An Analysis of the Uncoiling Effect of a BHT-Tween 80/Absolute Ethanol Mixture on Velban^R Arrested Human Metaphase Cells

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The white, crystalline, odorless solid called butylated hydroxytoluene (3,5-di-tert-butyl-4-hydroxytoluene; BHT) is an antioxidant for fats and oils, and packaged materials containing fats. This widely used preservative acts to accept free radicals which are formed on the autoxidation of unsaturated fatty acids. These free radicals, if unchecked, can lead to the breakdown of carbon-carbon double bonds and cause rancidity (Milner 1967). The agent's maximum concentration in foods is .02%, based on oil and fat content, and since its introduction as a food preservative in 1954, its effects *in vitro* and *in vivo* have been widely investigated.

Many workers have reported that high levels of BHT can induce an increase in the relative liver weights of rats (Brown *et al.* 1959, Deichman *et al.* 1955, Gaunt *et al.* 1963 and Johnson and Hewgill 1961), while others have examined the effect of this agent on the cells' protein synthesizing machinery. Gilbert and Goldberg (1965) and Nievel (1969) have performed experiments indicating increases in rat liver protein synthesis, while Milner (1967) has reported a depressing effect of BHT on RNA, DNA and protein synthesis in monkey kidney cells grown in monolayer cultures. Recently, Sciorra *et al.* (1974) have shown that the preservative causes disturbances in the timing of the human cell cycle of phytohemagglutinin-stimulated leukocytes, as well as producing an uncoiling effect in the chromatids of percentage of Velban^R arrested metaphase chromosomes when added in a solvent mixture of 1:4 (v/v) Tween 80/absolute ethanol.

This paper reports further investigations on this uncoiling effect on the human chromosomes, specifically analysing the pattern of condensed areas which are revealed along the lengths of the chromatids when this solvent-preservative is added to PHA stimulated leukocytes in their rapid growth phase.

Material and methods

Initiation and treatment of cultures

PHA-stimulated small lymphocyte cultures were set up according to the method of Moorehead *et al.* (1960), using blood from one donor and McCoys modified 5a media containing 12% human AB serum. These cultures were grown

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at 37°C in an atmosphere of 5% carbon dioxide. After the cultures had grown for 48 hours, .01 mls of a solution containing BHT dissolved in a mixture of Tween 80/absolute ethanol 1:4 (v/v) was injected with a microliter pipette into the cultures to give a final media concentration of either 50 or 100 µg/ml of culture media. After 22 hours exposure of the cultures to this mixture, vinblastine sulfate (.05 µg/ml of culture media) was added, with the harvesting of the cultures occurring 2 hours later. The harvesting techniques involved an initial suspension in a hypotonic balanced salt solution (Hanks) and fixation of the cell samples

Fig. 1. A typically uncoiled chromosome plate from a culture exposed to 50 µg of BHT (dissolved in 1:4 (v/v) Tween 80/absolute ethanol)/ml of culture media for 22 hours before the addition of Velban^R and subsequent harvest two hours later. Note the uncoiled nature of the chromatids of the metaphase chromosomes and the condensed areas along the arms. Aceto orcein stain 1000×, oil phase contrast.

in a 1:3 (v/v) solution of acetic acid/absolute methanol. The fixed, flame dried slides were then stained with 2% aceto orcein (2 g synthetic orcein dissolved in 100 mls of a 60% aqueous acetic acid solution) at 60°C for 45 mins, taken through successively increasing strengths of alcohol to absolute xylene, mounted in Permount and allowed to dry overnight on a slide warmer. The slide preparations were examined and photographed using Kodak high contrast copy film and a Zeiss photomicroscope II equipped with phase contrast optics. Of the more than 100 metaphase plates photographed, the six showing the highest degree of uncoiling

were analysed for number and position of condensed areas (knots) along the lengths of the chromatids. This was done by first karyotyping the plate, and then tracing the chromosomes, with emphasis placed on the position of any condensed areas observed in each chromatid. Such pencil tracings greatly facilitated counting and characterizing the knots.

Fig. 2. A comparison of the uncoiled pattern seen in the various a) control (solvent only) and b) BHT treated human karyotype groups (A, B, C, D, E, and F). Note the more condensed nature of the long and short arms of the smaller chromosomes relative to the large chromosome groups.

Results

Figure 1 illustrates a typically uncoiled metaphase cell obtained when a final media concentration of 50 μg of BHT/ml of media was added to the culture. At this concentration about 6% of the Velban^R arrested metaphase plates examined

could be found in a similar degree of uncoiling. Such a metaphase cell is partially karyotyped and compared to control chromosomes (solvent only), which show little if any uncoiling, in Fig. 2. The pencil tracings below the cut out chromosome photographs emphasize the condensed areas along each chromatid. As can be seen in such a picture, the large chromosomes display a greater degree of uncoiling than the smaller ones. Extensive uncoiling is seen in both the long and short arms

Fig. 3. Various chromosomes showing the centromere region highly uncoiled, with two condensed areas on each chromatid, sometimes joined by chromatin material into a boxlike structure.

Fig. 4. Various chromosomes showing two strands within each chromatid with condensed areas lying side to side on each strand.

of the larger chromosomes, with chromosomes of the C group showing more uncoiling in the long arms of the chromatids than in the short arms. While there is some uncoiling also observed in the smaller F and G chromosomes, the uncoiled pattern is not as dramatic as in the larger chromosomes.

In some of the more extremely unravelled chromosomes, the structure of the centromere is seen in which two condensed areas on each chromatid form a characteristic box structure in the metaphase chromosome. Figure 3 illustrates this pattern seen in the various chromosome groups. In some instances the four knots of the centromere seem to be joined by thin strands of chromatin material,

Fig. 5. Various chromosomes showing gaps in the arms of the chromosomes with either two, one or zero strands visible within each gap.

while in others this joining is incomplete. It has also been observed, that in those chromosomes with more extensive degrees of uncoiling, the centromere regions are themselves the most uncoiled portions of the chromosomes.

In some of the more extremely unravelled chromosomes, an examination of the chromatids indicated a doubled nature, with two condensed areas sometimes lying side by side along each of these parallel strands. Examples of this double stranded pattern in various chromosomes are shown in Figs. 4 and 5.

Table 1 summarizes counts of the number of condensed areas seen on each

Table 1. A comparison of the average number of condensed areas (knots)/chromosome in the different karyotype groups (A, B, C, D and E) of the six most uncoiled metaphase cells analysed

Karyotype group	Average number of knots/metaphase chromosome (BHT conc. in $\mu\text{g/ml}$ of culture media)						Pooled total
	II-2 (100 μg)	II-3 (100 μg)	V-9 (50 μg)	IV-4 (50 μg)	II-9 (100 μg)	I-2 (100 μg)	
A	18.0	20.0	18.8	16.2	16.2	15.5	17.4
B	11.0	12.7	13.0	10.7	11.3	11.5	11.7
C	11.8	11.5	10.9	9.9	10.6	9.1	10.5
D	8.2	9.4	8.0	9.0	8.3	7.9	8.5
E	7.0	8.2	6.2	6.2	6.4	6.0	6.6
Total cell	11.3	12.2	11.2	10.3	10.7	9.4	10.8

Table 2. A comparison of the number of condensed areas (knots) seen on the chromatids of metaphase chromosomes karyotyped in the six most uncoiled cells analysed

Karyotype group	Homologues	Metaphase cells analysed (BHT conc. in $\mu\text{g/ml}$ of culture media)											
		II-2 (100 μg)		II-3 (100 μg)		V-9 (50 μg)		IV-4 (50 μg)		II-9 (100 μg)		I-2 (100 μg)	
		Chromatids		Chromatids		Chromatids		Chromatids		Chromatids		Chromatids	
		L	R	L	R	L	R	L	R	L	R	L	R
A	1a	9	10	10	10	11	12	9	8	8	8	11	7
	1b	9	8	13	12	—	—	7	8	7	8	10	7
	2a	9	10	11	11	11	8	9	7	9	8	9	5
	2b	—	—	9	8	11	8	10	9	9	7	—	—
	3a	9	8	7	9	9	10	8	8	9	8	6	6
	3b	—	—	—	—	7	7	7	8	—	—	—	—
B	4a	5	6	8	7	7	7	5	4	6	5	7	6
	4b	—	—	4	5	7	7	—	—	5	5	6	5
	5a	5	6	7	7	7	6	6	5	7	6	5	5
	5b	5	6	—	—	5	6	7	5	—	—	6	6
C	6a	5	5	—	—	6	6	5	5	6	7	5	5
	6b	7	7	—	—	6	6	5	5	6	7	5	5
	7a	6	4	4	7	8	6	7	6	5	6	6	4
	7b	4	6	6	7	4	5	5	4	6	7	4	3
	8a	7	5	7	6	7	7	4	5	5	6	6	5
	8b	—	—	—	—	7	7	3	4	6	6	6	5
	9a	5	7	6	5	6	5	6	6	4	4	5	4
	9b	—	—	8	6	—	—	—	—	4	5	4	4
	10a	6	6	4	6	6	6	5	4	5	4	3	4
	10b	9	9	6	6	—	—	—	—	5	4	4	4
	11a	6	6	5	5	5	4	4	4	5	6	5	6
	11b	—	—	—	—	5	5	—	—	6	4	5	4
D	12a	4	4	4	5	5	4	5	6	7	4	4	4
	12b	—	—	—	—	5	4	—	—	—	—	4	4
	13a	3	4	5	4	4	4	3	4	—	—	4	3
	13b	—	—	—	—	5	4	5	4	—	—	5	4
	14a	4	5	5	7	3	4	6	6	4	4	3	3
	14b	4	6	6	3	4	4	—	—	—	—	3	4
E	15a	4	3	5	5	—	—	4	4	3	4	5	4
	15b	4	4	3	4	—	—	—	—	5	5	5	4
	16a	5	4	5	4	3	2	4	4	2	2	3	3
	16b	4	3	4	4	3	3	5	5	2	3	3	4
	17a	—	—	3	5	3	3	3	3	4	4	—	—
	17b	—	—	3	5	3	5	3	2	3	3	3	3
	18a	4	4	4	4	3	3	2	2	4	5	3	3
	18b	2	2	—	—	3	3	2	2	—	—	3	2

chromosome as determined from the pencil tracings of the six most uncoiled metaphase cells. Only chromosome groups A, B, C, D and E are recorded, since group F and G were usually too tightly coiled to analyse. As can be seen in the Table, the average number of knots/chromosome for each chromosome group is fairly constant in the six cells examined, with group A having an average number of knots/chromosome of 17.4, 11.7 for group B, 10.5 for C, 8.5 for D and 6.6 for E. This decreasing pattern is characteristic of both the greater degree of uncoiling one sees in the larger chromosomes and the decreasing size of the chromosomes within each group. The uncoiling pattern was also similar in cells exposed to either 50 or 100 μg of BHT/ml of media, and the average number of knots seen in each chromosome was remarkably similar from cell to cell.

Table 2 attempts to analyse the number of knots present on the chromatids of homologous chromosomes in the cells examined. The dashed lines indicate that one of the chromatids of the homologue was not analysed, usually because of overlap with another chromosome. In those chromosomes which were analysed, however, a good degree of consistency existed between the number of knots on the chromatids of homologous chromosomes. There was also a high degree of con-

Table 3. A comparison of the differences in the number of condensed areas (knots) found on sister chromatids of analysed metaphase chromosomes

Karyotype group	Number of chromosomes analysed	Differences in number of knots between sister chromatids				
		$\Delta 0$	$\Delta 1$	$\Delta 2$	$\Delta 3$	$\Delta 4$
A	29	6 (20.7%)	15 (51.7%)	3 (10.4%)	3 (10.4%)	2 (6.8%)
B	20	6 (30.0%)	13 (65.0%)	1 (5.0%)	—	—
C	68	26 (38.2%)	31 (45.6%)	9 (13.2%)	2 (2.9%)	—
D	27	9 (33.3%)	15 (55.6%)	2 (7.4%)	1 (3.7%)	—
E	31	19 (61.3%)	9 (29.0%)	3 (9.7%)	—	—
Total	175	66 (37.7%)	84 (47.4%)	18 (10.3%)	6 (3.4%)	2 (1.1%)

sistency in the number of knots observed on the sister chromatids of each chromosome as is seen in Table 3. Here the differences in the number of knots between the sister chromatids of chromosomes within each group is presented. As can be seen, 72.4% of group A chromosomes had sister chromatids with either the same or a variation of one knot between them. This percentage of 0 or 1 knot difference between sister chromatids was 95% in group B, 83.9% in group C, 88.5% in the D group and 90.3% in group E.

Discussion

Our data shows that 50 and 100 μg of BHT dissolved in a 1:4 (v/v) solution of Tween 80 to absolute ethanol/ml of culture media can produce an apparent uncoiling effect in human metaphase cells arrested with Velban^R. An analysis of this effects shows that: a) the larger chromosomes of groups A, B and C show a greater degree of uncoiling than those of the smaller E, F and G groups,

b) in extensively uncoiled chromosomes a characteristic box-like structure is seen in the centromere region, with the corners of the box made up of areas of condensed material, either totally or partially joined together by strands of chromatin material, c) in extensively uncoiled chromosomes a double stranded structure of the chromatids of the metaphase chromosome is sometimes visible, d) a remarkably high degree of correlation is seen in the average number of condensed areas (knots)/chromosome within each chromosome group from cell to cell, and e) a high degree of consistency existed between the number of knots present on homologous metaphase chromosomes and the number of knots present on sister chromatids of the same chromosome.

The causes of the uncoiling effect of the BHT-Tween 80/absolute ethanol mixture cannot at this time be definitely identified, and one could imagine many constituent parts of the cell and the chromosome being affected by such a mixture. Various workers using BHT have demonstrated many different biochemical changes occurring with addition of this agent *in vivo* and *in vitro*. Milner (1967) has illustrated a depressing effect of BHT on RNA, DNA and protein synthesis in monkey kidney cells grown in tissue culture, as well as decreases in the number of cells surviving BHT treatment. Neivale (1969) on the other hand, has shown increases in the rate of amino acid incorporation into proteins of microsomal fractions of liver and increased rough endoplasmic reticulum activity indicating increased protein synthesis. Differences in the timing of the cell cycle and increased cellular mortality has also been demonstrated by Sciorra *et al.* (1974) using BHT on PHA stimulated small lymphocyte cultures.

While none of these experiments has elucidated the exact mechanism of BHT's action on the biochemistry of the cell and the uncoiling effect one observes in some of the metaphase cells, this uncoiling effect itself might be helpful in investigating the fine structure of the human chromosome. Many theories on the unimene, bimene or multimene nature of chromosomes are found throughout the literature based on light (Iino 1970, Ohnuki 1968) and electron (DuPraw 1965, Bahr 1970, Stubblefield and Wray 1971) microscope examinations. Many researchers have suggested that the chromosome contains one strand or chromonemata per chromatid (DuPraw 1965, Whitehorse 1967, Ohnuki 1968) while others such as Kaufmann (1926), Nebel (1932), Cleveland (1949), Peacock (1963), and Trosko and Wolf (1965) support a polynemic chromosomal structure, with Stochert (1969) and Iino (1971) recently demonstrating a two chromonemata structure of the chromatid of a human metaphase chromosome. Our data obtained with the BHT-solvent uncoiling is also in agreement with a double stranded nature of the chromatid.

Several authors have examined the differentiation of special segments along the lengths of the chromatids of human metaphase chromosomes. Of note are two reports in the literature. In one, Ohnuki (1968) demonstrated the uncoiling effect of various pretreatments such as KCl (.055 M), KSCN (.062 M), NaNO₃ (.055 M) and CH₃COONa (.055 M). In the other study by Shiraishi (1970), a cold treatment of human leukocytes followed by a modified silver hexamethylenetransamine staining, revealed areas of differential staining along the lengths of the

chromatids. The number of condensed areas along the lengths of the chromatids using the BHT-solvent mixture does show a close similarity in the number of condensed areas observed on each chromosome in the karyotype and the number of stained areas on each chromosome as revealed by the cold treatment of Shiraishi. There was not, however, as close an agreement in the number of spirals observed in human somatic chromosomes after the uncoiling pretreatments of Ohnuki and the number of condensed areas on each chromosome as demonstrated in our experiments. This discrepancy, as well as the Ohnuki's interpretation of his results by a one chromonemata model, perhaps indicates a difference in the mechanisms by which the BHT-solvent mixture and the Ohnuki pretreatments are having their effect. In general there were more spirals reported on each chromosome using the Ohnuki pretreatments than the number of condensed areas observed with our mixture.

There has also been mentioned in the literature reports on the structure of the centromere region of the human chromosome following uncoiling pretreatments. Ohnuki reported that the centromere seemed to be a regionally elongated portion of the chromonemata, without showing any different structures, while Iino, using a hyaluronidase pretreatment, reported that the chromonemata display in general a fine and nonspiral structure, with some centromeres having a bar-like substance linking each sister chromatid and other centromeres showing granular matter. It is possible that the box-like nature of condensed material that we have observed in the centromere region is a clearer resolution of the bar-like nature of some of the centromeres which Iino has reported. It should also be noted that the box-like structure we have observed, as well as the double stranded nature of the chromatids, are both in agreement with the electron microscope pictures of the fine structure of Chinese hamster metaphase chromosomes (Stubblefield and Wray 1971). That the centromere region of human chromosomes does have some properties which distinguishes it from the rest of the chromosome is also indicated by the differential staining of the centromere illustrated by "C banding" (Arrighi and Hsu 1971) and the hybridization studies which have localized mouse satellite DNA in the centromere region (Pardu and Gall 1970).

It is thus possible that the unravelled chromatin fibers seen by this BHT-solvent treatment might lend themselves to a more careful examination under the electron microscope and help elucidate more clearly the fibrous and condensed nature of the human chromosomes. The fact that similar numbers of knots are seen between the sister chromatids of the metaphase chromosome and between homologues of the same and different cells also suggests a highly specific cellular mechanism for producing these condensed regions of the chromosome which deserves further examination.

Summary

Phytohemagglutinin stimulated human small lymphocyte cultures exposed to a final milliliter media concentration of 50 and 100 μg of butylated hydroxytoluene (BHT) dissolved in a 1:4 (v/v) solution of Tween 80/absolute ethanol, displayed

an uncoiling of the chromatids in a percentage of Velban^R arrested metaphase cells. These uncoiled chromosomes revealed areas of condensed staining knots along the lengths of the chromatids, with the larger chromosomes displaying a much greater degree of uncoiling than those of the smaller groups. In chromosomes with severe uncoiling, the centromere region revealed two highly condensed areas on each chromatid, giving a characteristic box formation. In some of these chromosomes, the chromatids seemed to have two strands parallel to each other, with condensed areas lying side by side along each of these strands. A remarkable degree of consistency existed in the average number of knots seen on the chromosomes of each chromosome group karyotyped in the different cells examined, with each chromosome group having a characteristic average number of knots/chromosome. There was also a high degree of correlation between the number of knots counted on sister chromatids on one chromosome, and the number of knots seen in the chromatids of homologous chromosome.

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