat liver DNA polymerase and *E. coli* DNA polymerase I

Incubations (60 min at 38 °C) of enzyme		Binding of (^{3}H) Acrolein to	
		Liver enzyme	*E. coli* enzyme
+ (^{3}H) acrolein		1434	151
Preincubation of enzyme	Incubation (30 min at 38 °C)		
+ iodoacetamide	(^{3}H) acrolein	881	138
+ 2-mercaptoethanol	(^{3}H) acrolein	64	0
+ (^{3}H) acrolein	2-mercapto-ethanol	1534	176

All reaction mixtures (0.5 ml) contained 100 µl of enzyme corresponding either to 5 units of regenerating rat liver DNA polymerase or to 30 units of *E. coli* DNA polymerase I. (^{3}H) acrolein was 6×10^{-4} *M*. The results are expressed in pmoles of (^{3}H) acrolein incorporated per ml of reaction mixture in 1 h.

respective chemical properties of 2-mercaptoethanol and acrolein, we suggested that acrolein inhibited the DNA polymerase by oxidizing the active thiol groups of the enzyme. This hypothesis was consistent with the fact that *E. coli* DNA polymerase I, devoid of SH groups in its active centre, was not inhibited but activated by acrolein. In agreement with these results, our present work demonstrates that (^{3}H) acrolein binds 10 to 20 times more to regenerating rat liver DNA polymerase than to *E. coli* enzyme. The antagonistic action of 2-mercaptoethanol and (^{3}H) acrolein with respect to the regenerating rat liver DNA polymerase and *E. coli* enzyme confirms that the unsaturated aldehyde attaches to the thiol groups of the enzymes and that the fixation is irreversible. Besides, for an (^{3}H) acrolein molarity similar to that measured in vivo in regenerating rat liver[3], the binding of (^{3}H) acrolein to the enzyme is at least 100 times higher than that measured in vivo for the total regenerating rat liver protein.

This result suggests a great affinity of acrolein for the regeneryting rat liver DNA polymerase. ALACRON[4] has demonstrated that acrolein was produced by enzymatically oxidized spermine and spermidine and also during the oxydative degradation of 2 antitumor agents[5,6]. He emphasized that the unsaturated aldehyde might be an effective cell growth inhibitor. Recently, CONNORS et al.[7] pointed out that phosphoramide mustard and acrolein, resulting from the in vitro metabolism of cyclophosphamide, had the highest cytotoxicity for Walker tumor cells. The inhibition of DNA and RNA synthesis that we observed in vivo and in vitro, the affinity of acrolein for regenerating rat liver DNA polymerase and also the ability of acrolein to bind DNA account for the cell growth inhibitory properties of acrolein[8].

Résumé. La fixation de l'acroléine ^{3}H à l'ADN polymérase de foie de rat en régénération et à l'ADN polymerase I de *E. coli* a été étudiée. L'acroléine ^{3}H inhibe l'activité de l'ADN polymérase de foie de rat en régénération en se fixant irréversiblement sur les groupements SH essentiels à son activité. Par ailleurs, la fixation de l'acroléine ^{3}H aux modèles, ADN ou polymères synthétiques, est de 1 molécule pour 10^3 nucléotides et correspond aux fixations à l'ADN de foie de rat mesurées in vivo.

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[3] N. MUNSCH and C. FRAYSSINET, manuscript in preparation.
[4] R. A. ALACRON, Arch. Biochem. Biophys. *137*, 365 (1970).
[5] R. A. ALACRON and J. MEIENHOFER, Nature New Biol. *233*, 250 (1971).
[6] R. A. ALACRON, J. MEIENHOFER and E. ATHERTON, Cancer Res. *32*, 2519 (1972).
[7] T. A. CONNORS, P. J. COX, P. B. FARMER, A. B. FOSTER and M. JARMAN, Biochem. Pharmac. *23*, 115 (1974).
[8] Acknowledgments. We are greatly indebted to Miss O. FICHOT for the rat liver enzyme purification. This work was supported by a grant from the C.E.A. and a subvention from Ministère de l'Environnement.

Multiple DNA-Dependent RNA Polymerases of *Neurospora*

Multiple DNA-dependent RNA polymerases have been demonstrated and characterized in fungi[1-4] as well as in other eucaryotes[5,6]. In a recent report[7], RNA polymerase activities were characterized from isolated nuclei of the *Neurospora crassa* mutant 'slime'. In that study, 4 peaks of enzyme activity were observed upon elution from DEAE-cellulose. All of these activities were insensitive to rifampicin, while one was completely sensitive to α-amanitin and one was partially sensitive.

In this study, DNA-dependent RNA polymerases were isolated from a crude preparation of nuclei from wild type *N. crassa* using different techniques, and when chromatographed on DEAE-sephadex, only 2 major peaks of RNA polymerase activity were resolved. These activities are identified as RNA polymerases I and II. The reason for the difference between these results and those obtained using *N. crassa* 'slime' are unclear at this time. *Neurospora crassa*, strain 853A, was grown in Vogel's medium N containing 2% (w/v) sucrose for 23 h at 25 °C with vigorous aeration. Conidia for obtaining large amounts of mycelia were produced as described by DAVIS and DESERRES[8]. Each 8 l culture was inoculated with conidia obtained from one 2.5 l Fernbach flask. Buffer H: 0.05 *M Tris*-HCl, pH 7.3 at 4 °C, 0.01 *M* $MgCl_2$, 1.0 m*M* $CaCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.1 m*M* EDTA, 0.5 *M* sucrose, 10% (v/v) glycerol. Buffer A: 0.05 *M Tris*-HCl, pH 7.5 at 4 °C, 0.01 *M* $MgCl_2$, 0.50 *M* $(NH_4)_2SO_4$, 0.5 m*M* DTT, 1.0 m*M* EDTA, 0.1 m*M* phenylmethylsulfonylfluoride (PMSF), 10% (v/v) glycerol. Buffer B: 0.05 *M Tris*-HCl, pH 8.2 at 4 °C, 0.01 *M* $MgCl_2$, 0.01 *M* $(NH_4)_2SO_4$, 0.5 m*M* DTT, 1.0 m*M* EDTA, 0.1 m*M* PMSF, 20% (v/v) glycerol.

The reaction mixture (0.25 ml) for the assay for RNA polymerase consisted of 0.04 *M Tris*-HCl, pH 7.9 at 30 °C, 0.1 m*M* EDTA, 2.0 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.5 mg/ml bovine serum albumin, 0.15 m*M* ATP, CTP, GTP, 0.015 m*M* 5-^{3}H UTP, specific activity 300 Ci/mole, 0.20 mg/ml calf thymus DNA, 0.4 m*M* potassium phosphate, pH 7.5, and 30% (v/v) glycerol. In addition, for assays for RNA polymerase I: 0.03 *M* $(NH_4)_2SO_4$, 1.0 m*M* $MnCl_2$, and for RNA polymerase II: 0.08 *M* $(NH_4)_2SO_4$ and 2.0 m*M* $MnCl_2$.

Reactions were started by the addition of enzyme, incubated 30 min at 30 °C, and stopped by the addition of 2 ml of cold 5% (w/v) trichloroacetic acid (TCA). After 30 min, precipitates were collected on Whatman GF/C filters and washed 10× with 5 ml of cold 5% TCA. Filters were dried at 80 °C and counted in a toluene based liquid

[1] T. M. BROGT and R. J. PLANTA, FEBS Lett. *20*, 47 (1972).
[2] H. PONTA, U. PONTA and E. WINTERBERGER, FEBS Lett. *18*, 204 (1971).
[3] P. A. HORGEN and D. H. GRIFFIN, Proc. natn. Acad. Sci., USA *68*, 338 (1971).
[4] H. PONTA, U. PONTA and E. WINTERBERGER, Eur. J. Biochem. *29*, *110* (1972).
[5] S. P. BLATTI, C. J. INGLES, T. J. LINDELL, P. W. MORRIS, R. F. WEAVER, F. WEINBERG and W. J. RUTTER, Cold Spring Harb. Symp. quant. Biol. *35*, 649 (1970).
[6] P. A. HORGEN and J. L. KEY, Biochim. biophys. Acta *294*, *227* (1973).
[7] M. T. TELLY DE INON, P. D. LEONI and H. N. TORRES, FEBS Lett. *39*, 91 (1974).
[8] R. H. DAVIS and F. J. DESERRES, Meth. Enzym. *27*, 79 (1970).