Biochemical Pharmacology, Vol. 31, No. 7, pp. 1425–1429, 1982.
Printed in Great Britain.

0006-2952/82/071425-05 $03.00/0

INHIBITION OF DNA SYNTHESIS AND ALTERATION TO DNA STRUCTURE BY THE PHENACETIN ANALOG *p*-AMINOPHENOL

NICHOLAS K. HAYWARD,* MARTIN F. LAVIN*‡ and PETER W. CRASWELL†
*Department of Biochemistry, University of Queensland, Brisbane 4067, Australia; and
†Department of Nephrology, Royal Brisbane Hospital, Brisbane 4006, Australia

(*Received* 21 *September* 1981; *accepted* 13 *November* 1981)

Abstract—*p*-Aminophenol a structural analog and minor metabolite of phenacetin has previously been shown to be a potent nephrotoxic agent. In this report we have shown that *p*-aminophenol has a marked effect on DNA function and structure. DNA synthesis was inhibited in a dose-dependent manner in human lymphoblastoid cells after exposure to *p*-aminophenol. Results suggest that DNA synthesis is inhibited by the action of *p*-aminophenol on DNA structure. At low concentrations of *p*-aminophenol a reduction in the degree of supercoiling of cellular DNA is observed, as determined by sedimentation under neutral conditions. However at higher concentrations an increase in sedimentation of nucleoids (supercoiled molecules) is obtained which is indicative of an increased level of supercoiling or a more compact structural form of DNA due to folding or aggregation.

The number of single strand breaks in DNA, when determined by sedimentation in alkaline sucrose gradients, increases with increasing dose of *p*-aminophenol. The increase in strand breakage observed at lower concentrations of *p*-aminophenol agrees with the reduced sedimentation rate obtained under neutral conditions. At higher concentrations of *p*-aminophenol the extent of breakage of DNA increases under alkaline conditions but an increase in sedimentation occurs under neutral conditions.

Prolonged use of analgesics containing phenacetin has been demonstrated to cause harmful side-effects such as blood dyscrasias, methaemoglobinaemia, gastro-duodenal ulceration, renal papillary necrosis and non-obstructive pyelonephritis [1–3]. In addition circumstantial evidence exists for an association between abuse with phenacetin containing drugs and transitional cell carcinoma of the renal pelvis and bladder [4–6]. In a number of studies from Sweden, 75% or greater of the patients with transitional cell carcinoma were found to be analgesic abusers [4, 7, 8]. Evidence for the association between analgesic abuse and renal cancer is also based on observations that the normal ratios of renal parenchymal and pelvic carcinoma (4–8:1) and of bladder and renal pelvic urothelial tumors (> 10:1) are reversed in analgesic abusers [9, 10].

A number of compounds, chemically related to phenacetin, have been shown to cause necrosis of the proximal convoluted tubule of the kidney when administered in a single intravenous dose [11, 12]. One of these nephrotoxic compounds, *p*-aminophenol, a minor metabolite of phenacetin [13, 14], has been used as an experimental model for analgesic-induced renal damage [15]. *p*-Aminophenol is a potent inhibitor of proximal tubular function, acting primarily on gluconeogenesis, ATP synthesis, and energy production [13]. Mitochondria isolated from kidneys of *p*-aminophenol treated rats exhibit an inhibition of respiration, oxidative phosphorylation, and ATPase activity [15].

In view of the diversity of effects of *p*-aminophenol on cell function we have investigated its possible interaction with nucleic acid, an approach which has not been considered to date. We have examined the effects of *p*-aminophenol on DNA synthesis and DNA structure in human lymphoblastoid cells in culture. Changes to DNA function and/or structure induced by *p*-aminophenol may provide information on the role of analgesic metabolites in nephrotoxicity and also in the onset of transitional cell carcinoma.

MATERIALS AND METHODS

Cell culture. EBV-transformed lymphoblastoid cells were used in this study and were obtained from the Queensland Institute of Medical Research, Herston, Queensland. The cells were diploid at the commencement of this study and had a doubling time of approximately 24 hr. The cells were always subcultured the day before being used to ensure that they were growing in log phase at the time of the experiment. Suspension cultures of cells were grown in RPMI 1640 medium (Gibco, Uxbridge, U.K.) supplemented with streptomycin (60 μg/ml), penicillin (100 I.U./ml) and 10% foetal calf serum (FCS) in an atmosphere of 5% CO_2 in air at 37°.

Inhibition of DNA synthesis. Aliquots (0.9 ml) of cells (10^6/ml in medium without FCS) were added to 2 ml plastic tissue culture plates. A 10 ml stock of 20 mM *p*-aminophenol (Ajax Chemicals, Sydney, Australia) in medium (without FCS) was prepared by first dissolving 21.8 mg of *p*-aminophenol in 0.5 ml dimethylsulfoxide (Ajax Chemicals). This stock solution was further diluted, with medium lacking FCS, to the appropriate concentrations. Samples (0.1 ml) of the various dilutions of *p*-aminophenol were added

‡ To whom reprint requests should be addressed.

to the cells to give final concentrations in the range of 0–2 mM. Cells were incubated for 60 min at 37° in an atmosphere of 5% CO_2 in air, collected, centrifuged and washed once with medium, and then reincubated at 37° for 30 min. Aliquots (100 μl) of the cell suspensions were dispensed into six wells of a microtitre plate. [^{3}H]Thymidine (100 μl) (5 μCi/ml, 25 Ci/mmole, Amersham, Arlington Heights, IL) was added to each microwell. Incubation was carried out for 90 min at 37° and terminated by placing the microtitre plates in a −70° Revco. Samples were subsequently thawed at room temperature and acid-precipitable DNA was collected on GF/A glass fibre filters (Whatman, Maidstone, U.K.) using a multicell harvesting device. These GF/A strips were then washed with water followed by ice-cold 5% trichloroacetic acid (TCA). The strips were washed in ethanol and dried before being counted in a Beckman LS-250 scintillation counter using toluene scintillation fluid. Incorporation of [^{3}H]thymidine into acid-precipitable DNA in treated samples was expressed as the percentage of that obtained in untreated samples.

Nucleoid sedimentation. Cells were treated with *p*-aminophenol as described above with the exception that after incubation they were washed once in phosphate buffered saline (PBS) and resuspended in PBS at 2–4 × 10^6/ml. A variation of the method of Cook and Brazell [16] was used to study the sedimentation of nucleoids from these cells [17]. Briefly, 100 μl aliquots of cell suspension were added to 300 μl of a lysis solution. This mixture was layered on top of 10–30% sucrose gradients. Lysis was allowed to proceed for 10 min and the gradients were centrifuged at 30,000–37,000 rpm for 15–30 min at 12° in a Beckman L2-65B ultracentrifuge using an SW41 rotor. In some experiments ethidium bromide was added to the gradients. Absorbance at 254 nm was used to determine the position of the nucleoids after pumping the gradient through an LKB Uvicord S absorbance monitor. Triplicate or duplicate sets of both untreated and treated cells were employed in each centrifugation run.

Alkaline sucrose gradient analysis. Cells were labelled with [^{3}H]thymidine (1 μCi/ml, 25 Ci/mmole) for 24 hr, then transferred to fresh unlabelled medium for 2 hr to deplete labelled precursor pools.

Cells were treated with *p*-aminophenol as described above with the exception that after incubation the cells were washed once in saline–EDTA and resuspended in saline–EDTA at 10^6/ml. Sedimentation was carried out on 5–24% isokinetic alkaline sucrose gradients as previously described [18]. Briefly, 200 μl of a lysis solution was applied to the top of each gradient and 100 μl of cells (10^6/ml) was added to this layer. After a 90 min lysis period, gradients were centrifuged at 27,000 rpm for 3 hr in an MSE superspeed 65 ultracentrifuge using a swing-out rotor. Fractions were collected from gradients by pumping onto strips of Whatman No. 1 chromatography paper. Strips were dipped in 5% TCA then washed in ethanol. After drying, strips were cut into fractions and counted in a Beckman LS-250 scintillation counter using toluene scintillation fluid. The molecular weight of DNA was determined with the aid of a computer programme [18].

RESULTS

A dose-dependent inhibition of DNA synthesis is observed when lymphoblastoid cells are preincubated for 1 hr with *p*-aminophenol (Fig. 1). A rapid decline in synthesis occurs at doses up to 0.5 mM *p*-aminophenol levelling off between 0.5 mM and 2 mM. The extent of inhibition with *p*-aminophenol was also found to be time-dependent over the first hour of incubation (results not shown).

Since *p*-aminophenol had a marked effect on DNA synthesis we have determined its ability to cause structural alterations in DNA. We have used the method of Cook and Brazell [16] to examine the integrity of DNA structure. This approach allows separation of DNA from most of the chromatin proteins while maintaining a high molecular weight supercoiled form (nucleoid). Figure 2 depicts the relative sedimentation positions of nucleoids from untreated and *p*-aminophenol treated cells. At the lower concentration of *p*-aminophenol (0.25 mM) a marked reduction in sedimentation, compared to the untreated value, is obtained. Surprisingly, as the concentration is increased to 2 mM, a sedimentation value greater than that for untreated cells is observed. More detailed studies, using a number of different concentrations of *p*-aminophenol, demonstrate that nucleoid sedimentation decreases over the range 0.05 mM–0.25 mM but increases at concentrations up to 2 mM (Fig. 3). The increased nucleoid sedimentation observed at higher concentrations of *p*-aminophenol was found to depend on incubation time. The results in Fig. 4 (2 mM *p*-aminophenol) show an initial decrease in nucleoid sedi-

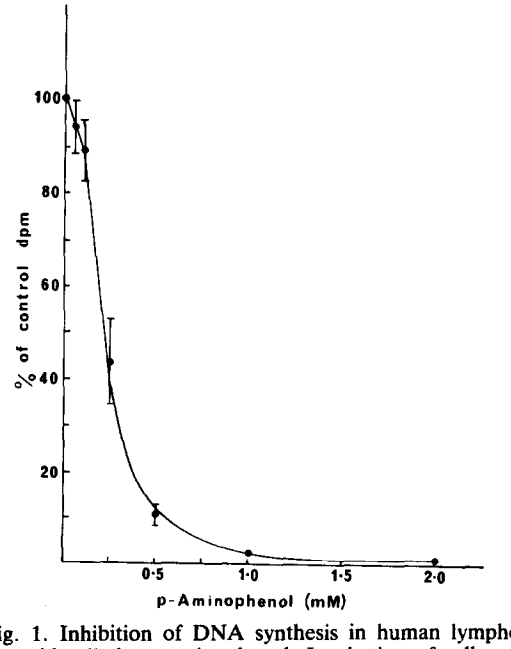

Fig. 1. Inhibition of DNA synthesis in human lymphoblastoid cells by *p*-aminophenol. Incubation of cells was carried out for 60 min in the presence of various concentrations of *p*-aminophenol. DNA synthesis was subsequently determined by incorporation of [^{3}H]thymidine over a 90 min period. Error bars represent ± the standard error of the mean (S.E.M.); at the highest concentrations errors are included within the symbol.

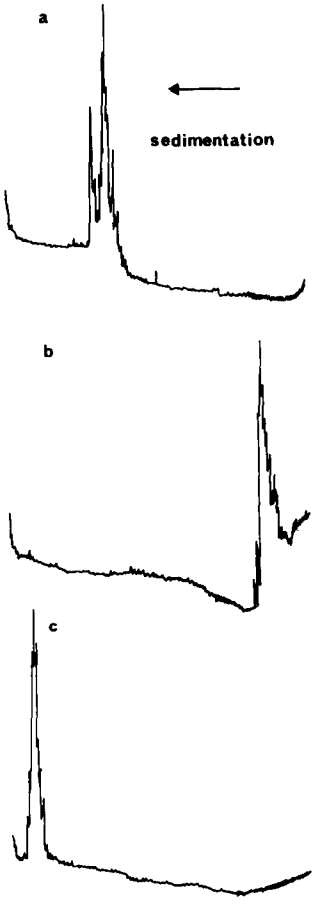

Fig. 2. Sedimentation of nucleoids from cells treated with *p*-aminophenol. (a) Untreated cells, (b) 0.25 mM *p*-aminophenol, and (c) 2 mM *p*-aminophenol. Peak positions on gradients were determined by absorbance at 254 nm.

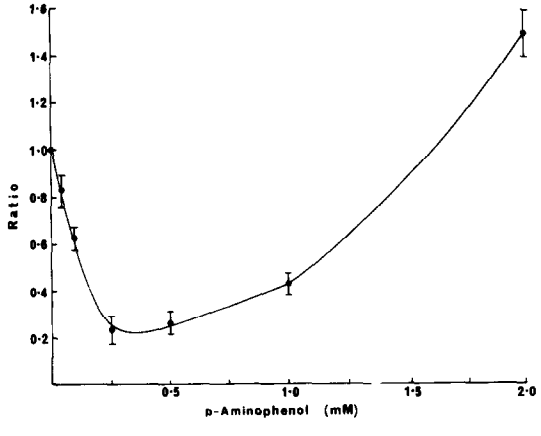

Fig. 3. Effect of *p*-aminophenol concentration on sedimentation of nucleoids. Incubation conditions were as described in Materials and Methods. The ratio refers to the distance sedimented by treated nucleoids divided by that of untreated nucleoids. Error bars represent S.E.M.

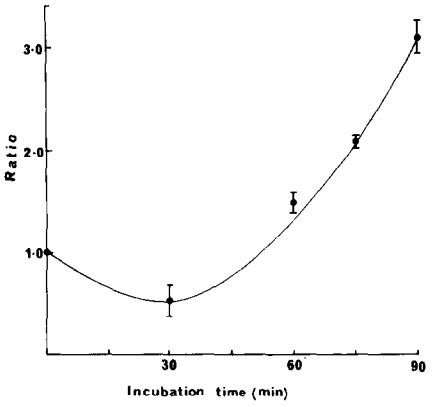

Fig. 4. The effect of incubation time with 2 mM *p*-aminophenol on sedimentation of nucleoids. Ratio is as in legend to Fig. 3. Error bars represent S.E.M.

mentation up to 30 min incubation time. Further incubation gives rise to a time-dependent increase in sedimentation reaching a value approximately three times that of the untreated cells after 90 min incubation. The increased sedimentation rate observed could be due to an increased degree of supercoiling of DNA in the treated cells. Sedimentation in sucrose gradients in the presence of ethidium bromide has been used to determine the degree of supercoiling of nucleoid DNA [19]. The concentration of ethidium used to give a minimum in the sedimentation rate provides a measure of the degree of supercoiling. The results in Fig. 5 compare the sedimentation of nucleoids in the presence of ethidium for untreated and 2 mM *p*-aminophenol treated cells. A characteristic biphasic response with increasing ethidium concentration is obtained for untreated cells reaching a minimum at a concentration of approximately 2 μg/ml ethidium. At this concentration of ethidium little or no effect on the sedimentation of treated nucleoids is observed. A minimum in sedimentation rate is achieved for treated nucleoids at 5 μg/ml ethidium and an increase in sedimentation rate is not observed up to 16 μg/ml ethidium. Furthermore the minimum reached at approximately 5 μg/ml is significantly higher than that obtained with untreated cells at 2 μg/ml ($P < 0.05$).

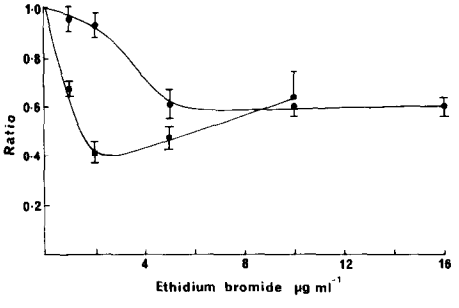

Fig. 5. Effect of ethidium bromide concentration on nucleoid sedimentation from (■) untreated cells, (●) cells treated with 2 mM *p*-aminophenol. Incubation with *p*-aminophenol was for 60 min followed by sedimentation analysis on gradients containing different concentrations of ethidium bromide. Ratio is as in legend to Fig. 3. Error bars represent S.E.M.

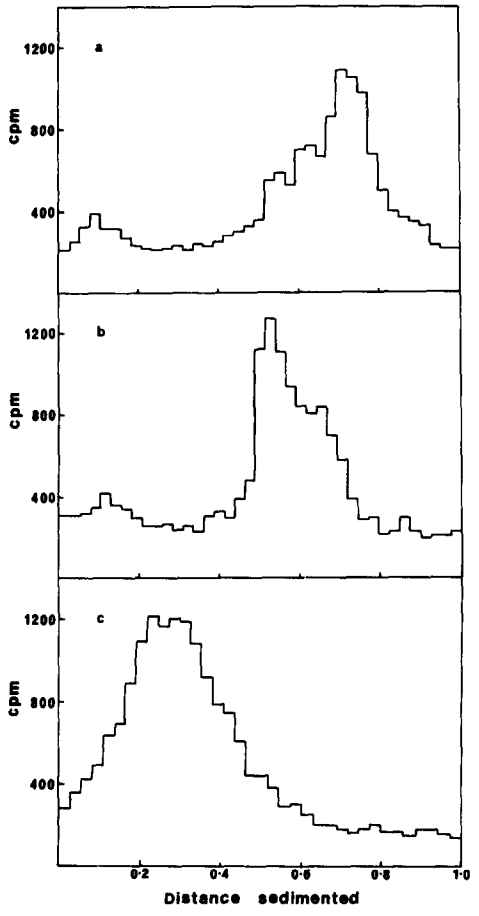

Fig. 6. Alkaline sucrose gradient profiles of DNA from cells treated with *p*-aminophenol. (a) Untreated cells, (b) 0.25 mM *p*-aminophenol, and (c) 2 mM *p*-aminophenol.

The effect of *p*-aminophenol on DNA structure was further investigated by determining the appearance of single strand breaks in DNA using alkaline sucrose gradient analysis. A typical set of gradient data appears in Fig. 6 showing a concentration-dependent decrease in DNA sedimentation compared to that from untreated cells. When single strand breakage is determined it is clear that a dose-dependent reduction in molecular weight of DNA occurs. The number of single strand breaks in DNA with increasing *p*-aminophenol concentration is presented in Table 1. A time course for production of breaks in DNA by *p*-aminophenol reveals that the majority of breakage has occurred after 30 min incubation (data not shown). The great majority of the cells were found to be intact and viable for the duration of the experiments. Trypan blue exclusion demonstrated that 95 ± 1% of cells were viable after incubation for 60 min with 2 mM *p*-aminophenol.

Table 1. Weight average molecular weight and number of breaks in DNA from untreated and *p*-aminophenol treated cells

p-Aminophenol (mM)	Molecular weight (MW × 10^{-8})	Breaks/10^{8}*
0	2.28 ± 0.25†	
0.25	1.20 ± 0.27	0.39
2.0	0.60 ± 0.23	1.23

Molecular weight was determined by sedimentation in alkaline sucrose gradients.

* Number of breaks is determined according to:

$$n \text{ (number of breaks)} = \left(\frac{1}{MW_{treated}} - \frac{1}{MW_{untreated}}\right) \times 10^{8}.$$

† Represents one standard deviation.

DISCUSSION

A rapid and marked inhibition of DNA synthesis was observed in human lymphoblastoid cells after incubation with *p*-aminophenol. This compound has previously been shown to be a potent nephrotoxin [11]. It appears that damage to mitochondria plays an important part in renal tubular necrosis induced by *p*-aminophenol [15]. Failure to observe the same effects in isolated mitochondria suggests that *p*-aminophenol must be metabolized before it becomes effective. It is possible that a derivative may play a role in the nephrotoxicity. Indeed some evidence exists which demonstrates that *p*-aminophenol is metabolized in rats [12]. The effect on DNA synthesis obtained in this study would suggest that *p*-aminophenol or a metabolic derivative may be effective in inducing renal damage through such an interaction, in addition to its observed effects on energy metabolism. Such a dramatic effect on DNA synthesis would be expected to give rise to a cytotoxic response in lymphoblastoid cells. In this respect it is somewhat surprising that *p*-aminophenol has been reported to be organ-specific [13]. While *p*-aminophenol inhibited gluconeogenesis and reduced ATP content in renal cells it left these processes unchanged in liver cells isolated from intravenously injected rats [13].

It seems likely that the inhibition of DNA synthesis observed after treatment of cells with *p*-aminophenol is due, at least in part, to alterations in chromatin structure. In this study we have demonstrated time-dependent and dose-dependent changes in the sedimentation characteristics of nucleoids from treated cells. The decrease in sedimentation at doses of *p*-aminophenol over the range of 0.05–0.5 mM is compatible with damage to DNA as observed with other chemicals [20], u.v. damage [20], and ionizing radiation damage [17]. However at higher concentrations of *p*-aminophenol an increase in sedimentation of nucleoids is observed (Fig. 3). Results with ethidium bromide would indicate that this increased sedimentation rate is due to an increased degree of negative supercoiling of the DNA [21]. A higher concentration of ethidium bromide, than that used in untreated cells is required to bring the DNA to the most relaxed state. However the extent of relaxation is significantly less that that observed in untreated cells. The latter observation could mean that the degree of supercoiling has not in fact increased but that other alterations to chromatin

structure, induced by *p*-aminophenol interfere with the binding of ethidium and contribute to the changes in sedimentation observed. The appearance of single strand breaks in DNA as determined by alkaline sucrose gradient analysis agrees with decreased sedimentation of nucleoids obtained after treatment with 0.25 mM *p*-aminophenol. As single strand breaks are introduced into DNA a decrease in the level of supercoiling would be expected [17]. The results using 2 mM *p*-aminophenol indicate a greater degree of breakage of DNA (Fig. 6). This finding appears to be contrary to that observed using nucleoid sedimentation analysis in which treated nucleoids (2 mM *p*-aminophenol) sediment more rapidly than untreated (Fig. 2). Increased sedimentation could arise due to direct interaction of *p*-aminophenol or a derivative with DNA or indirectly through interaction with chromatin proteins. These sites in turn may give rise to single strand breaks under alkaline conditions. Alternatively, single strand breaks may arise at alkali labile sites that play no role in the conformational changes that lead to an increase in sedimentation under neutral conditions.

The results reported here provide additional evidence for the toxicity of a compound which is chemically related to phenacetin, and which has been shown to occur in significant amounts as a metabolite of both phenacetin and paracetamol in different organisms [13, 14, 22, 23]. Previous results have demonstrated that similar concentrations of *p*-aminophenol interfere with energy metabolism in kidney mitochondria [13, 15]. While these results can explain the nephrotoxicity caused by analgesic abuse it is possible that the concentrations used are higher than those obtained in the renal tubule or the bladder. However repeated exposure to lower concentrations of *p*-aminophenol may have the same overall effect. In view of the effects of *p*-aminophenol on DNA synthesis and structure it is conceivable that this compound or a structural analog may also play a role in the development of transitional cell carcinoma which is reportedly high in analgesic abusers [7, 8].

Acknowledgements—The authors wish to thank Mark Davidson for the preparation of media. This work was supported by funds from the University of Queensland.

REFERENCES

1. O. Spuhler and H. U. Zollinger, *Z. clin. Med.* **151**, 1 (1953).
2. O. Norderfelt and N. Ringertz *Acta Med. Scand.* **170**, 385 (1961).
3. L. A. Jacobs and J. G. Morris, *Med. J. Aust.* **2**, 531 (1962).
4. U. Bengtsson, L. Angervall, H. Ekman and L. Lehmann, *Scand. J. Urol. Nephrol* **2**, 145 (1968).
5. J. S. Taylor, *Med. J. Aust.* **1**, 407 (1972).
6. U. Bengtsson, S. Johansson and L. Angervall, *Kidney Int.* **13**, 107 (1978).
7. N. Hultengren, L. Lagergren and A. Ljungvist, *Acta Chir. Scand.* **130**, 314 (1965).
8. L. Angervall, U. Bengtsson, C. G. Zetterlund and M. Zsigmond, *Br. J. Urol.* **41**, 401 (1969).
9. J. H. Stewart and E. D. M. Gallery, *Aust. N.Z. J. Med.* **6**, 498 (1976).
10. J. F. Mahoney, B. G. Storey, R. C. Ibanez and J. H. Stewart, *Aust. N.Z. J. Med.* **7**, 463 (1977).
11. C. R. Green, K. N. Ham and J. D. Tange, *Br. Med. J.* **1**, 162 (1969).
12. I. C. Calder, C. C. Funder, C. R. Green, K. N. Ham and J. D. Tange, *Br. Med. J.* **4**, 518 (1971).
13. J. D. Tange, B. D. Ross and J. G. G. Ledingham, *Clin. Sci. molec. Med.* **53**, 485 (1977).
14. B. B. Brodie and J. Axelrod, *J. Pharmac. exp. Ther.* **97**, 58 (1949).
15. C. A. Crowe, I. C. Calder, N. P. Madsen, C. C. Funder, C. R. Green, K. N. Ham and J. D. Tange, *Xenobiotica* **7**, 345 (1977).
16. P. R. Cook and I. A. Brazell, *J. cell. Sci.* **19**, 261 (1975).
17. M. F. Lavin and M. Davidson, *J. cell. Sci.* **48**, 383 (1981).
18. J. Houldsworth and M. F. Lavin, *Nucleic Acids Res.* **8**, 3709 (1980).
19. L. V. Crawford and M. J. Waring, *J. Molec. Biol.* **25**, 23 (1967).
20. P. R. Cook and I. A. Brazell, *Nature, Lond.* **263**, 679 (1976).
21. M. R. Mattern and R. B. Painter, *Biochim. biophys. Acta* **563**, 306 (1979).
22. R. H. Clothier, J. R. Dewar, M. A. Santos, A. D. North, S. Foster and M. Balls, *Xenobiotica* **11**, 149 (1981).
23. G. E. Smith and L. A. Griffiths, *Xenobiotica* **6**, 217 (1976).