sedimented under gravity, and the leucocyte layer was separated and diluted with minimal essential medium (MEM) and autologous plasma in order to obtain a cell concentration of $5{\cdot}0 \times 10^6$–$8{\cdot}0 \times 10^6$ per ml. with 20 per cent autologous plasma. Cultures (10 ml. each) were set up in rubber stoppered test tubes maintained at 37° C in a rotating system. After periods of incubation with the labelled precursors varying from 30 min to 6 h, the cells were harvested by chilling on frozen saline and centrifuged at 1,200 r.p.m. They were washed once with ice-cold saline. RNA was extracted by a modification[9] of the methods of Scherrer and Darnell[10] and of Kirby[11]. Because of the low number of blast cells used in each experiment, a suitable amount of unlabelled KB cell RNA was extracted simultaneously to act as a carrier. A quantity of RNA (15–30 *A* units) was centrifuged on 5–20 per cent linear sucrose gradients in 0·01 M Tris buffer, *p*H 7·4, at 16,000 r.p.m. for 16 h at 4° C in an '*SW* 25·1' rotor of a Spinco model *L* ultracentrifuge. Forty-five fractions were obtained with a gradient analyser (Isco), and absorption and radioactivity were assayed on alternate fractions[9]. The rate of 5-^{3}H-uridine incorporation was also studied by pipetting at 1 h intervals 200 µl. samples of the cultures in 50 volumes of 5 per cent trichloroacetic acid at 4° C. The precipitate was assayed for RNA radioactivity by a modification[9] of the Schneider[12] procedure.

In all cases studied, irrespective of the cytological type, the results were quite uniform. Incorporation of 5-^{3}H-uridine in acid-insoluble material gave a linear increase up to 7–8 h and decreased slowly afterwards. After short incubation periods (30–60 min) with 5-^{3}H-uridine, most of the radioactivity was associated with RNA with S values higher than 28 and with two main peaks at 32S–35S and 45S–50S (Fig. 1). Furthermore, in most cases, after 60 min incubation, a definite amount of radioactivity was associated with 18S RNA (Fig. 1). These findings indicated that the blast cells were synthesizing and processing ribosomal precursor RNA in a normal fashion, though at a somewhat slower rate. Normal behaviour of ribosomal RNA production would imply that, after longer periods of incubation, most of the radioactive label would be associated with 18S and 28S RNA, and the absorbance and radioactivity profiles would be superimposable. In no case could this be observed. On the contrary, exposure to 5-^{3}H-uridine up to 6 h resulted in an accumulation of radioactivity in the 30S–50S region of the gradient, the peak in 45S becoming extremely prominent (Figs. 1 and 2).

In a study of the distribution of RNA radioactivity after incubation with [^{3}H-*methyl*]-methionine definite peaks of radioactivity were observed at 45S, 32S–28S and 18S, closely corresponding to the peaks observed in the ^{3}H-uridine experiments, thus confirming that synthesis of ribosomal RNA took place in the cells studied. Contrary to the findings in the 5-^{3}H-uridine experiments, it was possible by prolongation of the incubation period to observe a gradual increase in radioactivity in the 32S–28S and 18S peaks, and a proportional decrease in the amount of label associated with the 45S RNA fraction.

We tentatively suggest that in the blast cells of acute leukaemia undermethylated 45S and 32S RNAs accumulate: a substantial amount of the former fails to undergo methylation and cleavage at a normal rate. This conclusion is supported by double-labelling experiments in which blast cells from three of our patients were incubated simultaneously with 5-^{3}H-uridine and [^{14}C-*methyl*]-methionine, and the two isotopes were evaluated in the same RNA fraction. It was observed that the ^{3}H-pyrimidine : ^{14}C-methyl ratio in 45S RNA was increased by prolonging the period of incubation (Fig. 3), thus indicating the accumulation of unmethylated unprocessed RNA molecules.

This investigation was supported by a grant from the Consiglio Nazionale delle Ricerche. We thank Dr Maria Soldati, Farmitalia Research Laboratories, Milan, for a generous gift of KB cells.

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Received January 27, 1970.

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Comparison of the Effects of LSD, Heliotrine and X-irradiation on Chromosome Breakage, and the Effects of LSD on the Rate of Cell Division

Cohen, Marinello and Back[1] (1967) first reported that lysergic acid diethylamide (LSD) increased chromosome abnormalities in human leucocytes. Although some later work failed to support this observation[2–4], others have confirmed that LSD damages chromosomes[5–10]. I have investigated the dose–effect relationship of LSD and the type of damage produced in chromosomes of leucocyte cultures of the marsupial *Potorous tridactylus* (rat-kangaroo), which has a few large chromosomes (2 n = 12 in ♀ and 13 in ♂) that make scoring of damage relatively easy and accurate.

I compared this damage with that caused by X-irradiation in the same cell system, and also by heliotrine, a pyrrolizidine alkaloid which is a dangerous hepatotoxin and chemical mutagen[11–14] which damages chromosomes in plants[15,16] and animals[17].

Methods for setting up cultures and assessing cell division have been described before[17]. Fully oxygenated blood was needed for irradiation experiments, and so, to maintain comparable conditions, blood for all experiments was first oxygenated for 30 min[17]. Whole blood was then irradiated (22° C) with 25 r./min (HVL, 0·5 mm Cu; 235 kV peak).

LSD ('Dylisid', Sandoz) was added when the cultures were set up. The smallest dose (0·4 µg/ml.) was chosen on the assumption that 2,000 µg (among the larger doses taken by LSD users) was completely absorbed into the blood stream.

Table 1 shows details of chromosome breakage produced by different concentrations of LSD. All cultures received colcemid at the 72nd hour and were terminated at the 74th hour, except those which received 6·64 µg/ml. of LSD. Because the suppression of cell division at this concentration precluded an adequate estimation of chromosome damage at the 74th hour, treatment with colcemid was prolonged to the 93rd or 96th hour.

Chromatid and isochromatid deletions increased with the size of the dose. All chromosome exchanges observed were of the chromatid type: chromatid dicentrics, triradials and chromatid intrachanges. With the larger

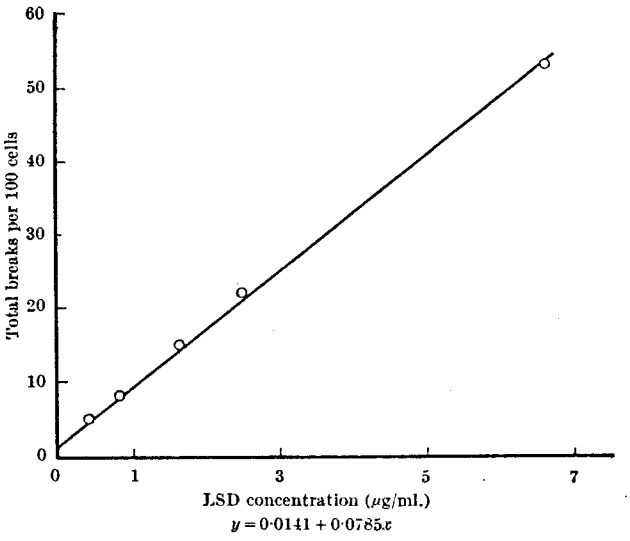

Fig. 1. Effect of LSD on chromosome breakage in leucocyte cultures of *Potorous tridactylus*. Aberrations due to culture conditions have been allowed for.

concentrations of LSD, there was an increase in elongation and attenuation of chromosome material, causing overlapping and difficulties in scoring some of the more damaged cells. This probably led to an underestimate of chromosome damage for the highest concentration of LSD (6·64 μg/ml.). For the range of concentrations used, a linear response was revealed when concentration was plotted against total chromosome breakage (Fig. 1).

Table 1. CHROMOSOME DAMAGE PRODUCED BY LSD IN LEUCOCYTE CULTURES FROM *Potorous tridactylus*

LSD (μg/ml.)	Abnormal nuclei (per cent)	Chromatid deletions (per cent)	Isochromatid deletions (per cent)	Chromatid exchanges (per cent)	Total breaks per cell	Cells scored
0	12	5	9	0	0·14	100
0·145	16	5	12	1·0	0·19	100
0·83	19	9	10	1·5	0·22	200
1·66	25	10	17	1·0	0·29	300
0	13	6	10	0	0·16	100
2·55	30	14	20	2·0	0·38	50
0*	24	15	16	1·0	0·33	100
6·64†	47	31	27	14·0	0·86	100
6·64*	52	39	27	12·0	0·90	100

Final concentration of colcemid was 0·004 per cent.

All cultures except with LSD concentration of 6·64 μg/ml. and the parallel control had colcemid treatment from the 72nd to the 74th hour.

* Cell division arrested from the 72nd to the 96th hour.

† Cell division arrested from the 72nd to the 93rd hour.

Because peripheral lymphocytes usually divide only when stimulated by phytohaemagglutinin, and because LSD was introduced when the cultures were set up, the drug must have been present for the whole cell cycle. Because only chromatid type exchanges were seen, LSD must act on chromosomes only during the period of DNA synthesis (S) or during the post-DNA synthetic period (G_2) when the chromosomes are already double structures. This means that chromosome damage in LSD users cannot be estimated from their peripheral leucocytes, because normally these cells *in vivo* are never at the appropriate stage. The actively dividing stem cells of the lymphoid tissue would be affected, but the more damaged cells would probably not survive to enter the blood stream. Furthermore, because the replenishment of the blood lymphocytes is dependent on many factors, there is no accurate way of determining the numbers of the less damaged cells or the time at which they enter the general circulation after administration of the drug.

Table 2 compares the total chromosome damage caused by LSD, heliotrine and X-irradiation. The total damage done by LSD was of the same order as that produced by heliotrine (1·66 μg/ml. of LSD produced 0·15 breaks/cell; 1·56 μg/ml. of heliotrine resulted in 0·10 breaks/cell). The high concentrations of LSD found in the liver[18] indicate that in habitual users of LSD, liver cells are continuously subjected to the effects of the drug and its breakdown products, for it is eliminated chiefly through this organ. In this connexion it is interesting that heliotrine causes liver lesions, and related pyrrolizidine alkaloids are known to be potent liver carcinogens[19].

Table 2. COMPARISON OF CHROMOSOME BREAKAGE PRODUCED BY LSD, HELIOTRINE AND X-IRRADIATION IN CULTURES OF LEUCOCYTES FROM *Potorous tridactylus*

Treatment	Total breaks/cell	Time of cell arrest (h)	Cells scored
μg/ml. of LSD			
0·415	0·05	72–74	100
0·83	0·08	72–74	200
1·66	0·15	72–74	300
2·50	0·22	72–74	50
6·64	0·53	72–96	100
6·64	0·57	72–93	100
μg of heliotrine*			
1·56	0·10	72–74	100
3·13	0·29	72–74	100
7·80	0·52	72–74	100
15·65	0·95	72–74	100
31·30	1·52	72–74	100
25 r./min of X-irradiation			
100 r.	0·36	72–74	100
200 r.	0·64	72–74	200
300 r.	0·92	72–74	100

* Results for heliotrine damage from ref. 17.

This table takes account of spontaneous aberration due to culture conditions.

LSD resembles heliotrine in preferentially attacking the heterochromatic regions of the chromosomes. With heliotrine, up to 30 per cent of the damaging events occurred in heterochromatin, but for LSD the figure was up to 50 per cent. By contrast, after X-irradiation only 5 per cent to 7 per cent of the total damage occurred in heterochromatin.

It is not possible to equate damage produced by a compound present for the whole of the culture period with that produced by an X-ray dose acting on the chromosomes only at the stage of the cell cycle at which it is delivered. But, as far as comparison is possible, a dose of 100 r. (at 25 r./min) produced a corresponding amount of damage as 4·2 μg/ml. of LSD (Fig. 1 and Table 2). A dose of 100 r. produced 0·0036 breaks/cell/r.[17]. A unit increase of 1 μg of LSD/ml. resulted in 0·078 breaks/cell.

Table 3. EFFECT OF LSD ON RATE OF CELL DIVISION IN LEUCOCYTE CULTURES FROM *Potorous tridactylus*

	Mitotic index as percentage of control				
	Time of arrest of cell division after initiation of cultures (h)				
μg/ml. of LSD	48–51	26–55	72–74	72–93	72–96
0·415	—	41	97	—	—
0·83	—	37	93	—	—
1·66	—	34	85	—	—
1·66 repeat	30	—	82	—	120
2·5	—	—	74	—	—
2·5 repeat	—	—	72	—	148
6·64	—	—	52	125	—
6·64 repeat	—	—	—	—	291

In all, 7,000 cells were scored for every mitotic index estimation.

The effect of LSD on the inhibition of cell division is shown in Table 3. At the 74th hour the higher the dose the lower was the mitotic index; however, in cultures stopped at the 53rd hour inhibition was more acute. At the 74th hour, the cells seem to have been overcoming, to some extent, this effect of LSD. Furthermore, when cultures were stopped in the 93rd or 96th hour, the mitotic index was higher than for corresponding controls. Repeat experiments (Table 3) confirmed that the inhibition of cellular division imposed by LSD was gradually overcome.

It is possible that the concentration of LSD decreases as a result of cellular metabolism, and that cells previously inhibited are released from the mitotic block. Cultures with 6·64 μg/ml. of LSD, however, had at the 96th hour a mitotic index three times that of controls

(Table 3), yet the curve for chromosome breakage did not decline for this concentration (Fig. 1).

These experiments do not rule out the possibility that at later stages of culture, LSD or a breakdown product (perhaps present in minute quantities) stimulates cells to divide. It is interesting that rats treated with LSD in early pregnancy produced, in addition to stunted and stillborn young, some unusually large offspring which weighed more than the young of controls ten days after birth[20].

To investigate this fully, peripheral lymphocytes would be inappropriate, for they have only a finite lifetime in culture. A more permanent cell line would be required.

The concentrations of LSD used in these experiments were greater than would be expected in the blood of users of the drug, for it passes quickly into the tissues and is subsequently eliminated. Some organs, however, contain a greater concentration than that originally present in the blood stream[18]. Human cells may well prove to be less sensitive than *Potorous* lymphocytes, but even a far lower level of damage than that observed here could have far reaching results.

I thank the Australian Institute of Nuclear Science and Engineering for a post-doctoral fellowship. This work was carried out at the Department of Zoology, University of Tasmania, during the tenure of that fellowship.

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Received October 20, 1969; revised April 21, 1970.

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In vitro Cytotoxic Test for detecting Cell Mediated Immunity to Soluble Antigen

The demonstration that lymphoid cells from immunized donors are cytotoxic for cells carrying antigens to which the cell donor is sensitized has led to the development of *in vitro* tests for cell-mediated immunity[1]. These systems measure directly the lytic activity of "sensitized" lymphocytes, and can be readily adapted to a quantitative approach by incorporating ^{51}Cr (as sodium chromate) into the antigen-carrying (or "target") cell, and following the kinetics of target cell destruction by measuring the release of ^{51}Cr. This approach has been used most effectively in studies of allograft immunity[2,3]. But in spite of many obvious advantages, these cytolytic tests are generally applicable only to systems involving cell surface antigens, and as such have found particular use in the study of histocompatibility systems. I now describe attempts to extend the applicability of these systems to antigens which are not an integral part of the cell membrane but which are covalently linked to it by the use of coupling agents. Similar previous attempts[4,5] have only yielded systems of low sensitivity.

Adult Hartley strain guinea-pigs were immunized, in the rear foot-pads, with 50 μg dinitrophenylated human IgG (DNP–HGG) in complete Freund's adjuvant. Spleens were removed at various times and a viable lymphocyte population was prepared and counted[2]. "Target" cells were prepared by coupling DNP–HGG to mouse mastocytoma cells (P815) of the DBA strain[6] using 1-ethyl - 3 - (3 - dimethylaminopropyl) carbodiimide–HCl (ECDI). These cells were especially chosen for their susceptibility to membrane permeability changes and destruction[7]. They have been found, by several criteria, to be more sensitive to lysis than are erythrocytes (ref. 8 and my unpublished observations).

Mastocytoma cells were collected from cultures in modified Eagle's medium supplemented with 10 per cent inactivated calf serum. The cells were washed twice in sterile Tris-buffered saline and 2·0 ml. of the cell suspension at a concentration of 2×10^6 cells/ml. was incubated with 0·2 ml. sodium ^{51}Cr-chromate (specific activity 100 μCi/μg chromium) for 30 min at 37° C. The suspension was then centrifuged at 1,500 r.p.m. for 5 min and washed three times with sterile Tris-buffered saline. Following haemocytometer counting, using a Trypan-blue exclusion test, 10^6 viable cells were suspended in 1 ml. Tris-buffered saline (*p*H 7·2). A solution of DNP–HGG was passed through a 'Millipore' filter (0·21 μm pore size) and 3 ml. of effluent containing 7·5 mg protein in Tris-buffered saline (*p*H 7·2) was mixed with the mastocytoma cell suspension. One minute later, 0·5 ml. of a 500 μg/ml. solution of ECDI was added. Following inversion, the suspension was left at room temperature for 1 h. The cells were sedimented by centrifugation at 1,500 r.p.m. for 5 min and then washed three times with Eagle's medium containing 10 per cent inactivated calf serum. These "coupled" cells were again counted and cell suspensions adjusted to appropriate concentrations.

"Target" cells (10^5 DNP–HGG coated mastocytoma cells) were incubated at 37° C with 5×10^7 lymphocytes from both normal and sensitized animals. The effects of antigen coupling were assessed by measuring ^{51}Cr release from untreated mastocytoma cells and from the same cells following "coating" with DNP–HGG. After incubation for 1, 3, 6 and 9 h at 37° C, 1 ml. of Tris-buffered saline was added to representative tubes which were then spun at 1,500 r.p.m. for 5 min; 1 ml. supernatant fluid was withdrawn and assayed for ^{51}Cr. The result was corrected for the volume left with the cell sediments, which were also counted for ^{51}Cr, and the amount of ^{51}Cr in the supernatant was calculated as a percentage of the total ^{51}Cr which was originally cell-associated. A typical result, using pooled lymphocyte preparations from three normal spleens and from three spleens obtained from animals 8 days after immunization with DNP–HGG, is shown in Fig. 1. Treatment of the mastocytoma cells with DNP–HGG and ECDI did not affect the rate of ^{51}Cr release over a 9 h period. A further study following ^{51}Cr release over a 20 h period similarly revealed no differences between untreated cells and those exposed to antigen and ECDI. The addition of normal lymphocytes caused only a slight increase in ^{51}Cr release (Fig. 1). On the other hand, the addition of lymphocytes from DNP–HGG immunized animals caused a marked increase in ^{51}Cr release from target cells, more than 30 per cent of radioactive label being released during the 9 h incubation period studied, compared with about 12 per cent for "normal" lymphocytes. (A recent adaptation