INVESTIGATION OF DECOMPOSITION OF ALKYLATING MUTAGENS OF THE ETHYLENIMINE SERIES IN HUMAN LYMPHOCYTE CULTURE

O. P. Kirichenko, A. N. Chebotarev, and K. N. Yakovenko

UDC 612.112.94-085.23:612.6.052

A simple method of determining the concentration of alkylating compounds in a culture medium with a high level of reproducibility is described. Thiophosphamide was shown not to decompose during 24 h either in the culture mixture or in a culture of human lymphocytes. Phosphemid, dipin, and fotrin underwent appreciable decomposition in culture in the course of 24 h, but these substances likewise were not decomposed during the first 6 h.

KEY WORDS: mutagenesis; ethylenimines; human lymphocytes.

Data on changes in the concentration of mutagens with time in experiments in vitro and in vivo are important for a correct understanding of the mechanisms of mutagenesis. However, these relationships are particularly important during the extrapolation of results obtained in experiments in vitro and on animals to processes taking place in vivo and in man, and also for testing chemical compounds for mutagenic activity. When human lymphocyte cultures are used as the test system, application of a test substance may be of short or long duration. To test the suitability of the various test objects, substances known to be mutagens are added in order to record the quantitative characteristics of these systems. Very often alkylating compounds, especially derivatives of the ethylenimine series, are used as such mutagens. The longer the contact between cells and mutagen, the greater the effect observed [3]. However, the longer the exposure, the more likely, it seems, is the mutagen to decompose by hydrolysis or through decomposition by the cells [2, 6]. The concentration of thiophosphamide and cyclophosphamide in vivo is known to fall very rapidly [4] and alkylating compounds are known to be broken down in the blood serum at different rates, characteristic of each substance [5]. Accordingly, when testing for mutagenic activity it is important to know the rate of decomposition of the substance used in the particular test system.

In the investigation described below the rate of change of concentration of thiophosphamide, phosphemid, dipin, and fotrin in human lymphocyte cultures was studied.

EXPERIMENTAL METHOD

The concentration of the alkylating substances in the culture medium was determined as follows. To 1 ml of the sample in a centrifuge tube 0.4 ml methanol, 1 ml of a 5% solution of zinc sulfate, 2 drops of phenolphthalein solution, and the equivalent amount of a saturated solution of barium peroxide were added. The contents were mixed and centrifuged (TsUM-1 centrifuge) for 10 min at 6000 rpm. The supernatant was then completely decanted into a glass centrifuge tube, treated with 0.1 ml molar acetate buffer (pH 4.6) and 0.2 ml of a 5% solution of 4-(p-nitrobenzyl)pyridine in acetone, mixed, and placed for 20 min in a boiling water bath. The tubes were then cooled on ice (the samples can be kept for a relatively long time on ice). To each tube were then added 0.6 ml acetone, 3.5 ml ethyl acetate, and 0.6 ml of 1 N NaOH solution. The contents were quickly shaken and centrifuged (TsLK-1 centrifuge) for 1 min at 3000 rpm. The top colored layer of ethyl acetate was drawn off into a cuvette and examined in the FÉK-56M photoelectric colorimeter with a No. 6 green filter (λ = 540 nm). Photometry was carried out during 3 min after the addition of the NaOH solution because of the rapid decomposition of the colored reaction product.

Laboratory of Mutagenesis, Institute of Medical Genetics, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. V. Zakusov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 81, No. 5, pp. 552-553, May, 1976. Original article submitted April 18, 1975.

TABLE 1. Decomposition of Phosphemid, Dipin, and Fotrin in Human Lymphocyte Cultures at 37°C (concentrations of substances 30 μg/ml, optical density values given)

Substance	Time (in h)				
	0	2	4	6	24
Phosphemid	0,247	0,247	0,250	0,245	0,225
Dipin	0,248	0,255	0,245	0,247	0,230
Fotrin	0,187	0,188	0,190	0,200	0,130

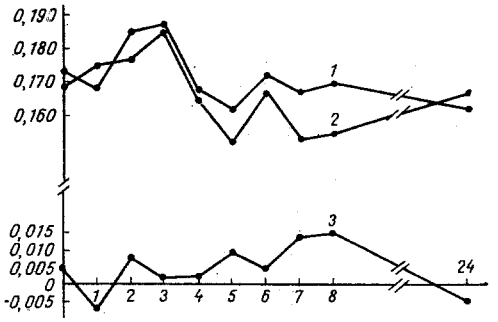

Fig. 1. Changes in concentration of thiophosphamide (10 μg/ml) plotted against incubation time at 37°C: 1) in culture mixture; 2) in lymphocyte culture; 3) difference between 1 and 2. Abscissa, time (in h); ordinate, optical density.

The concentration of alkylating substances was determined in the following culture mixture: Eagle's medium and bovine serum (in the ratio of 4 : 1), and in cultures of human lymphocytes: Eagle's medium, bovine serum, and whole blood (in the ratio of 12 : 3 : 1). Next, 1 ml of a solution of the mutagen in water was added to 9 ml of the culture or culture mixture, so that the concentration of the mutagen was reduced tenfold.

The experimental results were subjected to regression analysis [1].

EXPERIMENTAL RESULTS

The method of determining the concentration of alkylating compounds in the medium described above is less sensitive than methods described previously [5, 7] because of the larger volume of ethyl acetate for extraction of the colored reaction products. At the same time, the method enables the use of specially equipped spectrophotometers with microcuvettes to be dispensed with, and concentrations of alkylating compounds as used in chemical mutagenesis experiments can be reliably determined. The increase in the volume of ethyl acetate for extraction incidentally increases the reproducibility of the results. For instance, when the concentration of thiophosphamide (5 μg/ml in the culture mixture) was determined in eight samples the mean optical density was 0.06875 ± 0.00648, with an accuracy of determination of the mean of 1.7%.

To investigate the decomposition of thiophosphamide in the culture medium and the lymphocyte culture, it was used in a final concentration of 10 μg/ml, whereas for dipin, fotrin, and phosphemid the final concentration was 30 μg/ml. The results of these tests are given in Table 1 and Fig. 1. In each case there were three repetitions. Statistical analysis showed that in every case the change in concentration of the alkylating substances with time could be described satisfactorily by a linear model (in all cases deviation from satisfactory not significant; $P > 0.05$). Thiophosphamide, moreover, was virtually not decomposed for 24 h either in the culture medium or in the lymphocyte culture (the coefficient of linear regression did not differ significantly from 0). Meanwhile fotrin, phosphemid, and dipin were appreciably decomposed in culture during the first 24 h (all coefficients of linear regression were below 0 with a 5% level of significance). However, no appreciable decomposition of these substances likewise took place during the first 6 h ($P > 0.05$).

When experiments on chemical mutagenesis are organized, allowance must therefore be made for the degree of decomposition of the mutagen used in the cell culture during the time that the substance is present

in the culture. The method described can be used to determine easily and quickly the degree of decomposition of alkylating compounds in cell cultures.

LITERATURE CITED

1. N. R. Draper and H. Smith, Applied Regression Analysis, Wiley, New York (1966).
2. L. G. Dubinina, Genetika, 10, No. 11, 129 (1974).
3. O. P. Kirichenko, Genetika, 10, No. 9, 139 (1974).
4. V. S. Moiseenko and V. M. Pivnyuk, Vopr. Onkol., No. 5, 73 (1968).
5. V. A. Filov, Vopr. Onkol., No. 5, 69 (1968).
6. N. P. Bochkov and N. P. Kuleshov, Mutat. Res., 14, 345 (1972).
7. Y. L. Tan and D. R. Cole, Clin. Chem., 11, 58 (1965).