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Trans-membrane alkylation: a new method for studying irreversible binding of reactive metabolites to nucleic acids

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Examination of metabolites capable of alkylating nucleic acids has become an important point in assessment of possibly carcinogenic compounds. Alkylation of nucleic acids by metabolites of carcinogens can be demonstrated *in vivo* [1], but application of such methods is hampered by the necessity of administration of very large quantities of expensive radioactive material [2]. On the other hand, many carcinogens are converted by microsomal monooxygenases *in vitro* to the reactive metabolites which covalently bind to macromolecules [3, 4]. It has been proposed to add a protein, e.g. albumin, to incubations of rat liver microsomes, NADPH, and the labeled compound in question [5]. Re-isolation of the protein component after the incubation allows measurement of irreversible protein binding to a well defined molecule. Similarly, nucleic acids are often added to microsomal incubations to provide targets for alkylation [4, 5]. Besides DNA and RNA, the use of synthetic polynucleotides (polyadenylic acid, polycytidylic acid, etc.) gives invaluable insights into the type of nucleotide preferred for alkylation and into the possible mechanisms [6, 7]. A disadvantage of this method of using natural or artificial nucleic acids as trapping material for radioactive metabolites *in vitro* is the presence of nucleases in microsomal preparations, and the presence of ribosomal RNA in microsomes which often leads to difficulties in estimation, especially of DNA adducts. Also, nucleic acids re-isolated after incubations with microsomes may be contaminated with proteins originating from the incubation system; in general, proteins, according to their content of free sulfhydryl groups, are alkylated by electrophilic metabolites to a much higher degree than are nucleic acids. The presence of nucleases in microsomal incubations usually leads to unsatisfactory recoveries of nucleic acids on re-isolation from such incubations and to degradation of bioactive molecules. Especially if the alkylated re-isolated macromolecules are to be subsequently subjected to analyses using methods of molecular biology, a method is required which excludes direct action of microsomal enzymes on the macromolecule incubated.

This report describes a new method for alkylating nucleic acids by metabolites of xenobiotics in rat liver microsomal incubations which overcomes these difficulties. In this system, microsomal biotransformation and the binding target are separated into two compartments, separated from each other by polyamide molecular sieves. [1,2-^{14}C]Vinyl chloride is used as substrate to demonstrate the practicability of the method; this compound is known [6–8] to be transformed by rat liver microsomes to an alkylating metabolite. As a chemically well-defined target for alkylation, polyadenylic acid (poly-adenosine) is used because it gives a single well characterized alkylation product (1,N^6-ethenoadenosine) on reaction with vinyl chloride metabolites [6, 8]. Results obtained by the conventional method (method A) are compared with those of the new 'trans-membrane-alkylation' method (method B).

Microsomal incubations. Rat liver microsomal incubations were carried out under an atmosphere containing [1,2-^{14}C]vinyl chloride, using the all-glass incubation apparatus previously described [9]. Conditions of substrate saturation were chosen [9], i.e. vinyl chloride in the gas phase exceeded a partial pressure of 1 torr (0.13 kPa). Microsomes were prepared from livers of male Wistar rats according to standard procedures [9]. Incubations contained 1 mg microsomal protein/ml and an NADPH-regenerating system [9].

Method A (conventional). Polyadenylic acid (mol.wt = 100,000; Serva, Heidelberg) was directly added to the microsomal incubations, at a concentration of 2 mg/ml. After 45 min of incubation at 37° the incubation vessels [9] were removed, chilled on ice, and the contents were transferred to ultracentrifugation tubes [6]. After centrifuging at 100,000 *g* for 1 hr, the supernatants containing the soluble components were dialyzed in dialysis bags at 4° for 36 hr against 0.1 M ammonium acetate buffer, pH 6.5. After dialysis, an aliquot was taken for determination of the remaining concentration on polyadenylic acid by spectrophotometry at 260 nm [6]. Subsequently, the whole was lyophilized and subjected to enzymic hydrolysis and chromatography.

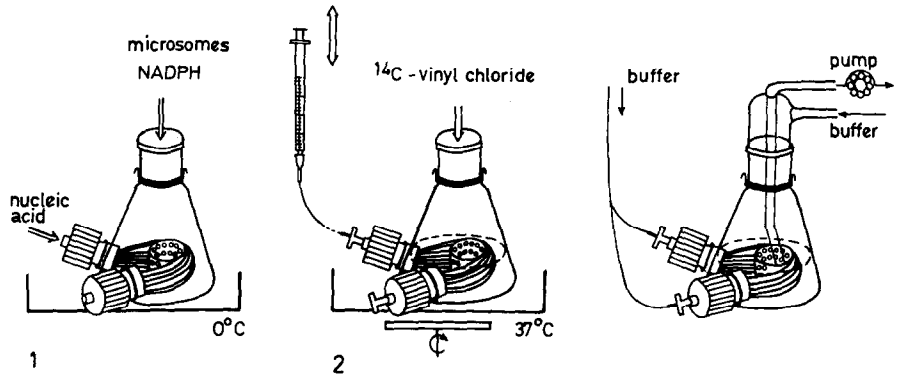

Fig. 1. Alkylation of nucleic acid by microsomal metabolites of [^{14}C]vinyl chloride (method B). (1) Introduction of the reaction components into the vessels. (2) Incubation under [^{14}C]vinyl chloride. (3) Diafiltration to remove the unbound material.

Method B. Incubations were performed in the vessels shown in Fig. 1; these vessels were connected to our incubation apparatus [9]. Microsomal biotransformation and the binding reaction were separated by polyamide molecular sieves. The 'nucleic acid solution' (2 mg polyadenylic acid per ml Tris/KCl buffer consisting of 4 parts 0.15 M KCl and 1 part 0.25 M Tris–HCl, pH 7.4) was filled into capillary membrane fascicles (4 ml inner volume). The membranes used (interchangeable capillaries for miniconcentrator BMU, Fa. Berghof, Tübingen-Lustnau) were asymmetric polyamide membranes pretreated with diazomethane. The membrane's skeleton was 200 μm thick and the active separation membrane was 0·5 μm thick. Membrane surface at 4 ml inner volume was 150 cm^2, the

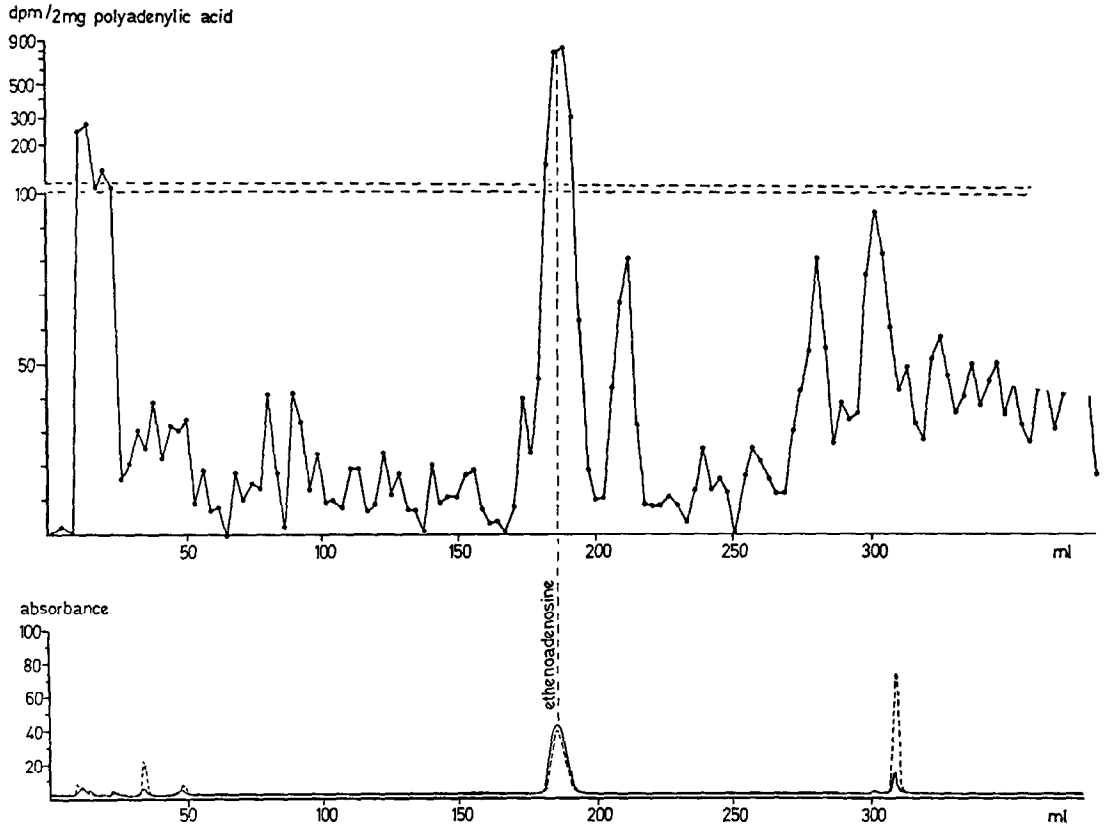

Fig. 2. Ion exchange chromatogram of hydrolysate of polyadenylic acid, incubated with rat liver microsomes, NADPH and [1,2-^{14}C]vinyl chloride (method A). Elution is photometrically recorded at 254 nm (– – –) and 280 nm (——). Absorbance is given in arbitrary scale units of the recorder. Radioactivity and optical peak of 'carrier' 1,N^6-ethenoadenosine (at 186 ml) are superimposed. Non-radioactive adenine is eluted at 325 ml.

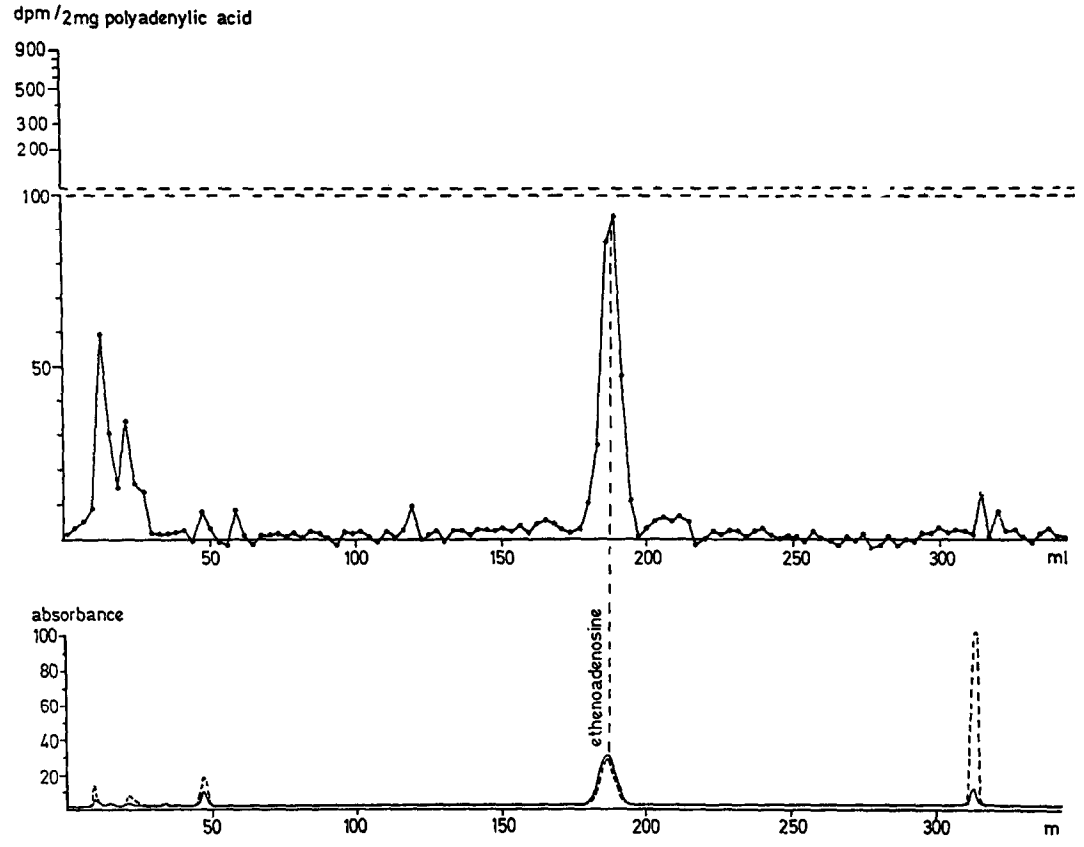

Fig. 3. Analysis of hydrolysate of polyadenylic acid after incubation according to method B. All other experimental details are as indicated in Fig. 2.

exclusion limit was 10,000 daltons. The fascicles (see Fig. 1) were bent around a perforated plastic ring which contained the stirring magnet [9] for the microsomal incubation. The whole was placed in an Erlenmeyer flask of 100 ml vol. After introduction of the 'nucleic acid solution' (while chilling on ice), 40 ml of ice-cold microsomal incubation mixture, containing the NADPH-regenerating system (see method A), was given to the flask. After connecting the flask with the [^{14}C]vinyl chloride containing atmosphere [9], reaction was started by heating to 37°. By means of a syringe connected to one of the lateral outlets (Fig. 1), alternating positive and negative pressures could be applied to the fascicles, thus facilitating fluid exchange between both compartments.

After 45 min of incubation the vessels were removed. Immediate diafiltration in the same system followed. The microsomal suspension was removed from the vessels and replaced by ice-cold dialysis buffer (0.2 M ammonium formate/0.02 M formic acid, set to pH 7.0 with conc. ammonia). A peristaltic pump (Fig. 1) supplied the system with fresh buffer to wash out all the removable metabolites. After diafiltration (3 hr at 4°) the contents of the membrane fascicles were removed (Fig. 1). An aliquot of the solution thus obtained served for determination of the remaining polyadenylic acid (spectrophotometry at 260 nm); subsequently, the whole was lyophilized and subjected to hydrolysis and to chromatography.

The 'nucleic acid' (polyadenylic acid) was hydrolysed by a two-step procedure in which endonuclease and exonuclease/phosphatase were used successively. This procedure has already been described in detail [6, 7]. After hydrolysis, 50 μl 10 mM 1, N^6-ethenoadenosine (Serva, Heidelberg) was added as a non-radioactive optical marker for the expected radioactive 1,N^6-ethenoadenosine. The subsequent chromatography of the nucleosides using Aminex A-6 columns has been previously described [6, 7, 10]. As the present procedure of hydrolysis [6] transformed adenosine into adenine (but not 1,N^6-ethenoadenosine into 1,N^6-ethenoadenine), the major peaks eluted from the column (Figs. 2 and 3) were 1,N^6-ethenoadenosine (radiolabeled and optically monitored) and adenine (optically monitored, non-radioactive).

Comparison of the chromatograms obtained using both methods of incubation (Figs. 2 and 3) shows that both procedures lead to formation of labeled 1,N^6-ethenoadenosine moieties in polyadenylic acid on rat liver microsomal incubation with ^{14}C vinyl chloride. Besides some radioactive material not completely hydrolysed by the enzymic procedure which appears in the breakthrough volume, ethenoadenosine is the only major radioactive peak. However, the insertion of the semipermeable membrane between the metabolizing microsomes and the nucleic acid target leads to a reduced formation of the alkylation product (ethenoadenosine) which is about 10 per cent that obtained with method A. The real advantage of method B is that it rules out the problems of recovery of the alkylated nucleic acid after incubation. Whilst using method A only 45.6 ± 5.7 per cent ($\bar{x}$ ± S.D.; 4 independent determinations) of the polyadenylic acid which was applied to the incubation could be recovered, with method B 86.0 ± 1.7 per cent ($N = 3$) was re-isolated. Single experiments with polycytidine and with DNA as targets confirmed the doubling of recovery by using method B. As in the past we often faced considerable difficulties in analysing DNA adducts from *in vitro* experiments, we expect that the new method in some cases may offer an advantageous alternative.

Also, this method presents a possibility to obtain alkylated nucleic acids in their native form, without contaminations, which may be suitable for further investigations, e.g. for translation experiments with alkylated RNA. The

use of *in vitro* methods to obtain nucleic acids alkylated with metabolites or labeled xenobiotics has a définite advantage over *in vivo* and organ per fusion methods, in that much lower amounts of radioactivity are necessary for an individual experiment.

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Institut für Toxikologie,
Universität Tübingen,
Wilhelmstr. 56, D-7400
Tübingen 1, F.R.G.

REINHOLD J. LAIB
HERMANN M. BOLT*

*Reprint requests should be directed to H.M.B. at his present address: Pharmakologisches Institut der Universität, Abteilung Toxikologie, Obere Zahlbacher Str. 67, D-6500 Mainz, F.R.G.

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An assay for alpha-adrenergic receptor subtypes using [^{3}H]dihydroergocryptine

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In 1948, Ahlquist [1] made the initial demarcation between alpha- and beta-adrenergic receptors. Subsequently, two subtypes of beta [2] and alpha [3, 4] receptors have been identified. A number of drugs have been proposed to discriminate in physiological experiments between these two alpha receptor subtypes by virtue of a relatively greater affinity for one or the other alpha receptor subtype [5–7]. We have recently described a method for quantitatively determining the alpha-adrenergic receptor subtypes using computer modelling of competition curves of prazosin with the non-selective antagonist [^{3}H]dihydroergocryptine ([^{3}H]DHE) [8]. Prazosin was found to be ~ 10,000-fold more potent at alpha$_1$ receptors, whereas yohimbine was ~ 500-fold more potent at alpha$_2$ receptors in rabbit uterus [8].

We now propose and validate a new and simpler method for quantifying the alpha-adrenergic receptor subtypes. This method has the advantage of not requiring complex computational techniques.

Rabbit uterine membranes were prepared and binding assays were performed as described previously [9]. Prazosin was a gift from Pfizer Inc., New York, NY. In each experiment a competition curve of [^{3}H]DHE (present at a concentration of ~ 5 nM) by prazosin was constructed; in the same membrane preparation, [^{3}H]DHE saturation curves (1–15 nM) were performed in the presence and the absence of a fixed concentration of prazosin (10^{-7}M). The data from the saturation curves were subjected to Scatchard analysis [10] to obtain estimates of the number of alpha receptor sites in the presence of prazosin (R_{alpha_2}) and the absence of prazosin (R_{total}). The difference between R_{total} and R_{alpha_2}, ($R_{total} - R_{alpha_2}$), was taken as an estimate of the number of alpha$_1$ receptors (R_{alpha_1}). Independent estimates of the proportion of alpha$_1$ and alpha$_2$ receptors were also generated by the computer modelling of prazosin competition curves. The computer modelling has been described previously in detail [8].

Briefly, using a PDP 11/45 computer, data were analyzed by a nonlinear least squares curve fitting technique [11] using a generalized model for complex ligand–receptor interactions [12] to determine the proportion of alpha receptor subtypes present in the prazosin competition curve, and to determine the number of alpha receptors in each of the saturation curves. The deviation of the observed data points from the predicted values was weighted according to the reciprocal of the predicted variance [13]. The computer simulations were based on the above computer techniques. All experiments were performed in duplicate.

The basic premises underlying the method to be described here include the following: (1) the affinity of prazosin for alpha$_1$ receptors ($K_{D_{alpha_1}} \sim 5 \times 10^{-10}$M) is so much higher than its affinity for alpha$_2$ receptors ($K_{D_{alpha_2}} \sim 5 \times 10^{-6}$M) [8] that, when it is present at a concentration of 10^{-7}M, essentially all the alpha$_1$ and virtually none of the alpha$_2$ receptors will be occupied by prazosin; (2) the binding of prazosin to the alpha$_1$ receptors is so tight that over the usual range of [^{3}H]DHE concentrations used in constructing saturation curves (1–15 nM) prazosin will not be displaced from the alpha$_1$ receptors; and hence (3) in the presence of 10^{-7}M prazosin, [^{3}H]DHE saturation curves will measure only the alpha$_2$ receptors in uterine membranes.

Computer simulations were done to test the validity of these premises. For the purposes of computer simulation, typical experimentally determined affinities of prazosin and [^{3}H]DHE were assumed: for prazosin these were $K_{D_{alpha_1}} = 5 \times {}^{-10}$M and $K_{D_{alpha_2}} = 5 \times 10^{-6}$M; and for the non-selective radioligand [^{3}H]DHE [9] the affinities were $K_{D_{alpha_1}} = K_{D_{alpha_2}} = 5 \times 10^{-9}$M. Also, the proportions of alpha receptor subtypes were set at alpha$_1$ = 20 per cent and alpha$_2$ = 80 per cent. The simulations revealed that even at the highest [^{3}H]DHE concentrations used in actual experiments (15 nM), prazosin filled more than 97 per cent of the alpha$_1$ sites and less than 5 per cent of the alpha$_2$ sites. Thus, the computer simulations substantiate the basic assumptions of the 'double' saturation curve or Scatchard plot technique proposed here.

Fig. 1 illustrates the results of experiments with the same membrane preparation, wherein both a detailed prazosin competition curve (Fig. 1A) and [^{3}H]DHE Scatchard plots