The minimum size of the ribosome-protected mRNA sections obtained after degradation of the interribosomal moieties with concomitant conversion of polysomes to monosomes correspond to 50 - 60 nucleotides. This compares favorably with values deduced from the ribosome number in polysomes formed with globin mRNA (15).

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During the preparation of the manuscript, a yet different method for the determination of mRNA labeling based on specific binding of poly A sections in mRNA has been published (16).

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INDUCTION OF ALKALI LABILE LINKS IN CELLULAR DNA BY CAMPTOTHECIN

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SUMMARY: Camptothecin has been reported to rapidly introduce single strand breaks into the DNA of cells exposed to the drug. The breaks are apparent when drug-treated cells are layered on alkaline-sarcosyl and released DNA sedimented in an alkaline gradient. In this report, we show that the DNA breaks are not found when formamide denaturation is substituted for high pH. Furthermore, if cells are lysed in neutral sarcosyl and the DNA then sedimented in alkali, no camptothecin-induced breaks are apparent. These results suggest that the DNA breaks induced by the drug result from alkali labile links in the DNA. The DNA fragmentation apparently only occurs if the DNA is exposed to high pH while the drug or some derivative is still present.

INTRODUCTION

Camptothecin is a potent alkaloid which rapidly inhibits both DNA and RNA synthesis [1-3]. This agent is useful for studying nucleic acid metabolism because of its rapid onset of action and its extraordinary reversibility [2,3]. It is selectively active against high molecular weight nuclear RNA synthesis, and has no effect on mitochondrial macromolecular processes [3]. It has been demonstrated and subsequently confirmed that camptothecin also has the ability to induce what appear to be single strand breaks in DNA [4,5]. However, DNA integrity is promptly restored after removal of the drug [5,6]. This behavior of the DNA would require the cell to repair all the single strand breaks within minutes of removing the drug.

In this report, we will demonstrate that the single strand breaks appear due to alkali labile links which appear only if the cellular DNA is exposed to high pH in the presence of the drug.

The experiments were suggested by the findings of Gaudin and Yielding, who reported that DNA exposed to nitrogen mustard behaved as small pieces under alkali denaturing conditions but not when denaturation took place in formamide.

MATERIALS AND METHODS

An S_3 clone of HeLa cells has been maintained in suspension culture in

Eagle's medium, as previously described [8]. Cells growing at a density of 4×10^5 cells/ml were labeled for 12-15 hours with [^{14}C]-thymidine (specific activity, 6.7 Ci/mmole, New England Nuclear) prior to utilization in these experiments. Prior to treatment with camptothecin, the thymidine-labeled cells were collected and concentrated to 2×10^6 cells/ml and resuspended in fresh media. Camptothecin (40 µg/ml) was added for 30 minutes. Incubations were terminated by addition of ice-cold Earle's solution, which included 5 µg/ml of camptothecin. Alkaline sucrose gradients were prepared by a modification of the method of Horwitz and Horwitz [4]. Briefly, gradients were prepared by placing 0.1 ml 35% (v/v) CsCl in the bottom of the centrifuge tube, overlaying this with alkaline sucrose gradient (15-30% sucrose, 1 M NaCl, 0.01 M EDTA, and 0.2 M NaOH). The alkaline sucrose was overlaid with 1% sarcosyl in 0.2 M NaOH with 5% sucrose added (lysing medium). Whole cells were layered on top of this lysing layer in Earle's saline containing 5 µg/ml of camptothecin. Formamide gradients contained 15-30% sucrose in 70% formamide (v/v) with 5 mM Tris, pH 7.4. Lysing medium was either 1% sarcosyl in alkaline or neutral buffer with 5% sucrose added. Centrifugations were performed in a Spinco SW40 rotor for 2 hours at 24° at 40,000 rpm. After centrifugation, gradients were collected by removal of successive 0.6-0.7 ml fractions from the top of the gradient. Each fraction was then precipitated with trichloroacetic acid and radioactivity measured on filters by liquid scintillation counting.

INHIBITORS

Camptothecin (NSC 100880) was obtained from the Drug Development Branch, Chemotherapy, of the National Cancer Institute.

RESULTS

Our results [6], as well as those of others [5], have confirmed the findings of Horwitz and Horwitz [4] that after exposing cells to camptothecin their DNA sediments in alkaline sucrose as short single strand pieces. To extend these observations, we sedimented camptothecin-treated DNA through denaturing formamide gradients. These results are shown in Fig. 1. In contrast

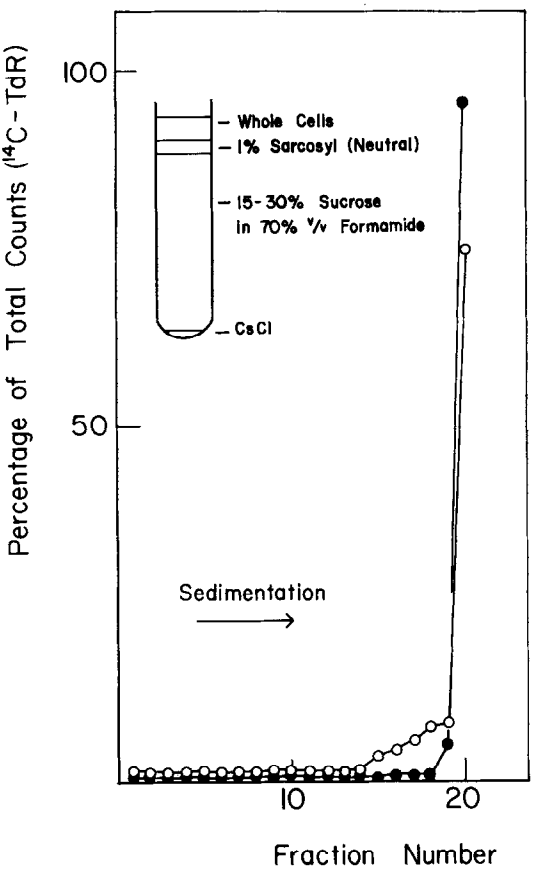

Fig. 1.

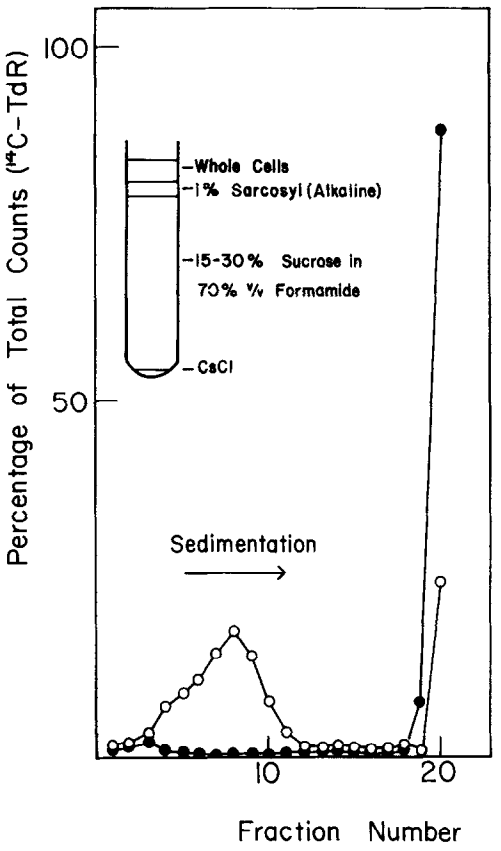

Fig. 2.

Figure 1. 4×10^5 cells/ml were labeled for 15 hours with [^{14}C]-thymidine. After concentrating to 2×10^6 cells/ml in fresh media, they were treated with camptothecin (40 µg/ml) for 30 minutes. Cells were collected in ice-cold Earle's saline with camptothecin (5 µg/ml) added. Whole cells suspended in Earle's saline with camptothecin (5 µg/ml) were layered directly onto gradients. Velocity sedimentation through 15-30% sucrose in 70% (v/v) formamide overlayered with neutral lysing medium was carried out in a Spinco SW40 rotor at 40,000 rpm for 2 hours at 24°C. ●—●—● control; 0—0—0 camptothecin.

Figure 2. As in Figure 1, except that the gradients are overlaid with alkaline lysing medium. Centrifugation as in Figure 1. ●—●—● control; 0—0—0 camptothecin.

to the distribution of DNA found after sedimentation through alkaline sucrose, the DNA from both untreated cells and DNA from camptothecin-treated cells sediments as large pieces in formamide. There is no evidence of the single-stranded

breaks such as are found after sedimentation in alkaline sucrose. In order to show that sedimentation in formamide does not in itself result in artifactually large DNA, cells were sedimented first through an alkaline layer and then into the formamide gradient, as shown in Fig. 2. In this situation, the DNA would be exposed first to alkali and therefore to whatever influences alkali exerts after treatment with camptothecin. These results show clearly that, in this situation, the DNA seems to sediment as small pieces. Therefore, under denaturing conditions in formamide after DNA has been exposed to alkali, there is evidence of single-stranded breaks.

The interpretation of these experiments is ambiguous because of the uncertainty about the behavior of DNA in formamide after deproteinization in sarcosyl. Conceivably, some proteins might remain which might prevent strand separation. However, the next experiments show the absolute requirement of high pH during cell lysis for DNA to show scissions even when subsequent sedimentation is in alkaline sucrose.

These last experiments show that if a neutral pH layer is interposed between the cell lysing layer and the alkaline sucrose, the single strand breaks are not manifested. If a layer of formamide is inserted between an alkali gradient and the lysing mixture (which has been adjusted to pH 7.4), the DNA now sediments rapidly. These results are shown in Fig. 3 and indicate that under these conditions DNA from both untreated and camptothecin-treated cells sediment as large pieces. To test the possibility that DNA aggregates in formamide in such a way as to make it inaccessible to alkali, we repeated the experiments in Fig. 3 but substituted 1 M Tris, pH 7.4, for formamide. These results, also shown in Fig. 3, indicate that, again in this instance, DNA sediments as large pieces. For comparison, the distribution of camptothecin-treated DNA sedimented directly into alkaline-sucrose is also shown in Fig. 3.

DISCUSSION

Camptothecin has become an important tool for the study of macromolecular processes because of its rapid onset of action and rapid reversibility, its

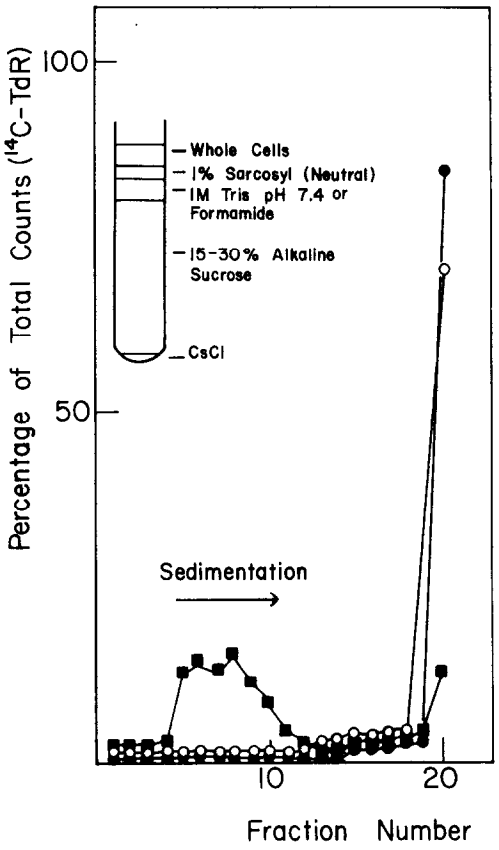

Figure 3. As in Figure 1, except the gradients are 15-30% sucrose in alkaline buffer. All samples are treated with camptothecin. The gradients are overlaid with either 1 M Tris, pH 7.4, or formamide. Included as well is the distribution of camptothecin-treated DNA sedimented directly into alkaline sucrose. Centrifugation as in Figure 1. ●—●—● formamide; 0—0—0 Tris; X—X—X sedimented directly.

selective inhibition of high molecular weight RNA synthesis, and its sparing of mitochondrial macromolecular processes. In addition, camptothecin seems to have an extraordinary effect on DNA, producing single-stranded pieces in alkaline sucrose, a process which is also rapidly reversible.

In order for treated DNA to sediment as shortened pieces in alkaline (i.e., single-stranded breaks), camptothecin must be continuously present in the preparation. Otherwise, the effect is vastly diminished and in fact can be completely reversed. This requirement is emphasized by our experiments in which the treated DNA is first sedimented through a small layer of either formamide or 1 M Tris

prior to encountering alkali. This brief "wash-out" of the drug is sufficient to completely reverse the effect of alkali on the sedimentation of treated DNA.

This does not alter the fact that in alkali there are apparent single-stranded breaks, but rather suggests that camptothecin (or a drug-induced intermediate) must be present with the DNA for this to be expressed.

The experiments using formamide suggest that unless alkali is present during cell lysis, the camptothecin-induced lesion cannot be demonstrated. Under denaturing conditions, no shortened DNA pieces are found, as would be expected if single strand breaks were present. These experiments, though, are not completely reliable, because the possible effects of proteins and non-specific aggregation are not totally excluded. However, the insertion of a layer of neutral buffer between the lysing medium and the alkaline sucrose apparently suppresses the appearance of DNA breaks. This suggests that the breaks are produced by high pH on the DNA in the presence of the drug, or, more likely, an activated form of the compound.

These results would explain the apparent rapid reversibility of the effect of the drug on DNA integrity. Rather than an instant repair of broken DNA, removal of the compound from cells apparently prevents the reaction at high pH from occurring.

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