Clayton, R. M. (1969) *Exp. Eye Res. 8*, 326.
Clayton, R. M. (1970) *Curr. Top. Dev. Biol. 5*, 115–180.
Clayton, R. M. (1974) *Eye 5*, 399–494.
Clayton, R. M., & Truman, D. E. S. (1967) *Nature (London) 214*, 1201.
Craig, S. P., & Piatigorsky, J. (1973) *Biochim. Biophys. Acta 299*, 642.
Delcour, J., & Papaconstantinou, J. (1970) *Biochem. Biophys. Res. Commun. 41*, 401.
Delcour, J., & Papaconstantinou, J. (1974) *Biochem. Biophys. Res. Commun. 57*, 134.
Genis-Galvez, J. M., Maisel, H., & Castro, J. (1968) *Exp. Eye Res. 7*, 593.
Ham, R. G. (1963) *Exp. Cell Res. 29*, 515.
Herbrink, P., & Bloemendal, H. (1974) *Biochim. Biophys. Acta 336*, 370.
Li, L. K., & Spector, A. (1972) *Exp. Eye Res. 13*, 110.
Li, L. K., & Spector, A. (1973) *Exp. Eye Res. 15*, 179.
Markert, C. L. (1963) *Science 140*, 1329.
Markert, C. L. (1968) *Ann. N.Y. Acad. Sci. 151*, 14.
Piatigorsky, J. (1976) *J. Biol. Chem. 251*, 4416.
Piatigorsky, J. (1978) *Exp. Eye Res. 27*, 227.
Piatigorsky, J., & Shinohara, T. (1977) *Science 196*, 1345.
Piatigorsky, J., Webster, H. deF., & Craig, S. P. (1972) *Dev. Biol. 27*, 176.
Piatigorsky, J., Zelenka, P., & Simpson, R. T. (1974) *Exp. Eye Res. 18*, 435.
Rabaey, M. (1962) *Exp. Eye Res. 1*, 310.
Rabaey, M. (1965) *Invest. Ophthalmol. 4*, 560.
Reszelbach, R., Shinohara, T., & Piatigorsky, J. (1977) *Exp. Eye Res. 25*, 583.
Söderholm, J., Allestam, P., & Wadström, T. (1972) *FEBS Lett. 24*, 89.
Thomson, I., Wilkinson, C. E., Burns, A. T. H., Truman, D. E. S., & Clayton, R. M. (1978) *Exp. Eye Res. 26*, 351.
Truman, D. E. S., & Clayton, R. M. (1974) *Exp. Eye Res. 18*, 485.
Truman, D. E. S., Brown, A. G., & Rao, K. V. (1971) *Exp. Eye Res. 12*, 304.
Truman, D. E. S., Brown, A. G., & Campbell, J. C. (1972) *Exp. Eye Res. 13*, 58.
Yoshida, K., & Katoh, A. (1971) *Exp. Eye Res. 11*, 184.
Zigler, J. S., Jr., & Sidbury, J. B., Jr. (1976a) *Comp. Biochem. Physiol. B 53*, 349.
Zigler, J. S., Jr., & Sidbury, J. B., Jr. (1976b) *Comp. Biochem. Physiol. B 55*, 19.

Accessibility of Deoxyribonucleic Acid in Chromatin to the Covalent Binding of the Chemical Carcinogen Benzo[*a*]pyrene†

Carolyn L. Jahn‡ and Gary W. Litman*

ABSTRACT: A model system utilizing rat liver microsomes to activate [^{3}H]benzo[*a*]pyrene (BP) in the presence of calf thymus nuclei was used to examine the ability of BP to bind regions of DNA which differ in their accessibility in chromatin. [^{3}H]BP-modified nuclei were digested with staphylococcal nuclease and DNase I, and the specific activity (cpm of [^{3}H]BP/A_{260} of DNA) of the DNA remaining undigested was determined. Both enzymes resulted in characteristic changes in specific activity as a function of digestion. No changes occurred during digestion of isolated [^{3}H]BP-DNA, and BP had no effect on the kinetics of digestion of DNA or nuclei, indicating that the specific activity changes seen in nuclear digests were due to preferential binding to DNA in regions of chromatin differing in enzyme susceptibility. The nucleosomal sites of [^{3}H]BP binding were determined by electrophoretic analysis of the resistant DNA and by examining the specific activity as a function of digestion of (1) nucleosome multimers isolated by sucrose gradient sedimentation of [^{3}H]BP-modified nuclei partially digested with staphylococcal nuclease and of (2) monomer subfractions obtained by KCl precipitation of H1-containing monomers. In addition, the distribution of [^{3}H]BP in fragments obtained from a DNase I digest of nuclei was compared to that of an isolated monomer fraction. These data led to the conclusion that BP binds to the spacer region and the outermost portions of the nucleosome core.

The polycyclic aromatic hydrocarbon benzo[*a*]pyrene (BP)[1] is ubiquitous as an environmental pollutant and is carcinogenic in animal test systems. Like most chemical carcinogens, it undergoes metabolic activation to form highly reactive intermediates capable of covalent binding to macromolecules. The 7,8-dihydrodiol 9,10-epoxide of BP has been identified as the major metabolite bound to DNA (Sims et al., 1974; King et al., 1976; Weinstein et al., 1976). By comparison to other metabolites, its increased reactivity with DNA has been correlated with its greater potency as a carcinogen (Jerina et al., 1976; Slaga et al., 1976; Levin et al., 1976), mutagen (Huberman et al., 1976; Jerina et al., 1976; Newbold & Brookes, 1976; Levin et al., 1977; Malaveille et al., 1977; Marquardt et al., 1977), and transforming agent (Marquardt et al., 1976, 1977). The in vivo covalent binding of BP has been shown to occur by derivitization of the 2-amino group

† From the Sloan-Kettering Institute, Walker Laboratory, Rye, New York 10580, and the Cornell University Graduate School of Medical Sciences, New York, New York 10021. *Received September 8, 1978; revised manuscript received January 3, 1979.* Supported by National Cancer Institute Grants CA 17085, CA 08748, and CA 24861 and U.S. Public Health Service Grant GM 01918.

* Address correspondence to this author at Sloan-Kettering Institute.
‡ Submitted by C.L.J. in partial fulfillment of the requirements for the Ph.D. degree at Cornell University Graduate School of Medical Sciences. Present address: Department of Bacteriology and Immunology, University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, NC.

[1] Abbreviations used: BP, benzo[*a*]pyrene; EDTA, ethylenediaminetetraacetate.

0006-2960/79/0418-1442$01.00/0 © 1979 American Chemical Society

of guanine by the 7,8-dihydrodiol 9,10-epoxide of BP. In addition, minor adducts consisting of the diol epoxide bound to the amino groups of adenine and cytosine are formed (King et al., 1976; Baird & Diamond, 1977; Jeffrey et al., 1977; Meehan et al., 1977; Koreeda et al., 1978).

The size of BP, its modification of adenine, guanine, and cytosine at positions that are normally involved in hydrogen bonding, and its planar aromatic structure, which makes it a possible intercalating agent, suggest that BP binding to DNA should affect the structure and function of DNA. The identification of the reactive form of BP has allowed further study of the effects of binding, since greater extents of modification (0.1–5% of the bases) can be obtained with BP-diol epoxide. Conformational changes in BP-DNA have been demonstrated and indicate that BP results in localized regions of denaturation (Pulkrabek et al., 1977). This has further been shown to occur primarily in the vicinity of BP-adenine adducts (Kakefuda & Yamamoto, 1978). BP also results in the loss of template function of DNA in transcription by *Escherichia coli* RNA polymerase (Leffler et al., 1977) and inhibition of replication of the phage ϕx 174 (Hsu et al., 1977). In both cases, the carcinogen appears to prevent the further progression of the enzymes.

Further elucidation of the role of covalent binding is essential to the understanding of the initiation process of carcinogenesis. The reactive nature of carcinogens suggests that where a carcinogen binds will be dependent on both the specific structure of the activated carcinogen and the structure of the macromolecular target and that both aspects of the binding will determine the nature of the functional alterations produced. To date, most research has centered on identifying the amino acid or nucleotide specificity of carcinogens. An approach which has received relatively little attention is to examine how higher orders of structure determine where a carcinogen binds. Because DNA is the most likely target for initiation of carcinogenesis, it is a logical choice for such an investigation. Furthermore, the structure of DNA in chromatin has recently become an aspect of DNA structure which is particularly amenable to study. Thus, the purpose of this investigation was to examine the role of chromatin structure in determination of where carcinogens bind to DNA.

For this purpose, we have utilized incubation of nuclei with radioactive BP in the presence of an isolated rat liver microsome fraction and digestion of BP-modified nuclei with staphylococcal nuclease and DNase I, two enzymes which have been instrumental in defining the nucleosome structure of chromatin. The sites which are accessible to these enzymes can be probed for their reactivity with BP by determining the amount of BP bound to the DNA fragments obtained in the course of digestion. Interpretation of the binding distribution relative to the digestion patterns can be made in the context of the known preferential digestion of nucleosome spacer regions (vs. core) by staphylococcal nuclease and of transcriptionally active DNA by DNase I [reviewed by Felsenfeld (1978)].

Experimental Procedures

Preparation of Microsomes. Male, Fisher rats obtained from Charles River and weighing approximately 150 g were injected ip with 3-methylcholanthrene (20 mg/kg) in peanut oil and killed 18 h later. The livers were removed, and the microsome fraction was isolated as described by Pietrapaolo & Weinstein (1975). The microsomes were suspended in 0.25 M sucrose at 10–20 mg/mL of protein and stored at −20 °C. Protein concentration was determined by the method of Lowry (Lowry et al., 1951).

Isolation of Nuclei. Nuclei were isolated from frozen calf thymus by modification of the procedure of Johnson et al. (1975). Homogenization in a Virtis Model 60 homogenizer was carried out for 3 min at 1200 rpm in 0.32 M sucrose, 3 mM $MgCl_2$, and 0.5 mM $CaCl_2$. Nuclei were washed twice with 0.25 M sucrose, 3 mM $MgCl_2$, and 0.5 mM $CaCl_2$. All steps were performed at 0 °C.

[^{3}H]BP Binding. The reaction of [^{3}H]BP (Amersham/Searle) with nuclei was carried out in the 0.25 M sucrose solution at 2×10^8 nuclei/mL, 1 mg of microsomal protein/mL, and 1 mM NADPH at 37 °C for 30 min essentially as described (Jahn & Litman, 1977). [^{3}H]BP was added in ethanol to give a final concentration of 0.2 mCi/mL (8–24 μM). Following incubation, nuclei were washed twice in the 0.25 M sucrose solution containing 0.5% Triton X-100 and twice with the 0.25 M sucrose solution and then resuspended in digestion buffer, consisting of 3% sucrose, 1 mM Tris-HCl, and 0.1 mM $CaCl_2$, pH 7.9, for digestion with staphylococcal nuclease (Worthington or Sigma) and of 3% sucrose, 1 mM Tris-HCl, and 0.1 mM $MgCl_2$ for DNase I (Worthington) digestion. (Digestion with DNase I was also carried out in the $CaCl_2$ buffer with no apparent effect on the results.)

Digestion of Nuclei. Staphylococcal nuclease or DNase I was added at a concentration of 5–20 μg/mL to nuclei suspended at a concentration of $(1–2) \times 10^8$/mL. Digestion was allowed to proceed at 37 °C for up to 1 h. Aliquots were periodically removed, and digestion was stopped by the addition of EDTA to a final concentration of 5 mM and by cooling on ice. Acid solubility was determined by diluting an aliquot in 1 M $HClO_4$–1 M NaCl and determining the A_{260} of the supernatant. Total A_{260} was determined by suspending an equal sample in 2 M NaCl–5 M urea. Percent digestion was determined by subtracting the acid-soluble A_{260} at $t = 0$ from the A_{260} at each time point and dividing this by the total A_{260} at $t = 0$. This term was corrected for hyperchromicity by dividing by 1.3.

DNA Extraction. The undigested DNA was extracted by adding NaCl to 0.4 M and sodium dodecyl sulfate to 1% and incubating with 100 μg/mL of proteinase K (E. Merck) overnight at 37 °C. Samples were extracted once with phenol, chloroform, and isoamyl alcohol (25:24:1) and twice with chloroform and isoamyl alcohol (24:1), followed by precipitation with ethanol. Samples were resuspended in 0.15 M NaCl–0.015 M sodium citrate and treated with pancreatic ribonuclease (Worthington; heated to inactive DNase's), followed by a repeat of the extraction procedure and two ethanol precipitations. Monitoring of the specific activities at each point in the extraction procedure demonstrated that no changes in the ratio of [^{3}H]BP to A_{260} occur in the final two ethanol precipitations. This procedure for the extraction of DNA containing both noncovalently and covalently bound BP was found to be necessary for the precise determination of specific activity. The use of a less rigorous extraction procedure (Jahn & Litman, 1977) accounted for an opposite effect for DNase I than that illustrated under Results.

Specific Activity Determinations. The A_{260} of a dilution of each sample was determined, and duplicate aliquots of the dilution were counted in Biofluor (New England Nuclear) in a Beckman LS-230 scintillation counter. The ratio of A_{260} to A_{280} was used as an indication of DNA purity. To determine the statistical deviations in specific activity, triplicate samples, triplicate dilutions, and triplicate aliquots of dilutions were analyzed.

Digestion of DNA. DNA extracted from [^{3}H]BP-modified nuclei was digested with staphylococcal nuclease or DNase

I under conditions identical with those of nuclei, with 0.5–1 μg/mL of either enzyme and a DNA concentration of 0.5 mg/mL. Aliquots were either precipitated directly by dilution in 1 M $HClO_4$–1 M NaCl or treated the same as for nuclei, with digestion terminated by addition of EDTA, followed by the extraction procedure.

Sucrose Gradients. Aliquots of the digestion (0.5–1 mL) were layered on 5–20% (w/v) sucrose gradients (5 mM in EDTA) and were centrifuged at 25000 rpm for 16 h in a SW27 rotor with a Beckman L2-65B ultracentrifuge. Gradients were collected as 1-mL fractions from the bottom of the tube, and the A_{260} was monitored, while collecting, with a Pharmacia UV monitor. Pooled fractions from gradients were extracted as described above.

Monomer Fractionation. Monomer fractions pooled from six simultaneously run gradients of the same partial digest were concentrated by ultrafiltration. EDTA (0.5 mM) was added while the sample was concentrating to lower the sucrose concentration below 5%. Fractionation into H1-containing and -noncontaining monomers was performed according to Olins et al. (1976), modified for use with smaller amounts of material. KCl was added dropwise to the sample (10–20 A_{260}/mL) with continuous mixing to a concentration of 0.2 M. The precipitate was allowed to develop overnight at 4 °C and was separated by centrifugation at 12000*g* for 10 min.

Digestion of Monomers. Monomers fractionated as described above (supernatant fraction) were dialyzed against 10 mM Tris-HCl–0.25 mM EDTA, pH 7.9, followed by dialysis against 10 mM Tris-HCl–0.25 mM EDTA and 1 mM $MgCl_2$, pH 7.9. DNase I was added at 0.5–5 μg/mL, and digestion was allowed to proceed for up to 10 min at 37 °C.

Gel Electrophoresis. Samples from staphylococcal nuclease digests and isolated nucleosome multimers were analyzed on 3% acrylamide–0.5% agarose or 6% acrylamide tube or slab gels with 0.04 M Tris–0.02 M sodium acetate and 0.002 M EDTA, pH 7.0, buffer. Samples from DNase I digests were electrophoresed on 12% acrylamide and 7 M urea tube or slab gels prepared and run according to the procedure of Maniatis et al. (1975). Bromophenol blue and xylene cyanol were included as size markers as described. Gels were stained overnight in 0.005% Stains All (Canalco) in 50% formamide and destained in running water. Tube gels were scanned with a Beckman Acta CIII at 550 nm. Gels were sliced into 1.5-mm sections, solubilized in 3% Protosol in Econofluor (New England Nuclear) by heating overnight at 37 °C, and counted.

Results

Digestion of [^{3}H]BP-Modified DNA. In order to analyze the distribution of BP in chromatin by enzyme digestion, it was first necessary to examine the effect of BP binding on the digestion process. For these experiments BP-DNA was obtained either by modification of calf thymus DNA directly or by modification of nuclei followed by isolation of DNA. The kinetics of digestion of modified and unmodified DNA and the relative amount of [^{3}H]BP in digested and undigested fractions were determined. DNA modified by either procedure gave identical results. With either enzyme the kinetics of digestion of modified and unmodified DNA were the same (Figure 1a); the release of BP paralleled the release of nucleotides (Figure 1a), and the specific activity of the undigested DNA remained the same at various times throughout digestion (Figure 1b). It was concluded that modification with BP did not alter the sensitivity of DNA to digestion by either enzyme and that the distribution of BP in DNA was random with respect to digestion.

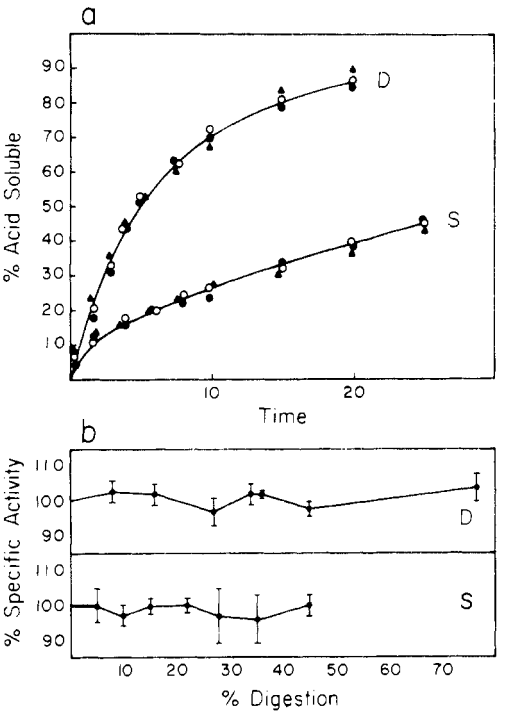

FIGURE 1: Digestion of BP-modified DNA. DNA extracted from [^{3}H]BP-modified nuclei and unmodified calf thymus DNA were digested with DNase I (D) and staphylococcal nuclease (S). The kinetics of digestion were determined and are plotted in (a) as the percent of the total A_{260} or the percent of total cpm which was acid soluble as a function of time. (○) Unmodified; (●) BP modified; (▲) [^{3}H]BP in cpm. In (b), triplicate samples of DNA at each time point were extracted and precipitated, and the specific activity was determined.

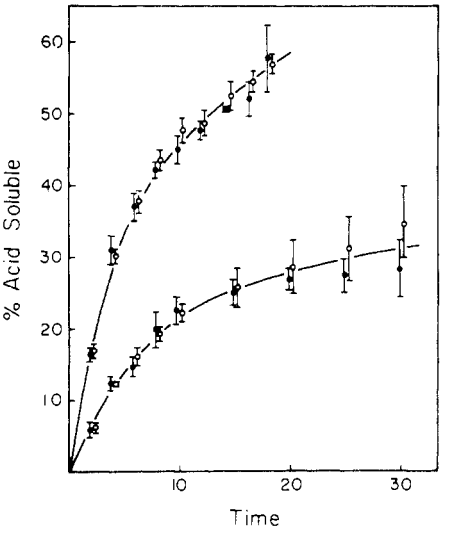

FIGURE 2: Kinetics of digestion of BP-modified nuclei. Modified and unmodified nuclei were digested with DNase I (top curve) and staphylococcal nuclease (bottom), and the percent of the total A_{260} which was acid soluble was determined as a function of time. (○) Unmodified; (●) modified.

Digestion of [^{3}H]BP-Modified Nuclei. To determine whether BP binding affected digestion of nuclei, the kinetics of digestion of unmodified and modified nuclei were examined. As seen in Figure 2, modification with BP did not change the kinetics of production of acid-soluble nucleotides. Sucrose gradient profiles of lysed nuclei and electrophoretic profiles of extracted, undigested DNA at various times of digestion indicated that modified and unmodified nuclei yielded identical digestion products.

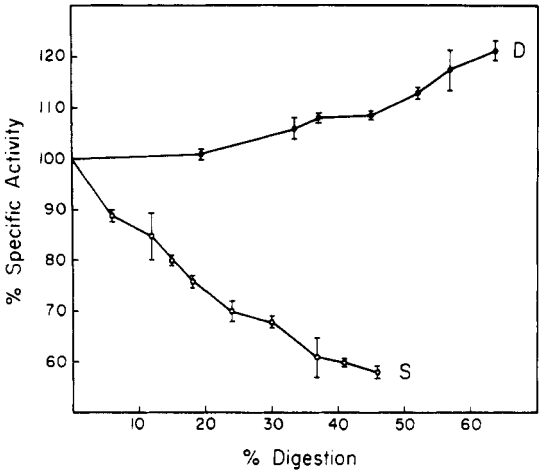

FIGURE 3: Specific activity of DNA as a function of digestion of [^{3}H]BP-modified nuclei. Triplicate samples of nuclei digested to increasing extents with DNase I (D) or staphylococcal nuclease (S) were extracted and precipitated, and the specific activity (cpm of [^{3}H]BP/A_{260} of DNA) was determined. The results are plotted as the percent of the initial specific activity as a function of the percent of the DNA digested (determined from the acid solubility).

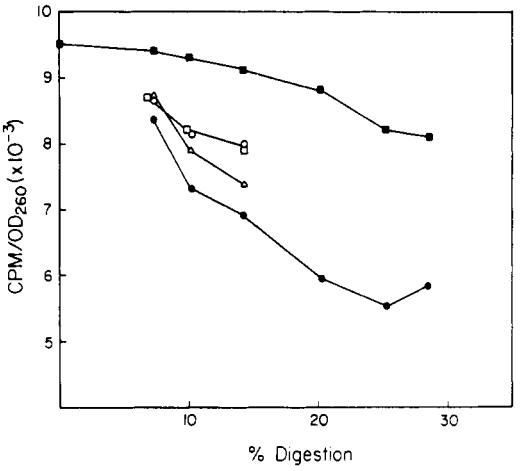

FIGURE 4: Specific activity of nucleosome multimers as a function of digestion with staphylococcal nuclease. Gradient fractions pooled as shown in Figure 8 were extracted, and the specific activities (cpm of [^{3}H]BP/A_{260} of DNA) were determined. The results are presented as the specific activity of the total sample (without fractionation, as in Figure 7) and each of the multimers as a function of the percent of the DNA digested (determined as the acid-soluble A_{260} of the total sample). (■) Total; (□) tetramer; (○) trimer; (△) dimer; (●) monomer.

Figure 3 illustrates that in contrast to the results obtained with [^{3}H]BP-DNA (Figure 1b), the specific activity of the DNA remaining after digestion of [^{3}H]BP nuclei with staphylococcal nuclease or DNase I changes as a function of digestion. In the experiment shown, staphylococcal nuclease resulted in a decrease in specific activity equivalent to approximately 40% of the initial specific activity by 50% digestion. Results of a number of experiments showed decreases in specific activity ranging from 30 to 60% by 50% digestion. DNase I consistently results in an 8–14% increase in specific activity by 50% digestion (up to 20% as digestion approached completion). However, the DNase I results were not strictly linear; instead, the specific activity was fairly constant early in the digestion and then linearly increased (beyond 15% digestion).

The dependence of the specific activities of digested and undigested regions of DNA on the extent of microsomal activation and binding was investigated by modifying nuclei for varying periods of time (10, 20, or 30 min) or with varying concentrations of BP (for the standard 30 min). The binding of BP was linear with time of incubation and with concentration, and the levels of modification obtained ranged from one BP per 10^5–10^6 bases, equivalent to one BP per 500–5000 nucleosomes. In all cases, the same pattern of change in specific activity was apparent.

Analysis of Staphylococcal Nuclease Digestion Products. The loss of BP with digestion by staphylococcal nuclease indicated that BP was preferentially bound in DNA regions sensitive to digestion by staphylococcal nuclease (i.e., spacer regions). Since the kinetics of digestion are indicative of both cleavage to produce smaller repeats and removal of spacer from each size repeat, the specific activity of each multimer was examined to determine if spacer removal resulted in a specific activity change. Modified nuclei were partially digested with staphylococcal nuclease, and, following sedimentation, the fractions corresponding to monomer, dimer, trimer, and tetramer were pooled, and the specific activity of the extracted DNA was determined. As seen in Figure 4, increasing sizes (monomer, dimer, trimer, and tetramer) had increasing specific activities at any given time point during digestion, and all of the isolated multimers had specific activities less than the total sample. The specific activity of each multimer decreased as a function of digestion, and the decrease occurred at a different rate for each size. The specific activity of the monomer fraction leveled off after 20% of the DNA was digested. Continued digestion of the total sample to 50% yielded DNA of the same specific activity as the plateau in the monomer curve. These results were consistent with the specific activity change of the total sample occurring as a result of spacer removal from each size repeat.

The purity of the sucrose gradient fractions was determined by electrophoretic analysis of each fraction used in the determination of specific activity. This indicated that dimer, trimer, and tetramers were contaminated with their neighboring peaks, while monomer was free of DNA migrating at other sizes. Since isolated multimers obtained at different percentages of digestion had the same proportion of stain in each peak, the differences observed as a function of digestion could not be a result of contaminating fractions. To further assess the extent to which the pooling affected the results, the pooled fractions were concentrated and resedimented. The resulting fractions still contained components from the adjacent peaks but in reduced amounts. This purification step did not appreciably alter the specific activity results (not illustrated).

Electrophoresis of the total DNA from various time points of digestion was also used to distinguish the amount of BP bound to different size DNA fragments and to determine if the loss of BP was correlated with decrease in size due to removal of spacer. This method complemented the sucrose gradient experiments described above since it allowed analysis of the entire sample at each time point (as shown in Figure 3) instead of relying on pooled subpopulations. Since random labeling of the DNA would result in coincident profiles of [^{3}H]BP and stain, any lack of coincidence would indicate differences in specific activity and could be interpreted in terms of preferential binding of BP.

Staphylococcal nuclease digested samples were examined on both 3% acrylamide–0.5% agarose gels and 6% acrylamide gels, since the fragments larger than monomer and smaller than monomer, respectively, are better resolved with the two gel systems. Both analyses demonstrated that [^{3}H]BP was associated with fragments of all sizes, including those smaller than monomer in size. If these submonomer fragments were a result of internal cleavage of monomers after removal of

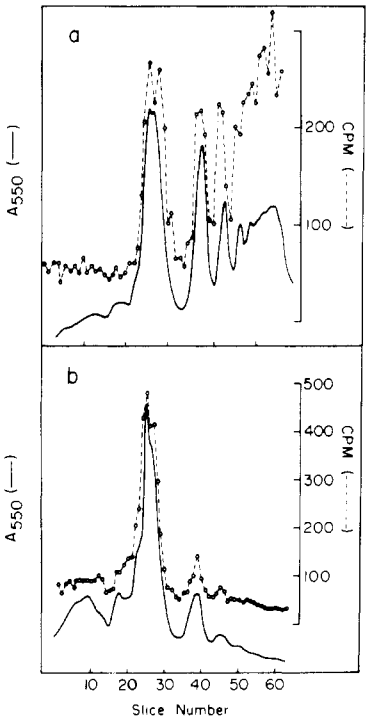

FIGURE 5: Electrophoretic analysis of staphylococcal nuclease digests. Samples at two different time points of digestion with staphylococcal nuclease, analyzed on 6% acrylamide tube gels, are shown. Electrophoresis was from right to left. (a) and (b) correspond to 17 and 33% digests, respectively. (—) A_{550} scan of stained gel; (●) cpm of [^{3}H]BP per slice.

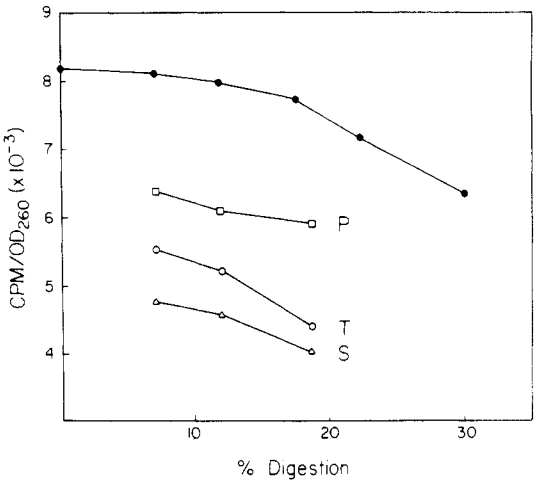

FIGURE 6: Specific activity as a function of digestion (monomer fractions obtained by KCl precipitation). The DNA was extracted from the total staphylococcal nuclease digest and from each of the monomer fractions obtained at three time points of digestion, and the specific activity (cpm of [^{3}H]BP/A_{260} of DNA) was determined. (●) Total digest of nuclei; (○) total monomer (T, unfractionated); (□) precipitated (P) monomer fraction; (△) supernatant (S) monomer fraction.

spacer, the association of counts with fragments of this size indicates that some BP must be bound to the core DNA. The profiles also indicated that fewer counts relative to stain were associated with submonomer-sized fragments than in sizes greater than monomer. Representative profiles of two different times of digestion analyzed on 6% acrylamide gels are shown in Figure 5 and illustrate the finding that, in general, the counts, relative to stain, decreased in decreasing size fragments. This is particularly clear in the monomer, dimer, and trimer peaks in Figure 5a. Although the two monomer peaks in Figure 5a did not appear to be appreciably different in specific activity, in Figure 5b there was a distinct difference in the counts profile in the 160 base-pair peak (shoulder) vs. the 140 base-pair peak. In most cases, these two peaks, although very well defined in the stain profiles, were not well separated in the cpm profiles (only one slice between peaks). The overlapping of the slices prevented us from making any general conclusions concerning the relative specific activities of the 140 and 160 base-pair peaks.

The electrophoretic profiles demonstrated the same specific activity changes determined by sucrose gradient fractionation since the counts relative to stain decreased in each size repeat with increasing digestion, and the counts decreased in proportion to size at any given time point. In addition, the specific activities of all of the multimers above trimer appeared to be equal.

Fractionation of Monomers by KCl Precipitation. To further explore the preferential binding of BP to spacer DNA, the relationship of histone H1 binding to spacer (vs. core) was utilized as a means of examining monomers with and without spacer DNA (i.e., with and without H1). Several groups have described differential precipitability of monomers with KCl, whereby monomers containing H1 are selectively precipitated (Olins et al., 1976; Wittig & Wittig, 1977). Thus, it was expected that the fractions obtained by KCl precipitation would differ in specific activity in proportion to the amount of spacer region which remained associated with H1.

Pooled monomer fractions from sucrose gradients of samples from differing times of digestion with staphylococcal nuclease were precipitated with KCl, and three fractions were characterized: (1) total (before KCl), (2) pellet (H1 containing), and (3) supernatant (non-H1). The amount of monomer which was precipitated decreased with increasing digestion, and it was apparent that, beyond 15% digestion, the amount of monomer precipitated plateaued and also was more variable. Thus, at 7–9% digestion, 64–67% of the monomer was precipitated, at 12–14% digestion, 30–37% was precipitated, and at 16–18% digestion, a range of 20–50% was precipitated (three experiments). The results in Figure 6 illustrate the specific activities of each monomer fraction as well as the total sample. The monomer fractions had relative specific activities in the order pellet > total ≥ supernatant at all times of digestion. The specific activity of the total monomer fraction converged with the supernatant fraction, reflecting the decrease in the amount of monomer which was precipitable as digestion proceeded. The specific activity differences between these three fractions would support the contention that DNA fragments from monomers which contain proportionately more spacer DNA (and remain associated with H1) have a greater amount of BP bound than monomer DNA fragments.

Analysis of DNase I Digestion Products. The increase in specific activity as a function of digestion with DNase I suggested that BP was preferentially located in a region of DNA which was protected from DNase I digestion. The location of BP relative to sites of digestion by DNase I was investigated by examining the distribution of [^{3}H]BP in different size DNA fragments at various time points of digestion. Again, a nonrandom association of BP with any of the fragments produced would be indicated by a lack of correspondence of the counts profile with the staining. Samples from DNase I digested nuclei were analyzed on denaturing 7 M urea and 12% acrylamide gels. The amount of counts and stain in each of three regions of the gels shown in Figure 7 was calculated: 10–30 nucleotides (fractions 0–20), 30–120 nucleotides (fractions 20–50), and greater than 120 nucleotides (fractions 50–65). From these results it was apparent that

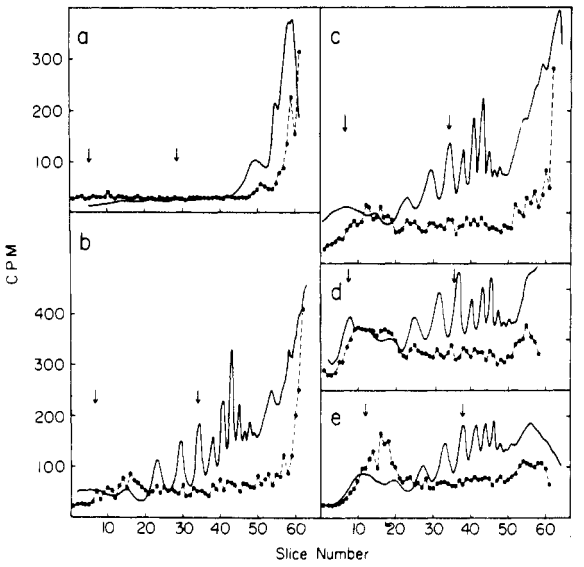

FIGURE 7: Electrophoretic analysis of DNase I digests. Samples of the extracted, undigested DNA at different percentages of digestion with DNase I were analyzed on 12% acrylamide and 7 M urea tube gels. The gels were stained and scanned [(—) A_{550}] and sliced and counted [(●) cpm of [^{3}H]BP]. Electrophoresis was from right to left, and the arrows designate the mobility of bromophenol blue (left) and xylene cyanol (right), which correspond to single-stranded DNA fragments approximately 10 and 50 nucleotides in length, respectively. (a), (b), (c), (d), and (e) represent 0, 19, 37, 50, and 64% digestion.

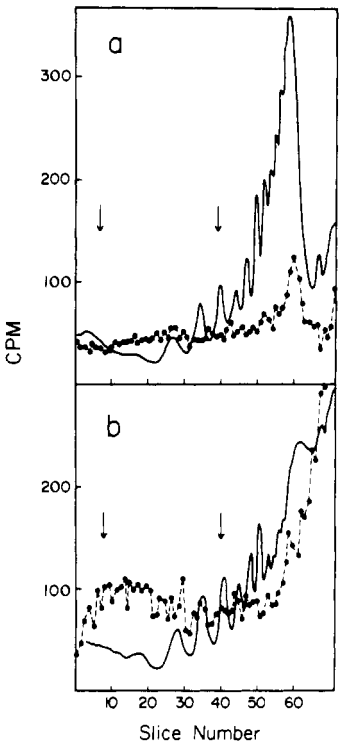

FIGURE 8: Electrophoretic analysis of DNase I digested monomers. Samples from a DNase I digest of monomers (supernatant fraction from KCl precipitation) and from a DNase I digest of nuclei (same preparation as used in the staphylococcal nuclease digest and isolation of the monomer fractions) were analyzed on 12% acrylamide, and 7 M urea tube gels (as described for Figure 7). (a) Monomer digest; (b) total digest; (—) A_{550}; (●) cpm of [^{3}H]BP.

most of the DNA larger than monomer disappeared with increasing digestion, while the small and intermediate size fragments accumulated. The specific activities of the large fragments and the intermediate fragments were approximately the same, but the small fragments were three times higher in specific activity. Thus, as the large fragments disappeared, the small fragments made a greater contribution to the total specific activity. The high specific activity of the smallest fragments suggested that the highly modified spacer region which was preferentially digested by staphylococcal nuclease was digested primarily to fragments 10–30 nucleotides in length by DNase I. Comparison of the cpm distribution of BP to that obtained when core monomers are digested after terminal labeling of the 140 base pairs with ^{32}P (Simpson & Whitlock, 1976; Sollner-Webb & Felsenfeld, 1977; Whitlock et al., 1977) indicated that the profile of BP relative to stain was very similar to the profiles of the 5′ ^{32}P label relative to stain (i.e., highest in fragments 10–30 nucleotides in length). This further indicated that the highly modified 10–30 nucleotide fragments might be a result of cleavage of spacer DNA and/or the end of nucleosome cores. This possibility was further investigated by digesting the KCl-soluble monomers, fractionated as above, with DNase I and examining the extracted DNA by electrophoresis.

Figure 8 shows the stain and cpm profiles comparing a total DNase I digest of nuclei and the digest of monomer prepared from the same nuclei. As a result of the staphylococcal nuclease digestion and removal of modified spacer, the specific activity of fragments produced from core monomer was approximately one-third of the specific activity of fragments produced from the total sample. In both cases the distribution of counts and stain in each of three regions, 10–30 nucleotides, 30–120 nucleotides, and 120–160 nucleotides, made up the same percentage of the total counts or stain in fragments 10–160 nucleotides in length (sum of the three regions), and the ratio of [^{3}H]BP/stain in the small fragments (10–30 nucleotides) was three times the ratio in the larger fragments. Thus, the BP remaining after removal of most of the spacer region by staphylococcal nuclease (Figure 8a) had the same location with respect to sites susceptible to digestion by DNase I as the total sample. This suggests that the remaining BP must be associated with the ends of the nucleosome core.

Discussion

The purpose of this study was to determine whether carcinogen binding was random or nonrandom in DNA modified in nuclei, that is, whether the distribution of BP reflected the accessibility of binding sites in chromatin. To use enzymes as probes of carcinogen binding, it was first necessary to determine if the digestion of DNA was random with respect to sites of binding. Although BP is relatively small in comparison to intercalating antibiotics, these compounds have been shown to inhibit nucleases (Facchinetti et al., 1978). This, in addition to the ability of BP to inhibit both transcription and replication, suggested that BP might affect the digestion process. Other possible deviations from random digestion could result from the conformational changes induced by BP modification or from the base sequence preference of the enzymes (Ehrlich et al., 1973) (which might result in faster or slower removal of guanine residues relative to the total DNA). The results of digestion of [^{3}H]BP-modified DNA indicated that digestion occurred randomly with respect to the binding of BP. Furthermore, a random pattern of digestion relative to binding was obtained when chromatin which was isolated from [^{3}H]BP nuclei was sheared and then digested with either enzyme (not illustrated). In this case, the absence of changes in specific activity seen with digestion of nuclei was accompanied by the absence of a pattern of nucleosome repeats when the samples were analyzed by gel electrophoresis. This further indicated that the specific activity changes seen by digestion of nuclei were a result of differential binding to

regions of DNA in chromatin which differed in their accessibility to enzymes. Because of the low levels of modification with BP used in these studies, it is possible that inhibition of digestion would not be observable. However, these results did demonstrate that at low levels of binding such effects would not interfere with the use of specific activity as a means of determining nonrandom distributions of BP in chromatin.

The specific activity changes as a function of digestion of [^{3}H]BP-modified nuclei were indicative of BP being preferentially bound in regions digested by staphylococcal nuclease and resistant to DNase I. To further determine where the carcinogen was located would require information on what these enzymes removed in the course of digestion as well as what the products of digestion were. Although the latter has been described in some detail, the former is only known by determining what is missing from the products (such as the absence of active genes in products of digestion with DNase I). In the case of staphylococcal nuclease, it is apparent that most of the digestion process involves removal of spacer DNA because there is a continuous conversion of multimers to monomers, and each repeat decreases in size as if the enzyme continues to digest spacer until it reaches the nucleosome core (Johnson et al., 1976; Shaw et al., 1976; Gaubatz & Chalkley, 1977; Lohr et al., 1977; Martin et al., 1977; Noll & Kornberg, 1977).

The results of the analyses of staphylococcal nuclease digestion of BP-modified nuclei are consistent with BP being preferentially bound in the spacer region, as suggested by earlier results (Jahn & Litman, 1977). Determination of the amount of BP bound in nucleosome multimers and in monomer subpopulations as a function of digestion demonstrates that the specific activity decrease of the total nuclear digests is indicative of higher extents of binding in the spacer region.

The DNase I results further confirmed the BP-spacer region relationship determined in the staphylococcal nuclease experiments, because the highly modified region digested by staphylococcal nuclease was localized to the nucleosome regions most susceptible to digestion by DNase I. Furthermore, the DNase I results demonstrated that all of the BP binding was clustered in the spacer region or at the very ends of the nucleosome core, since removal of 60–70% of the BP by staphylococcal nuclease yielded fragments reduced in specific activity by this amount relative to fragments produced from the total sample and with the same distribution according to size. Comparison of these results to those obtained by ^{32}P labeling of the 5′ ends of monomers (Simpson & Whitlock, 1976; Sollner-Webb & Felsenfeld, 1977; Whitlock et al., 1977) argues that the observed distribution of counts relative to stain are a function of binding in the spacer region and the termini of the core.

These studies have defined the binding pattern of a carcinogen in a static system and will aid in the interpretation of differences observed (1) in in vivo systems, where the state of chromatin is dynamic and may be changing as a function of carcinogen binding, and (2) with other carcinogens where other types of metabolic activation or adduct formation may result in a different distribution. Previous studies of acetylaminofluorene and nitrosamine binding in vivo were suggestive of a binding distribution similar to that observed in this study (Ramanathan et al., 1976; Metzger et al., 1977). However, because no attempt was made to correlate specific activity differences with the time course of digestion of chromatin by staphylococcal nuclease and DNase I or with the production of specific DNA fragments, several possible differences in the in vivo binding (particularly as a function of repair) require further evaluation of these results.

Acknowledgments

We thank Dr. Rosemary Bachvarova for helpful suggestions.

References

Baird, W., & Diamond, L. (1977) *Biochem. Biophys. Res. Commun. 77*, 162–167.

Ehrlich, S., Bertazzoni, U., & Bernardi, G. (1973) *Eur. J. Biochem. 40*, 143–147.

Facchinetti, T., Mantovani, A., Cantoni, L., Cantoni, R., & Salmona, M. (1978) *Chem.-Biol. Interact. 20*, 97–102.

Felsenfeld, G. (1978) *Nature (London) 271*, 115–122.

Gaubatz, J. W., & Chalkley, R. (1977) *Nucleic Acids Res. 4*, 3281–3301.

Hsu, W. T., Lin, E. J. S., Harvey, R. G., & Weiss, S. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3335–3339.

Huberman, E., Sachs, L., Yang, S. K., & Gelboin, H. V. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 607–611.

Jahn, C. L., & Litman, G. W. (1977) *Biochem. Biophys. Res. Commun. 76*, 534–539.

Jeffrey, A. M., Weinstein, I. B., Jennette, K. W., Grzeskowiak, K., Nakanishi, K., Harvey, R. G., Autrup, H., & Harris, C. (1977) *Nature (London) 269*, 348–350.

Jerina, D. M., Yagi, H., Hernandez, O., Dansette, P. M., Wood, A. W., Levin, W., Chang, R. L., Wislocki, P. G., & Conney, A. H. (1976) in *Polynuclear Aromatic Hydrocarbons Chemistry, Metabolism and Carcinogenesis* (Freudenthal, R., & Jones, P., Eds.) pp 91–113, Raven Press, New York.

Johnson, E. M., Hadden, J. W., Inoue, A., & Allfrey, V. G. (1975) *Biochemistry 14*, 3873–3884.

Johnson, E. M., Littau, V. C., Allfrey, V. G., Bradbury, E. M., & Matthews, H. R. (1976) *Nucleic Acids Res. 3*, 3313–3329.

Kakefuda, T., & Yamamoto, H.-A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 415–419.

King, H. W. S., Osborne, M. R., Beland, F. A., Harvey, R. G., & Brookes, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2679–2681.

Koreeda, M., Moore, P. D., Wislocki, P. G., Levin, W., Conney, A. H., Yagi, H., & Jerina, D. M. (1978) *Science 199*, 778–781.

Leffler, S., Pulkrabek, P., Grunberger, D., & Weinstein, I. B. (1977) *Biochemistry 16*, 3133–3136.

Levin, W., Wood, A. W., Yagi, H., Dansette, P. M., Jerina, D. M., & Conney, A. H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 243–247.

Levin, W., Wood, A. W., Lu, A. Y. H., Ryan, D., West, S., Conney, A. H., Thakker, D. R., Yagi, H., & Jerina, D. M. (1977) *ACS Symp. Ser. No. 44*, 99–126.

Lohr, D., Corden, J., Tatchell, K., Kovacic, R. T., & Van Holde, K. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 79–83.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Malaveille, C., Kuroki, T., Sims, P., Grover, P. L., & Bartsch, H. (1977) *Mutat. Res. 44*, 313–326.

Maniatis, T., Jeffrey, A., & van de Sande, H. (1975) *Biochemistry 14*, 3787–3794.

Marquardt, H., Grover, P. L., & Sims, P. (1976) *Cancer Res. 36*, 2059–2064.

Marquardt, H., Baker, S., Grover, P. L., & Sims, P. (1977) *Cancer Lett. (Amsterdam) 3*, 31–36.

Martin, D. Z., Todd, R. D., Lang, D., Pei, P. N., & Garrard, W. T. (1977) *J. Biol. Chem. 252*, 8269–8277.

Meehan, T., Straub, K., & Calvin, M. (1977) *Nature (London) 269*, 725–727.
Metzger, G., Wilhelm, F. X., & Wilhelm, M. L. (1977) *Biochem. Biophys. Res. Commun. 75*, 703–710.
Newbold, R. F., & Brookes, P. (1976) *Nature (London) 261*, 52–54.
Noll, M., & Kornberg, R. D. (1977) *J. Mol. Biol. 109*, 393–404.
Olins, A. L., Carlson, R. D., Wright, E. B., & Olins, D. E. (1976) *Nucleic Acids Res. 3*, 3271–3291.
Pietropaolo, C., & Weinstein, I. B. (1975) *Cancer Res. 35*, 2191–2198.
Pulkrabek, P., Leffler, S., Weinstein, I. B., & Grunberger, D. (1977) *Biochemistry 16*, 3127–3132.
Ramanathan, R., Rajalakshmi, S., Sarma, D. S. R., & Farber, E. (1976) *Cancer Res. 36*, 2073–2079.
Shaw, B. R., Herman, T. M., Kovacic, R. T., Beaudreau, G. S., & Van Holde, K. E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 505–509.
Simpson, R. T., & Whitlock, J. P., Jr. (1976) *Cell 9*, 347–353.
Sims, P., Grover, P. L., Swaisland, A., Pal, K., & Hewer, A. (1974) *Nature (London) 252*, 326–328.
Slaga, T. H., Viajc, A., Berry, D. L., Bracken, W., Buty, S. G., & Scribner, J. D. (1976) *Cancer Lett. (Amsterdam) 2*, 115–122.
Sollner-Webb, B., & Felsenfeld, G. (1977) *Cell 10*, 537–547.
Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H., & Nakanishi, K. (1976) *Science 193*, 592–595.
Whitlock, J. P., Jr., Rushizky, G. W., & Simpson, R. T. (1977) *J. Biol. Chem. 252*, 3003–3006.
Wittig, B., & Wittig, S. (1977) *Nucleic Acid Res. 4*, 3901–3917.

Formation of 7-*cis*- and 13-*cis*-Retinal Pigments by Irradiating Squid Rhodopsin†

Akio Maeda,* Yoshinori Shichida,‡ and Tôru Yoshizawa

ABSTRACT: Squid rhodopsin was irradiated with orange light (>530 nm) at various temperatures from −190 to 10 °C until a photo-steady-state mixture was formed. Then the chromophoric retinals were extracted from the photo-steady-state mixtures and their isomer composition was analyzed by high-performance liquid chromatography. In the case of a photo-steady-state mixture formed at −85 °C, large peaks in the chromatogram were found at the positions of both 7-*cis*- and 13-*cis*-retinals. Each peak was further identified by synthesizing the pigments from these retinals with cattle opsin or apobacteriorhodopsin. Both 7-*cis*- and 13-*cis*-retinals were also extracted from a photo-steady-state mixture formed by irradiation at −40, at 0, or at 10 °C. These isomers were scarcely detected in a photo-steady-state mixture formed by irradiation at −190 °C, though 9-*cis*-retinal was found as a major constituent in this mixture. Irradiation of lumirhodopsin at −190 °C, however, produced 7-*cis*-retinal pigment. These findings suggest that bathorhodopsin may have a conformation to prevent the formation of 7-*cis*-retinal from the all-trans form and that this particular conformation may be relaxed by the conversion of bathorhodopsin to lumirhodopsin.

Squid rhodopsin has 11-*cis*-retinal as its chromophore (Hubbard & St. George, 1958). Upon absorbing light it converts into the all-trans form and the conformation of rhodopsin changes through several kinds of intermediates, hypsorhodopsin (Shichida et al., 1979), bathorhodopsin (Yoshizawa & Wald, 1964), lumirhodopsin (Kropf et al., 1959), and LM-rhodopsin (Tokunaga et al., 1975; Shichida et al., 1978). Depending on pH, the final product is acid or alkaline metarhodopsin (Hubbard & St. George, 1958). Each intermediate is stable at its characteristic temperatures (Shichida et al., 1978, 1979). The excitation of visual cells is believed to be caused by one of the intermediates.

Besides this primary action of light, there is a secondary photoprocess which leads to the formation of isorhodopsin, a stable isomeric form of rhodopsin. Isorhodopsin can be produced by irradiation of rhodopsin with orange light at liquid nitrogen temperatures (Yoshizawa & Wald, 1964). Recently, the chromophore of isorhodopsin was identified as 9-*cis*-retinal in cattle and frog rhodopsin systems by means of high-performance liquid chromatography (LC)[1] (Kawamura et al., 1978). Isorhodopsin can also be synthesized from authentic 9-*cis*-retinal with cattle opsin (Hubbard & Wald, 1952–1953).

Since all the mono-*cis*-retinals can be produced by simply irradiating *all-trans*-retinal in polar organic solvents (Denny & Liu, 1977; Maeda et al., 1978b), it is of interest to test the possibility that 7-*cis*- and 13-*cis*-retinals can be formed at the retinal binding site of rhodopsin. Maeda et al. (1978a) previously observed the formation of a 7-*cis*-retinal pigment by irradiating cattle rhodopsin at −75 °C.

The present report gives data on the isomeric composition of retinals which can be extracted from photo-steady-state mixtures formed by irradiating squid rhodopsin at various temperatures where the different intermediates are stable.

Materials and Methods

Chemicals. All the solvents used were reagent grade of Nakarai Chemicals, Ltd., Kyoto, Japan. Petroleum ether (bp 30–50 °C) and diethyl ether were refined as described by Rotmans & Kropf (1975).

Preparation of Microvilli (Rhodopsin). A microvilli fraction from squid (*Todarodes pacificus*) retina was prepared by a slight modification of a method described previously

† From the Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan. *Received October 18, 1978; revised manuscript received January 3, 1979.* This research was supported partly by a grant from the Ministry of Education of Japan (148315).

‡ Predoctoral fellow of the Japan Society of the Promotion of Science.

[1] Abbreviation used: LC, high-performance liquid chromatography.

0006-2960/79/0418-1449$01.00/0 © 1979 American Chemical Society