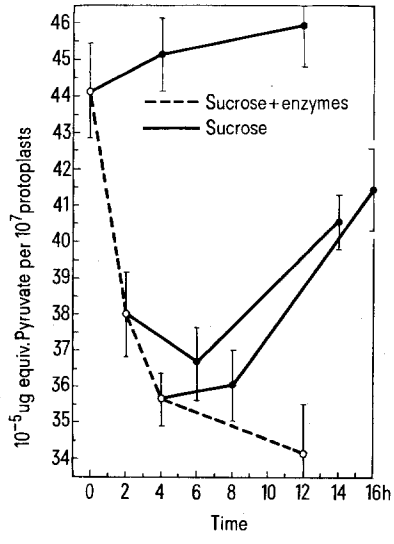
The comparison (Table) between the protoplasts, mechanically and enzymatically prepared, indicated that, for the first type, the volume was lower and the transaminase activity higher than for the second type; all data were found to be significant. Results (Figure) call for a few comments. 1. Keeping the mechanically obtained protoplasts for 12 h in the sucrose solution did not change their transaminase activity significantly. 2. When incubating these protoplasts in the cell-wall-degrading enzymes mixture, a significant decrease of the transaminase activity was observed. Such effect seemed to be very rapid since the depressed transaminase activity remained the same after 4 and 12 h of enzyme incubation. 3. When protoplasts were replaced in the sucrose solution, after 2 or 4 h in the enzymes mixture, their transaminase activity was partly restored. 4. The difference in the transaminase activity between the control and the treated protoplasts was found to be much higher than that observed between the protoplasts mechanically and enzymatically prepared (see Table). It may be noticed that, in the first case, the direct action of the enzymes on the protoplasm transaminases was obviously very much stronger than in the assays with the cells still enveloped by their walls.

Comparative volume and transaminase activity of protoplasts mechanically (M) and enzymatically (E) prepared from *Allium* roots (30 mm) previously decapitated at 3 mm

	Protoplasts	
	M	E
Volume[a] in μ^3 per protoplast	2370 ± 304	9109 ± 832
Transaminase activity[b] per 10^7 protoplasts	44,27 ± 1,34	40,31 ± 1,52

[a] The diameter of respectively 212 and 201 protoplasts was measured.
[b] In µg equiv. ($\times 10^{-5}$) of pyruvate formed.



Changes with time in transaminase activity (in pyruvate formed) of the root (*Allium*) protoplasts mechanically prepared. Continuous lines: assays with protoplasts kept in 20% sucrose solution. Broken lines: protoplasts incubated in the enzyme mixture. Vertical lines extending on one side of the points show the standard error of the mean.

In conclusion, the cell wall-degrading enzymes (or the impurities present in the active mixture) – used for preparing enzymatically the protoplasts – change some of the properties of the protoplasts obtained mechanically. Consequently, it can be supposed that the enzymes preparation may act on the protoplasts produced enzymatically. It is clear that such enzymes mixture depress the transaminase activity with an optimum action after 4 h incubation. From these results, it is not possible to determine at what level of the protoplasts, transaminase is lost, and to decide whether the enzymes act on the formation, the degradation or the activation of transaminase. But it is not excluded that some transaminase co-factors may be destroyed during the enzymatical preparation of protoplasts. Such stimulating agents may be progressively reformed when the protoplasts were replaced in the stored medium.

Résumé. Les enzymes employées pour l'obtention enzymatique des protoplastes de racines (*Allium*) altèrent les transaminases (EC. 2.6.1.1.) de protoplastes mécaniquement préparés. Cette inhibition transaminasique est partiellement levée lorsque les protoplastes ne sont plus en contact avec ces enzymes. Les effets des enzymes dégradant les parois cellulaires sont discutés relativement aux propriétés biochimiques des protoplastes.

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Can the C_4-Dicarboxylate Transporter be Specifically Labelled?

Although the use of affinity labelling substrate analogues has not proved especially useful for studies on membrane systems[1], the non-specific alkylating reagent, N-ethylmaleimide (NEM), has been used, in conjunction with substrate protection, to specifically label the lactose permease of *E. coli*[2–4] and the adenosine triphosphatase (ATPase) of sarcoplasmic reticulum[5].

A transport system for the uptake of C^4-dicarboxylic acids in *Escherichia coli* has been described and characterized[6]. We have recently tried to label some of the membrane component(s) comprising this uptake system.

Materials and methods. E. coli, wild type strain AT 2752 were obtained from Professor H. Kornberg and grown and induced for C_4-uptake as previously de-

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scribed[6,7]. C_4-acid uptakes were carried out and assayed as described[6]. Double labelling experiments were done as described[2].

The reaction with NEM was done according to Fox and KENNEDY[2]. For the kinetic studies, cells were suspended in minimal salts buffer[7] to an absorbance of 0.2 at 680 nm (2×10^8 cells per ml) with or without substrate, and shaken at 28 °C. At the indicated intervals, 2.5 ml aliquots were removed and mixed with 2.5 ml aliquots of minimal salts buffer containing a concentration of 0.02 *M* 2-mercaptoethanol. The resulting 5 ml samples were then incubated in a 37 °C shaking water bath for 10 min, succinate added, and 5 min C_4-uptakes carried out.

Membrane fractions were prepared by sonification in a Branson sonifier followed by centrifugation at $105{,}000 \times g$ for 30 min, in a Spinco Model L ultracentrifuge. Protein was estimated by the Lowry modification of the Folin technique using bovine serum albumin as standard[8]. Polyacrylamide gel electrophoresis was done as described by JONES and KENNEDY[4].

Succinic acid, (1,4-^{14}C) was purchased from Amersham Searle, Chicago, Illinois, and diluted to the desired specific activities with unlabelled succinic acid. NEM (1-C^{14}) and NEM (Ethyl-2^{3}H) were purchased from New England Nuclear Corporation, Boston, Mass., and diluted to the desired specific activity. All other chemicals were either of reagent grade or the highest available grade.

Results and discussion. 1. Inhibitors of C_4-uptake. At 10^{-3} *M* and 10^{-4} *M*, NEM caused nearly complete inhibition of C_4-uptake, but the effect was not prevented by substrate. At 10^{-5} *M*, NEM gave reasonable amounts of inhibition which could be partially blocked in the presence of a large excess of substrate (Table I). At substrate concentrations lower than 10^{-2} *M*, the amount of substrate protection was variable and lower. The amount of inhibition appeared to be maximal after 10 min incubation and longer incubation times caused impaired uptake in control cells. Thus, a relatively restricted set of conditions was required for both significant inhibition and substrate protection.

2. Kinetics of inhibition by NEM. The rate of inhibition of C_4-transport by NEM alone or by NEM in the presence of succinate is shown in the Figure. In these kinetic experiments most of the inhibition occurred within the first 2 min. The apparent first-order rate constants for inhibition have been calculated, assuming that the reaction follows pseudo-first order kinetics (Table II). The rate of reaction of the inhibitor with the transport system is only modestly reduced, even though the concentration of substrate is 4 orders of magnitude greater than that of inhibitor.

The data in Table II may be used to calculate[3] a dissociation constant (K_D) for the transporter-substrate complex equal to 1.35×10^{-1} *M*, which is almost 4 orders of magnitude higher than the K_M for this system as measured directly[6]. The discrepancy between these 2 values suggests that one or more of the assumptions formulated and apparently valid for the lactose permease[3] are not valid for the C_4-transport system.

3. Labelling of particulate fraction. The amount of ^{3}H NEM covalently bound to particulate fractions of *E. coli* in presence (protected) or absence (unprotected) of substrate in shown in Table III. Although there is some variability in the values from one set of experiments to another, there are no significant differences between protected and unprotected samples within each experiment.

Table I. Inhibition of ^{14}C-succinate uptake by NEM alone or in presence of substrate

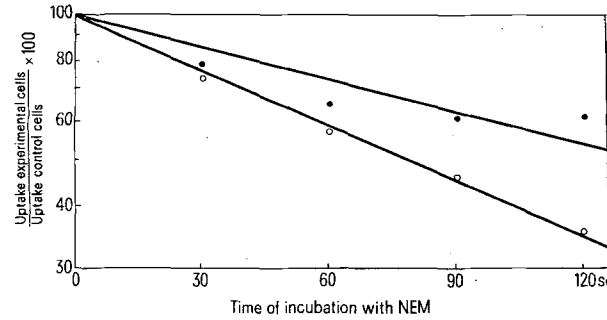
NEM alone[a]	Uptakes (%)	Mean (%)
Control		100
Inhibited	21–43 ($n = 7$)	36
NEM and substrate[b]		
Control		100
Inhibited	37–83 ($n = 7$)	60

Experiments 160672, 230672, 300572, 010672, 120672, 230572, 250572. [a] NEM $10^{-5}M$, 10 min, 28 °C, before uptakes. [b] Malate $10^{-1}M$, 2 min, 28 °C, followed by NEM $10^{-5}M$ 10 min, 28 °C, before uptakes.

Table II. Rates of inactivation by NEM

	Substrate absent	Substrate present
Half-time of inactivation[a] (sec)	79	135
k[b] (sec^{-1})	0.0087 ± 0.0008	0.0051 ± 0.0011

[a] Time at which 50% inactivation is achieved. [b] Assuming pseudo-first order kinetics (k) the apparent first order rate constant is calculated from the gradient of the inactivation curve[4].



Inhibition of ^{14}C succinate uptake by NEM expressed as a percentage of the control value. Points on the graph show the means of logarithms of experimental points. Each point represents the arithmetical mean for 3 experiments. The lines are the regression lines calculated for all points ($n = 20$). ○, NEM alone; ●, NEM and substrate.

Table III. Uptake of NEM by particulate fraction, nmol mg^{-1} protein

Experiment	Protected	Unprotected
040972	0.39	0.45
230572	0.43	0.43
240572	0.27	0.24
250572	0.30	0.32
260572	0.51	0.46
300572	0.21	0.22
Mean	0.35	0.35

Each pair of experiments is the mean of 3 determinations.

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Although the total amount of label in 2 samples may not be very different, the qualitative distribution of the label may be such as to permit identification of specific patterns[1]. In the double-labelling experiments, induced and uninduced cells were reacted with ^{3}H NEM and ^{14}C NEM respectively; both isotopes were distributed uniformly throughout the gel. A uniform distribution was obtained as well when the cells were reacted with unlabelled NEM in the presence of substrate, followed by ^{3}H NEM after the substrate was removed by washing. Thus, in spite of satisfactory functional protection, no evidence for specific labelling of a component of the C_4-transport system was found.

Fox and KENNEDY[2] have estimated that although there is a significant difference in the amount of NEM incorporated between protected and unprotected fractions, 0.15 nmoles mg^{-1} respectively, only about 20% of this difference can be ascribed to labelling on the M protein. KARLIN et al.[9] have calculated that only 10–20% of the labelling of Electroplax electric organ by an alkylating substrate analogue was on the acetylcholine receptor, and that the amount of bound label was not significantly decreased by prior treatment with unlabelled alkylating reagent.

In spite of these difficulties, 3 specific membrane proteins have now been labelled and apparently located electrophoretically[4,5,10]. The reason for the present negative are not known[11].

Résumé. Le transporteur des C-4 acides est inhibé par le N-éthylmaléimide. L'inhibition est partiellement bloquée par le substrat, mais ce n'est pas évident en regardant la définition spécifique du transporteur.

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[11] Acknowledgments. The present studies were supported by funds from the Department of Medicine, Duke University. I wish to thank Mr. S. EISEMANN for technical assistance.

Effect of some Derivatives of Naphthalene on Aryl Hydrocarbon (Benzo[a]pyrene) Hydroxylase in vitro

The inducible enzyme system, aryl hydrocarbon (benzo[a]pyrene) hydroxylase, is found in many tissues of numerous mammalian species. It was first described in rat liver by CONNEY and MILLER[1] and called benzo[a] pyrene hydroxylase. The enzyme converts polycyclic hydrocarbons to phenols, dihydrodiols, quinones and epoxides[2,3]. In addition to its function as a detoxification agent, this enzyme complex has been found to activate polycyclic hydrocarbons to toxic and carcinogenic metabolites[4]. The enzyme also catalyzes the formation of covalently bound complexes of hydrocarbon with DNA[5,6], RNA[5] and protein[6]. In this work, we examined the affinity of certain isomeric derivatives of naphthalene for this enzyme and studied their effect on benzo[a]pyrene hydroxylation. The inhibition shown by some compounds suggests competition for the benzo[a]pyrene hydroxylation enzyme site[7].

All chemicals were obtained from Calbiochem or Sigma. The naphthalene compounds were purified by recrystallization. Male Wistar rats (Commentry strain) weighing from 100 to 120 g were injected i.p. with methylcholanthrene (20 mg/kg) in 0.5 ml corn oil. Control animals received corn oil only. Animals were sacrificed by decapitation and the livers were quickly removed, chilled at 0 °C and homogenized with 0.25 *M* sucrose in 0.05 *M Tris*-HCl, pH 7.5 in a Perspex homogenizer. Microsomes were obtained from the 10% homogenate according to SCHNEIDER[8]. Aryl hydrocarbon (benzo[a]pyrene) hydroxylase was assayed as described by GELBOIN[7]. Alkali-extractable metabolites of benzo[a]pyrene were measured with a Jobin-Yvon Model spectrophotofluorometer to determine enzyme activity. Enzyme activities were determined in duplicate or triplicate. The activity was compared to a blank to which acetone had been added prior to incubation. The various naphthalene hydrocarbons were dissolved in ethanol and added in 0.010 ml amounts to yield a final concentration equimolar or 1/10 equimolar to B(a)P. This blank gave no fluorescent readings. The addition of ethanol containing the various polycyclic hydrocarbons (0.010 ml) to the reaction mixture after the incubation period did not affect the subsequent fluorometric measurements.

As shown in the Table, the addition of naphthalene and its 4 derivatives (naphthol-(1), naphthol-(2), naphthonitril-(1), naphthonitril-(2)), at equimolar or 1/10 equimolar concentrations to the benzo[a]pyrene substrate, had no significant effect on benzo[a]pyrene hydroxylation when the medium contained either control or induced microsomes.

The 2 isomers, naphthyl-phosphordicloridat- (1) and naphthyl-phosphordicloridate-(2), inhibit hydroxylation of benzo[a]pyrene incubated with control microsomes by 14% and 21% respectively, and 2-methyl-β-naphthothiazol by 45%. These 3 compounds also inhibit aryl hydrocarbon (benzo[a]pyrene) hydroxylase in methylcholanthrene-induced microsomes (22%, 22%, and 55% respectively). The addition of other compounds to control or methylcholanthrene-induced microsomes resulted in either negligible or no inhibition of benzo[a]pyrene hydroxylation. With the exception of 2-methyl-β-naphthothiazol, and the 2 isomers of naphthyl-phosphordicloridate, the results suggest that none of the other compounds studied has an affinity for the benzo[a]pyrene hydroxylation site equal to benzo[a]pyrene. The inhibition of

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