Oxidation of Cyclophosphamide by Means of $KMnO_4$

After reporting the successful preparative separation of the dominant urine metabolite of cyclophosphamide from different alkylating agents[1-4], we found further information concerning the major metabolic pathway of this antitumor agent. Careful oxidation of cyclophosphamide by means of $KMnO_4$ yielded a product which is identical with the dominant urine metabolite of the rat and with a synthetic product the structure of which is well known.

Methods. For supplying us with the necessary amounts of cyclophosphamide and the synthetic product N,N-*bis*-(2-chloroethyl)-diamidophosphoric acid (2-carboxyethylester), we are obliged to Prof. Dr. N. BROCK, Asta-Werke AG, Brackwede. The 4-(*p*-Nitrobencyle)pyridine was obtained from Dr. TH. SCHUCHARDT GmbH, Munich.

Oxidation of cyclophosphamide in vitro was carried out using 280 mg ($10^{-3}M$) dissolved in 30 ml aqua bidest. By adding of 0.1 *N* HCl this solution was adjusted to a pH-level of 2.5 and kept at 4 °C until the added quantity of 210 mg $KMnO_4$ was consumed. Any residual MnO_4 was separated by repeated filtration. The unmodified cyclophosphamide was extracted with chloroform; all other compounds remaining in the aqueous phase were dry frozen. The separation was effected by thin layer chromatography on aluminium foils with silica gel (Merck, reagent No. 5553/0025) utilizing a mixture of chloroform, methanol, 0.1*N* H_2SO_4 (60:35:5) as solvent. After cutting off and developing the side-stripes according to[5], the major alkylating fraction was extracted from the silica gel with methanol. The solvent was evaporated in vacuo at 22 °C. Then 1.0 ml methanol was added and the solution was given to the following thin layer chromatography systems:

I. Chloroform, methanol, 0.1*N* H_2SO_4 (60:35:5). II. Chloroform, methanol, acetic acid 96% (90:24:6). III. Cyclohexan, chloroform, methanol, acetic acid 96% (10:75:10:5). IV. Ethyl acetate, acetone, tetrahydrofuran, H_2O (40:20:20:20). V. Butanol, water saturated. VI. Chloroform, methanol, formic acid (35:35:10).

R_{st}-values which were identical for both compounds investigated. In further investigations the same values were found concerning the synthetic product N,N-*bis*(2-chloroethyl)-diamidophosphoric acid (2-carboxyethylester). In the systems V and VI the common R_{st}-values were 1.40 and 1.25 if *Bis*-(2-chloroethyl)-amin was used as reference substance. Therefore it is demonstrated by comparative examination of the product, which was synthesized in our suggestion, that our repeatedly reported hypothesis postulating a preferential oxidation on C_4 of cyclophosphamide[1,6-8] was correct. As indicated in the Figure, the products of in vitro and in vivo oxidation correspond with regard to their rate of spontaneous heterolytic passage in non alkylating derivatives. These rates, depending upon the kind of buffer solution employed and the pH-level, confirm our earlier statement that the metabolite is less

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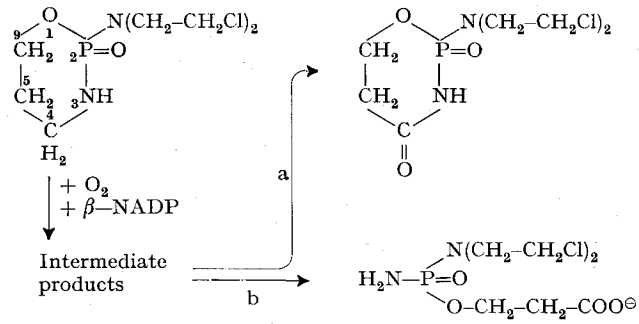
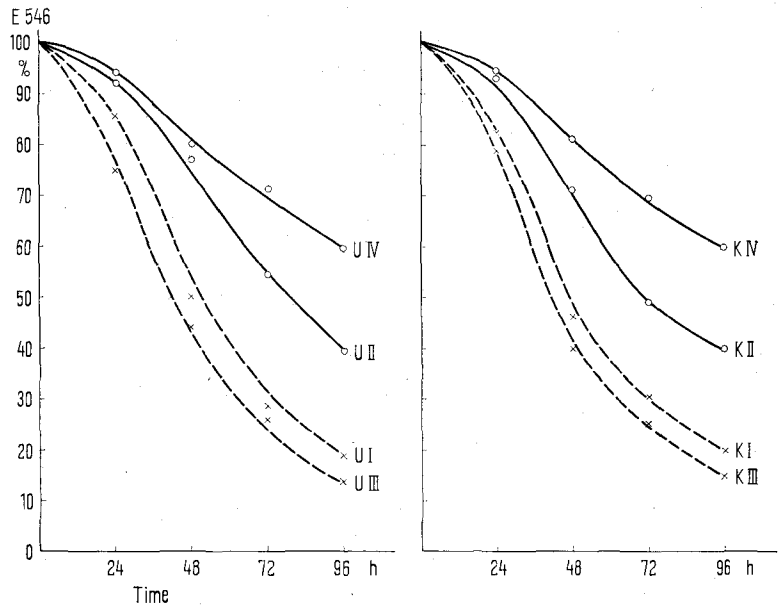


Biological transformation of cyclophosphamide by $NADPH_2$-dependent hydroxylation. a) minor metabolic pathway. b) major pathway.

Methodical procedures used to get large amounts of urine from rats treated with cyclophosphamide were described earlier[1,2]. The metabolite was separated in the same way as the $KMnO_4$-oxidation product. The stability of both oxidation products in different buffers was examined during a 90 h period of incubation in a water bath at 37 °C by utilizing the NBP-reaction. Prior to the test, all preparations were adjusted to an uniform NBP-extinction of ca. 8000 as measured by Eppendorf's photometer at 546 nm.

Results and discussion. The Table shows the R_{st}-values of the major metabolite and the oxidation product as ascertained in 4 thin layer systems. Cyclophosphamide served as the reference substance for the determination of the rapidly transformed by hydrolysis than the analogous open-ring compound of cyclophosphamide, as synthesized and reported by ARNOLD and BOURSEAUX[9]. As shown in the Formulae we assume a second metabolic pathway in accordance with the experimental data of HILL et al[10] and BENCKHUIJSEN et al.[11]. Since both derivatives, the 4-Keto-cyclophosphamide and the carboxylic open ring compound are non toxic[3,4,6] the effect of cyclophosphamide must depend on another metabolite. Presently we are examining the question as to wether this is a precursor of the known products. This problem may be solved and also the metabolism of new analogous compounds of cyclophosphamide may be clarified not only by in vivo investigations but also by methods of in vitro oxidation.



Decrease of an initial NBP-extinction of 8000/ml (=100%) in buffer solutions containing the major urine-metabolite of cyclophosphamide (U), respectively, the product of in-vitro oxidation (K). Incubation-temperature 37 °C. I and II ethylene-diamine-acetate buffer (I pH 6,15; II pH 7,15). III and IV: Sörensen phosphate buffer (III pH 6,2; IV pH 7,2).

R_{st}-values of the major urine metabolite of cyclophosphamide and a cyclophosphamide derivative received by $KMnO_4$-oxidation. Reference substance: Cyclophosphamide

System	I	II	III	IV
Urine metabolite	0.46	0.70	0.52	0.45
$KMnO_4$-product	0.46	0.70	0.52	0.44

Zusammenfassung. Nach Oxidation von Cyclophosphamid mit $KMnO_4$ lässt sich eine Verbindung gewinnen, die dünnschichtchromatographisch und reaktionskinetisch mit dem vorherrschenden Urinmetaboliten übereinstimmt. Es handelt sich um N,N-bis(2-chloräthyl)diamidophosphorsäure(2-carboxyäthylester). Da weder diese Verbindung noch das bereits als Nebenmetabolit bekannte 4-Keto-cyclophosphamid zelltoxisch wirkt, muss der Effekt des Cyclophosphamids von einem anderen Stoffwechselprodukt ausgehen. Durch modifizierte oxidative Umsetzung in vitro sollte es möglich sein, Cyclophosphamid auch in den wirksamen Metaboliten zu überführen.

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Choline Acetyltransferase and Acetylcholinesterase in Spinal Motor Neurons Cultured in vitro

Recent tissue culture studies on central nervous tissue have demonstrated the continued differentiation and maturation of the tissue in vitro evidenced by formation of synapses and myelin sheaths[1,2]. The development of synaptic connections in tissue culture was observed by silver impregnation method[3,4] and by electron microscopy[2,5]. The operation of functional synapses in such cultures was also demonstrated by the neurophysiological method[6]. Since the presence of acetylcholinesterase (AChE) in central nervous structure is regarded as an indication of cholinergic transmission in that area[7], we have studied previously the localization of AChE in neurons of mouse cerebellum and in motor neurons of chick spinal cord cultured in vitro[8,9]. The presence of acetylcholine (Ach) or choline acetyltransferase (ChAc) is generally regarded as being a more reliable indicator of cholinergic mechanisms than is the presence of AChE[7]; hence, it is more desirable to demonstrate ChAc activity in neuronal elements of the cultures in order to provide further evidence of the operation of cholinergic transmission in central nervous tissue maintained in vitro.

Cross sectioned explants from cervical level of 10–12-day chick embryo spinal cords were placed on collagen coated coverslips and maintained in Maximow's slides. The feeding medium composed of equal parts of horse serum, Hanks' balanced salt solution (BSS) and medium-199. Supplementary glucose was added in the final concentration of 600 mg percent. Cultures were incubated at 36 °C for 7–180 days. Twice a week the cultures were washed briefly in BSS and fed with fresh feeding medium.

For the histochemical demonstration of ChAc activity, Kása, Mann and Hebb's method[10] was used in modified conditions. The incubating mixture contained the following: acetyl CoA, 0.3 mM; choline chloride, 10.0 mM; lead nitrate, 0.3 mM; cacodylate buffer (pH6.0), 33.0 mM; and BW 284C51, 0.01 mM. The inclusion of BW 284C51, a specific inhibitor for AChE, is to prevent the hydrolysis

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