Rotterdam, 11th June 1982

To : K. Beutel - Horgen
cc : L. Rinzema - Horgen
C. Loefgren - Horgen

Re : VINYL CHLORIDE TOX STUDIES

In addition to my telex of April 26 1982 and the Shell letter of May 11 (CMCF/1) I am coming back to the above subject.
Our 1980 committment to pay 50.000 Dfl. to Shell as Dow's contribution to the project has not yet resulted in an invoice, but I think this will come soon.
The progress of the Shell studies has been slower than expected. The main reason was that the major adduct, 7-(2'oxoethyl)guanine did not produce detectable levels of antibodies in rabbits. Therefore another detection method had to be developed. In this method an unknown quantity of unlabeled adduct is spiked with ^{14}C-labeled adduct. A subsequent reduction of the adducts involves the incorporation of tritiated sodium borohydride. The purified reduced material is counted for ^{3}H and ^{14}C. The ratio $^{3}H/^{14}C$ will then give the amount of adduct in the sample. The method involved in identifying adducts will be at our disposal if we continue over and beyond the 50.000 Dfl. to participate in this project.

In the particular case of vinyl chloride the method will enable the detection Of the amount of DNA adducts, if any, at levels that are not tumorigenic in rats, with a sensitivity of less then 1 adduct in 10^{7} DNA molecules.
If at no-tumor levels there are no DNA adducts either, we can make a significant step ahead in dealing with a possible no effect level for carcinogens.

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In the case of vinyl chloride this would be important for the food packaging materials made of vinyl chloride.
But beyond vinyl chloride these methods could be useful for other foodpackaging materials.

I realise that a request for sponsoring may come at a bad time for a product that has no great interest for Dow Chemical Europe. Nevertheless I request that you as product steward discuss this proposal with the appropriate product business team. Our colleagues in the USA may be willing to consider to take over from us this additional sponsoring, because of more business interest and the methodology involved. I therefore recommend that you also contact Berny Schwetz and the USA product steward for vinyl chloride.

In reviewing the proposal one should consider that the other 3 sponsors, AKZO / DSM and Shell have equally bad times in the plastic business and some moral obligation in these circumstances can not be denied.
The appropriate amount for additional sponsoring would be 50 - 75.000 Dfl. or 20 to 30 thousand dollars.

I would appreciate an answer from you before August 1.

Regards,

H.G. Verschuuren

result DCE is not interested in further sponsoring

Encl. 1. telex of April 26
2. proposal by Shell

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2nd Interim Report

Molecular Dosimetry Studies with Vinyl Chloride Monomer

The objectives of the study are stated in the Protocol, dated 14th October, 1980, and were re-stated in the 1st Interim Report, dated 16th June, 1981. This report addresses the points, listed on page 5 of the Protocol, covering the procedure to determine the quantitative relationship between Vinyl Chloride exposure and DNA dose in rats in the CIVO study. Progress to date is summarised below.

1) Synthesis of deoxyriboside - vinyl chloride adducts

The major adduct, 7-(2'-oxoethyl)guanine, and the two minor ones, 1,N^6-ethenodeoxyadenosine and 3,N^4-ethenodeoxycytidine, have been chemically synthesised and structurally characterised.

2) Development of an hplc method to separate the adducts

Conditions have been developed using reversed-phase hplc under which the three adducts are resolved from each other and from the four natural deoxyribosides present in a DNA hydrolysate.

3) A high dose pilot study using ^{14}C-labelled vinyl chloride

This was not carried out for two reasons:

a) ^{14}C-Vinyl chloride of high specific activity is extremely expensive to produce, and its stability is not guaranteed.

b) At the start of this study, Professor Bolt from the University of Mainz published the results of an experiment very similar to the one which we were planning, which gave us the results we required.

4) Development of methods to assay DNA - vinyl chloride adducts in tissues of exposed animals

The main criteria for such assays are sensitivity and specificity. The level of DNA alkylation which can be detected following administration of a ^{14}C-labelled carcinogen to a mammal is of the order of 1 adduct in 10^7 nucleotides, which gives an idea of the sensitivity required for the assays under development. The analytical method chosen to develop assays for the three DNA adducts of vinyl chloride is radioimmunoassay. The principle of this technique and the steps involved in its development are outlined in the 1st Interim Report.

Since the DNA adducts are small molecules, they must first be bound to a carrier macromolecule in order to stimulate antibody production when injected into animals. The two minor adducts were both successfully bound to bovine serum albumin, a carrier protein, by application of a standard method. A modification of the standard method had to be developed to bind the reduced (stable) form of the major adduct to a carrier protein. This was successfully achieved, and conjugates of the reduced major adduct

to two carrier proteins, bovine serum albumin and keyhole limpet haemocyanin, and to a third carrier, activated CH-Sepharose 4B, were obtained. The three adducts were also prepared in tritium-labelled form as a requirement for the radioimmunoassay procedures.

The conjugates of the two minor adducts, and the three conjugates of the reduced major adduct were injected into rabbits in order to produce antibodies. Antibodies against the two minor adducts were produced in substantial quantities in a matter of weeks. These antibodies were characterised for sensitivity, avidity of binding to substrate and cross-reactivity in a radioimmunoassay procedure. A modification of the radioimmunoassays for the two minor adducts, called ultrasensitive enzymatic radioimmunoassay was investigated, but didn't result in any increase in sensitivity. The levels of sensitivity achieved with radioimmunoassays for these two minor adducts are such that in 10 mg DNA isolated from the tissues of exposed animals we can expect to detect one molecule of ethenodeoxyadenosine in 3×10^8 nucleotides and one molecule of ethenodeoxycytidine in 1.2×10^9 nucleotides. The sensitivities of both these assays can possibly be increased by 1-2 orders of magnitidue using ^{125}I-labelled substrates which we have recently synthesised. Two recently-published studies have described experiments in which ^{14}C-labelled vinyl chloride was administered to rats[1] and mice[2]. In the rat study neither of the two minor adducts was detected in liver DNA. The limit of sensitivity for this study can be calculated at one molecule of adduct in 1.5×10^7 nucleotides. In the mouse study tentative identification of ethenodeoxyadenosine was made in liver DNA at a level of one molecule in 2.4×10^6 nucleotides.

Injection of the three conjugates of the reduced major adduct into rabbits did not produce detectable levels of antibodies directed against this adduct. This result was completely unexpected. A possible reason is that on injection into rabbits, the slightly basic physiological conditions caused a chemical modification of the adduct. If this were the case then any antibodies raised would have been produced in response to the chemically modified adduct.

It was thus apparent that the radioimmunoassay technique could not be applied to the major adduct and that an alternative assay would have to be developed, leading inevitably to a delay in the progress of the study. The assay currently being developed is based on a radiolabelled derivatisation procedure. The sample, containing an unknown amount of the adduct is 'spiked' with a known amount of ^{14}C-labelled adduct of high specific activity. The mixture of labelled and non-labelled adduct is reduced with tritiated sodium borohydride, a reaction which will introduce tritium into both species. The reduced material is purified to constant specific activity and counted for ^3H and ^{14}C. The $^3H/^{14}C$ ratio will give the amount of adduct in the sample. In theory this method will enable us to detect one adduct in 1.5×10^7 nucleotides. In the rat study[1] mentioned above 7-(2'-oxoethyl)guanine was detected in liver DNA at a level of approximately 1 adduct in 7.5×10^5 nucleotides. It is anticipated that a further 2-3 months of effort will be required to develop this assay to

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a stage where it can be applied to samples from the CIVO study.

5) Collection of samples from the CIVO study

Samples of liver, lung, brain, kidney and testis taken after six weeks, nine months and eighteen months from rats in all exposure groups in the CIVO study have been collected. These are currently being stored at -80°C in Shell Toxicology Laboratory pending assay.

References

1. R.J. Laib et al, Chem.-Biol. Interactions, 37, 219 (1981).

2. K. Bergman, Arch. Toxicol. 49, 117 (1982).

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STL, 22nd April, 1982

Proposal for Project Finalisation

Objectives/Justification

The aims of the studies described in this proposal are to bring the current project to an expeditious and satisfactory conclusion in respect of the principal objectives of the project. These objectives are as follows:-

1. To determine the quantitative relationships between exposure dose of vinyl chloride and primary DNA damage (amounts of DNA adducts) in the target (liver) and in selected non-target tissue(s) of rats.

2. To determine the quantitative relationships between primary DNA damage and carcinogenic response in rats (second CIVO/TNO study).

The results of these studies would directly address important issues such as the question of thresholds in respect of the induction of primary DNA damage and also in respect of the development of neoplasia as a response to this DNA damage. These studies (including comparisons of DNA alkylation in liver and a non-target tissue(s)) may also yield important insights into the mechanism of action of vinyl chloride in rat liver, e.g. the results may signify whether or not factors other than DNA alkylation are operative in determining the response of rat liver to this chemical.

Experimental Programme

The proposed programme comprises three discrete studies to be conducted in the following sequence:-

1. Completion of Method Development

The development phase of the project will be completed by providing a method for the assay of the major DNA adduct (7-(2'-oxoethyl)guanine). The method is currently being developed and is based on double-radiolabelled derivatisation procedures. It is anticipated that 0.3 dmy will be required for the completion of the assay. (Immunochemical assays for the two minor DNA adducts (see 2nd Interim Report) have been developed). This aspect of the work will be reported to the sponsors during August, 1982.

2. Pilot Study

The implementation of this study is dependent upon the satisfactory completion of item 1. Prior to commiting the valuable CIVO/TNO samples to analysis, it is important to gauge whether or not the amounts of DNA adducts in the livers of the CIVO/TNO rats are likely to be below the threshold for detection using the methods developed/described in item 1, above. Therefore, a pilot study is proposed in which vinyl chloride will be administered at a dose of 1.7 mg Kg^{-1} body weight to a group of five male rats for five days by oral intubation. (The need or desirability of conducting analogous studies at lower or higher exposures will be reviewed at the appropriate time). Following exposure, the rats will be killed, livers excised and liver DNA isolated. The liver DNA from the exposed

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animals will be assayed for the three adducts.

An estimated 0.1 dmy will be required for this aspect.

3. Application of Methods to the CIVO/TNO Samples

Following successful application of the three assays to the pilot study, the CIVO samples will be assayed. The first tissues to be examined will be livers from the high dose animals for all three exposure times. Assuming that detectable levels of adducts are found, then liver samples from the other two dose groups will be assayed. The feasibility of assaying other (non-target) tissues for DNA adducts will depend on the levels found in the liver.

Accurate estimates of effort are dependent upon information to be gleaned from item 1. However, it is anticipated that all liver samples and a representative number of non-target tissues from the CIVO study can be assayed for the three DNA adducts using 0.6 dmy effort.

It is therefore estimated that the project can be concluded in 1982 in respect of the principal objectives with the expenditure of 1.0 dmy of effort.

STL, 22nd April, 1982

Toxicological studies with Vinylchloride

When it became known that vinylchloride (VC) was the suspected agent to cause liver cancers (angiosarcoma's) not only in experimental animals but also in humans occupationally exposed to VC by inhalation, limits were introduced to safeguard health. These included limits for food packaging materials and foodstuffs themselves although VC had not been tested orally.

A feeding study was then carried out at CIVO-TNO to attempt to establish a no toxic effect level (NTEL) for regulatory purposes. The study was initiated by the German Plastics Producers Industry federation (VKE) who invited other than German Companies to co-sponsor. AKZO, DOW, DSM and Shell joined. This study failed to yield a NTEL and the VKE had to commit themselves to a second study to avoid the introduction of very stringent regulations on PVC by German authorities. CIVO-TNO again was contracted for the second study and VKE invited the same sponsors to participate.

This second study, however, would be with much lower dose levels and the number of animals could not realistically be increased to take account of the statistical requirements. This, taken together with the fact that a "no effect level" for a carcinogen was not accepted in principle by certain scientific bodies, prompted us to propose a DNA alkylation dosimetry study to establish the non-lineairity of the dose-response relation i.e. the existence of a practical no effect level. This study would at the same time be of much wider interest in that it would help the case against the "one hit theory". The proposal was supported by AKZO, DOW and DSM but not by VKE who only intended to fulfill the commitment towards their regulatory authorities. It was agreed that two sponsor groups would be formed to conduct the studies independently. The results would be exchanged on the basis that costs would be similar.

Since the start of the dosimetry study two interim reports have been produced. Progress was not as easy as envisaged and costs are higher than originally estimated. For those reasons it was decided to review the situation and discuss it with the sponsors. The reasons in favour of proceeding with the study even though the costs will be substantially higher are as follows:

1. The work has advanced to a stage that the methods can soon be applied to obtain the actual results (two thirds).

2. The subject is of high scientific interest in that it complements other work at reputed toxicology research institutes. It should yield new insights on finalisation.

3. The results should be of commercial value: Industry will be in a much better position to negotiate acceptable levels of residual VC in PVC for food packaging with regulatory authorities.

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4. The work is complementary to the CIVO-TNO lifetime animal feed studies. Exchange will give us access to the results of the CIVO-TNO work.

5. The study will have interesting side benefits. If required it will be possible to develop a biological monitoring method for workers exposed to VC.

6. On a more general basis it is our Companies' contribution to the pool of knowledge on VC toxicology to which the first important input came from the sponsors of the Maltoni studies. (Italian, French, Belgian and UK PVC producers.)

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SICM, 6th May, 1982

Molecular dosimetry studies with vinylchloride

Costs

	£	@	N.fls.
Original project cost estimate	84000	4.1	345000
STL reported actual costs 1980	38000	4.62	175600
STL reported actual costs 1981	66000	5.04	332600
CIVO-TNO costs 1981			62800
Total to end 1981			571000
Estimated costs to complete the programme	50000	4.77	238500
Total for project			809500

SICM, 6th May, 1982

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