INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International
300 N. Zeeb Road
Ann Arbor, MI 48106

Brothman, Arthur Richard

AUTORADIOGRAPHIC AND IMMUNOFLUORESCENT DETECTION OF LOW CONCENTRATIONS OF ACTINOMYCIN D BOUND TO HUMAN METAPHASE CHROMOSOMES

The University of Arizona PH.D. 1982

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __✓__.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print _____
3. Photographs with dark background ✓
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

AUTORADIOGRAPHIC AND IMMUNOFLUORESCENT DETECTION OF LOW CONCENTRATIONS OF ACTINOMYCIN D BOUND TO HUMAN METAPHASE CHROMOSOMES

by

Arthur Richard Brothman

A Dissertation Submitted to the Faculty of the

COMMITTEE ON GENETICS

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 8 2

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Arthur R. Brothman

entitled Autoradiographic and Immunofluorescent Detection of Low Concentrations of Actinomycin D Bound to Human Metaphase Chromosomes

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

	6/4/82 Date
	6/4/82 Date
	6-4-82 Date
	6-4-82 Date
	6/4/82 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

	6/14/82
Dissertation Director	Date

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: 

This dissertation is dedicated to the loving memory of my parents, who instilled in me a quest for knowledge.

ACKNOWLEDGMENTS

With heartfelt gratitude, I thank my advisor, Dr. Thomas J. Lindell for his continuous guidance and inspiration during the course of my graduate research.

I will be forever grateful to Dr. Robert M. Harris, chairman of the Committee on Genetics, for his encouragement and constant enthusiasm. To the other members of my committee, Drs. John R. Davis, John E. Endrizzi and Klaus Brendel, I convey my most sincere appreciation for all of their generous time and suggestions. I thank Dr. John J. Duffy for his helpful discussions and advice.

I acknowledge Ms. Iris Veomett and Dr. Raymond Nagle for their counsel in cytogenetics and immunology, respectively. A special note of thanks to Drs. Carol and Harris Bernstein for the extended use of their light microscope, and to Dr. Mary Hendrix for the use of her fluorescent microscope.

Finally, a very special thank you to my dear wife, Lisa, for all of her never-ending support and encouragement throughout my graduate studies.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS--Continued

LIST OF TABLES

ABSTRACT

The binding of low concentrations of actinomycin D (Act D) to fixed human metaphase chromosomes was studied using both autoradiographic and immunofluorescent techniques. At the concentration range of 0.001 - 0.1 μg/ml Act D is known to selectively inhibit rRNA synthesis. Although it was previously suggested that at these low concentrations Act D would selectively bind to the ribosomal cistrons, evidence also exists that the drug binds to non-ribosomal DNA, and inhibits rRNA transcription in an indirect fashion. Because of the conflicting data on Act D binding and a lack of focus on biologically relevant concentrations of drug, it was decided to systematically investigate the distribution of the drug binding in low concentrations to chromosomes from 72-hr human lymphocyte cultures.

Autoradiographic detection of [^{3}H]Act D bound to chromosomes showed no selective binding of the drug at concentrations that maximally inhibit rRNA synthesis. A new technique was employed using Formvar and potassium chromium sulfate as a pretreatment to autoradiography. This technique permitted simultaneous detection of silver grains and chromosome identification by G-banding. With autoradiographic exposure times of 1 and 7 days, there was a positive correlation of autoradiographic grains with chromosome length.

To increase sensitivity in detection of Act D bound to chromosomes, a specific anti-Act D antibody was generated in rabbits.

Antibody avidity was evaluated on the basis of a rapid charcoal assay. This charcoal assay was then used in development of a radioimmunoassay for Act D which is sensitive in quantitating the drug down to 0.005 μg/ml. The anti-Act D antibody was characterized to be IgG, and was shown to be specific for the pentapeptide lactone portion of the Act D molecule.

Indirect immunofluorescence of Protein A-purified IgG containing anti-Act D was used to detect drug bound to fixed human chromosomes. The antibody was shown to be specific for drug bound to chromatin. When 0.1 μg/ml Act D was bound to chromosomes, the drug was observed bound throughout the genome, with no selective binding at the ribosomal cistrons. This confirms the autoradiographic data and supports the model of extranucleolar regulation of rRNA synthesis. Preliminary results suggest that Act D binds to GC-rich DNA, since an R-banding pattern was observed in 5% of the immunofluorescent metaphases examined.

CHAPTER 1

AUTORADIOGRAPHIC DETECTION OF ACTINOMYCIN D BOUND TO CHROMOSOMES

Introduction

The naturally occurring antibiotic actinomycin D (Act D) is known to selectively inhibit rRNA synthesis in the concentration range of 0.001 - 0.1 µg/ml (Perry, 1962). Act D is proposed to intercalate into the DNA helix (Muller and Crothers, 1968) or "pseudo intercalate" as recently described by Takusagawa et al. (1982) and interfere with transcription by blocking the function of the eukaryotic RNA polymerases (Muller and Crothers, 1968). Perry (1964, 1973) has proposed that low concentrations of Act D, which inhibit rRNA synthesis, act selectively at the ribosomal cistrons, and therefore functions in a direct manner. Evidence for an alternative theory (Tsurugi, Morita, and Ogata, 1972; Lindell, 1976; Lindell, O'Malley, and Puglisi, 1978) indicates that the inhibitory function of low concentrations of Act D may be more complex, and that the drug may not act directly at the ribosomal cistrons. This mechanism predicts that low concentrations of Act D may bind to extranucleolar chromatin and inhibit mRNA synthesis, which codes for some regulatory protein(s) necessary for rRNA synthesis.

The latter model is supported by the action of a number of other inhibitors that are known to affect rRNA synthesis by a variety

of mechanisms (Lindell, 1980). α-Amanitin, a peptide toxin from the mushroom Amanita phalloides, was shown to be a specific inhibitor of RNA polymerase II (Lindell et al. 1970), the enzyme that catalyzes the synthesis of precursor mRNA. α-Amanitin has been shown to have no effect on RNA polymerase I, the enzyme that catalyzes the synthesis of ribosomal RNA (Roeder and Rutter, 1970). Tata, Hamilton, and Shields (1972) report that even though α-amanitin binds to RNA polymerase II, following exposure to this toxin, there is a marked reduction of newly synthesized 45S rRNA. This was also observed by Jacob, Sajdel, and Munro (1970), who suggest that some extranucleolar factors are controlling the synthesis of nucleolar RNA. The nucleoside analog, cordycepin (3'deoxyadenosine) was originally believed to have a selective effect on rRNA transcription in vivo (Siev, Weinberg, and Penman, 1969), but was later found to inhibit polyadenylation at the 3' end of most eukaryotic messenger RNAs (Darnel et al. 1971). The lack of polyadenylation of this presumably regulatory mRNA may be the cause of the inhibition of rRNA synthesis. Lindell (1976) showed that endogenous RNA polymerase I in isolated nucleoli is not inhibited by concentrations of Act D that inhibit rRNA synthesis in vivo (0.001 - 0.1 μg/ml), but that nucleoplasmic RNA polymerase II is partially inhibited in this concentration range of drug. All of these observations with various inhibitors support the notion that some regulatory protein is needed for the maintenance of rRNA synthesis in eukaryotic cells. Actinomycin D may inhibit the transcription of the mRNA that codes for this protein(s).

The nucleolus organizer regions (NORs) of chromosomes contain rDNA and are the sites of rRNA synthesis. They have been mapped in humans to the short arms of the D and G group chromosomes (13, 14, and 15; 21 and 22) by Evans et al. (1974) and Tantravahi et al. (1976). If Act D binds preferentially to the ribosomal cistrons at concentrations where the drug selectively inhibits rRNA synthesis, [^{3}H]Act D should be detected at these sites as observed by autoradiography. If the alternate theory is true, and Act D is also bound to extranucleolar DNA, no significant autoradiographic grains would be preferentially observed at the ribosomal cistrons at the low concentrations known to inhibit rRNA synthesis in intact cells.

Several investigators have previously examined the binding of [^{3}H]Act D to chromatin using autoradiography. Ebstein (1967, 1969) has shown that 10 µg/ml [^{3}H]Act D specifically labels the DNA of amphibian oocyte and salivary gland chromosomes. Camargo and Plaut (1967) have shown that 3.3 µg/ml [^{3}H]Act D labels the polytene chromosomes of Drosophila, and formalin fixation appears to decrease the drug binding. Simard (1967) labeled hamster fibroblasts with 25 µg/ml [^{3}H]Act D and claimed that the drug bound to a greater extent to the compact nuclear heterochromatin than it did to the euchromatin or the nucleolus. The opposite result was reported by Berlowitz, Pallotta, and Sibley (1969) since their data indicated that [^{3}H]Act D (0.74 µg/ml) bound less to the paternally derived, heterochromatic set of chromosomes in the male mealy bug than the drug bound to the maternally derived, euchromatic set. Sieger, Garweg, and Schwarzacher (1971) claimed that Act D (7 µg/ml) binds equally as well to both euchromatin and the more condensed

heterochromatin, and any differences observed are due mainly to the amount of DNA present. Cionini and Avanzi (1972) showed that autoradiographic detection of 3.5 μg/ml [^{3}H]Act D bound to polytene chromosomes of Phaseolus coccineus gave a constantly reproducible "banding" pattern, presumably due to the differential DNA distribution. This pattern was useful in identification of individual chromosomes.

Most of the studies of Act D binding to human chromosomes involved the use of drug concentrations greater than those that selectively inhibit rRNA synthesis. Ringertz, Darzynkiewicz, and Bolund (1969) labeled human lymphocyte cultures with 2.5 μg/ml [^{3}H]Act D and found that 60% of the binding sites were blocked by deoxyribonucleoprotein, suggesting that the protein content of chromatin plays a significant role in access to the DNA by Act D. Much of this protein is presumably lost after acetic acid-methanol fixation, and an increased number of sites was observed when lymphocytes were labeled after fixation.

Rocchi et al. (1974) labeled acetic acid-methanol fixed human chromosomes with 10 μg/ml [^{3}H]Act D. Of the chromosomes that could be identified, a nonrandom labeling distribution with little drug bound to the centromeric regions was observed. Hubner and Kononowicz (1977) labeled fixed human chromosomes with 7 μg/ml [^{3}H]Act D and observed a banding pattern of autoradiographic grains over chromosomes. They also postulate that Act D binds to the less condensed chromatin.

Miles (1970) labeled fixed human chromosomes at concentrations down to 0.04 μg/ml [^{3}H]Act D. This was the only study reported to date

in which the investigator examined chromosomes labeled with the concentration of Act D that selectively inhibits rRNA synthesis. Miles' data indicate that the long arm telomeres labeled more than the short arm telomeres, with the exception of the acrocentric chromosomes, which showed a light labeling on the short arms (the locations of rDNA). Miles also confirmed that acetic acid-methanol fixation of chromosomes causes an increased amount of Act D binding as no binding was observed on chromosomes fixed only in methanol. Miles' data did show some label on the short arms of the acrocentric chromosomes; however, it was the first evidence that biologically significant concentrations of Act D do not selectively bind to the ribosomal cistrons.

A summary of some of the previous observations of Act D-chromosome binding is given in Table 1-1. It should be noted that the drug concentrations studied vary significantly. With the exception of the work of Miles, no investigator studied the binding of Act D at concentrations which, in vivo, selectively inhibit rRNA synthesis. It can also be seen from the earlier literature that few conclusions can be drawn with regard to the binding of Act D to chromatin by autoradiographic techniques. The only consistent observation appears to be that acetic-alcohol fixation of chromosomes increases the accessibility of DNA to Act D. Acetic-alcohol fixation has been shown by Dick and John (1968) to remove histones from chromatin. It has been suggested that this histone extraction causes the increased Act D binding to DNA (Berlowitz et al. 1969). This chapter describes a series of experiments that were designed to critically examine and characterize Act D binding at low concentrations to fixed human chromosomes by autoradiography.

Table 1-1. Autoradiographic Detection of Act D Binding

Reference	Concentration Act D (g/ml)	Major Observations
Ebstein (1967, 1969)	10	Act D bound to DNA of Newt oocyte and salivary gland chromosomes.
Camargo and Plaut (1967)	3.3	Act D binds to DNA in Drosophila polytene chromosomes. Act D binding reduced if formalin used in fixation.
Simard (1967)	25	In hamster fibroblasts, Act D bound more to heterochromatin than to euchromatin.
Berlowitz, Pallotta, and Sibley (1969)	0.74	In the male mealy bug, Act D bound to euchromatic chromosomes more than heterochromatic chromosomes.
Sieger, Garvey, and Schwarzacher (1971)	7	Act D bound equally to heterochromatin and euchromatin in field voles.
Cionini and Avanzi (1972)	3.5	In Phaseolus coccineus Act D bound to polytene chromosomes in a distinct and reproducible band-like pattern.
Ringertz, Darzynkiewicz, and Bolund (1969)	2.5	More Act D bound to human chromosomes post-fixation than pre-fixation (acetic-methanol).
Rocchi et al. (1974)	10	Little Act D bound to centromeric regions of human chromosomes.
*Miles (1970)	0.04, 0.2, 0.4	In human chromosomes, long-arm telomeres labeled more than short-arm telomeres. Confirmed increased binding post fixation. At 0.04 µg/ml, Act D bound to G and D group short-arm, but not exclusively.
Hubner and Kononowicz (1977)	7	Act D bound to areas of lower chromatin condensation in human chromosomes.

*Miles was the only one to study concentrations of Act D that are in the selective rRNA synthesis inhibitory range (0.001-0.1 µg/ml).

Materials and Methods

Chromosome preparation. Human lymphocyte cultures, obtained from six male and six female volunteers, were grown for 72 hr in RPMI-1640 media (Gibco) supplemented with 25% fetal bovine serum (Gibco), and 120 µg/ml phytohaemagglutinin (Burroughs-Wellcome), with penicillin-streptomycin (Gibco) as a bacteriostatic agent. Cultures were grown in sterile disposable centrifuge tubes (Falcon Plastics) at 37°. Chromosomes were harvested following standard procedures as described by Moorhead et al. (1969), and fixed in 3 parts methanol:1 part acetic acid. (See Appendix A.)

Labeling chromosomes with Act D. Freshly prepared slides containing nicely-spread metaphase chromosomes were layered with 300 µL [^{3}H]Act D (Amersham) (0.004, 0.01, 0.03, 0.06, 0.1, 0.13, 0.2, or 1.0 µg/ml) and incubated with a coverslip in the horizontal position for 15 min in a humid chamber. Unbound Act D was removed by washing the slides three times in 95% ethanol (50 ml/wash). Studies performed by Jones (1976) and in this laboratory (Duffy and Lindell, in preparation) have shown that the Act D-DNA complex is stabilized in ethanol. A set of slides which were not exposed to [^{3}H]Act D were employed as controls. Slides which were expected to have a number of autoradiographic grains too great to identify individual G-banded chromosomes were first autoradiographed and then stained with Geimsa after development (see Appendix B). After labeling with [^{3}H]Act D, the slides on which individual chromosomes were to be identified were dried overnight in an oven at 56°, and the chromosomes were G-banded by the method of Muller and Rosenkranz (1972). (See Appendix A.)

Preparation of G-banded chromosomes for autoradiography. The G-banded chromosome slides were dipped into a 1% solution of Formvar (E. F. Fullam, Inc.) dissolved in 1,2 dichloroethane and excess liquid was drained on absorbent paper (Rutledge, 1979). Slides were then placed (face up) into a saturated atmosphere of 1,2 dichloroethane for 5 min to enhance uniform Formvar coating. After slides have air-dried, they were dipped into a freshly prepared solution of 1% gelatin containing 0.01% chromium potassium sulfate (Brothman and Lindell, submitted) and drained of excess liquid by blotting on absorbent paper. Once dry, the slides were ready for autoradiography.

Autoradiography of slides. Slides were dipped in Kodak NTB-3 emulsion (42^{o}) diluted 1:1 with 1% Tween 80 detergent (Sigma). Slides were air-dried (in the dark) and stored in a light-proof box at -70^{o} for 1, 7, or 30 days. The slides were then developed in D-19 developer (Eastman Kodak). (See Appendix B.)

Photomicroscopy. Chromosomes were photographed on a Zeiss Photomicroscope II equipped with a Planapo 100x objective/1.3 oel. Photographs were taken on Kodak Technical Pan Film 2415.

Scoring autoradiographic grain locations. Autoradiographic grains from tritium were localized to chromosomal regions in the following manner. Grains directly over a particular region of a chromosome were scored as were grains which were less than or equal to one-half the distance of the chromosomal width from the edge of the chromosome. This system was used to maximize the efficiency of grain

location with correction for beta particle migration as described by Rogers (1979). Efficiency of autoradiographic tritium counting was determined by the methods of Ron and Prescott (1970) and Ada et al. (1966).

Results

The technique of G-banding with simultaneous autoradiographic grain analysis proved to be an effective method of identification of chromosomes that bind [^{3}H]Act D as evidenced in Figs. 1-1 and 1-2. 1.0 µg/ml [^{3}H]Act D was used to label slides to be G-banded. This was followed with the Formvar-potassium chromium sulfate technique and a subsequent 1-day autoradiographic exposure (Fig. 1-1). The use of potassium chromium sulfate (Fig. 1-1b) effectively eliminated the problem of slippage of the nuclear track emulsion which was observed when slides were only pretreated with Formvar (Fig. 1-1a). G-banded chromosomes labeled with 0.1 µg/ml [^{3}H]Act D were easily identified and grains scored in association with individual chromosomes as shown in Fig. 1-2.

Of 120 cells examined after 24-hr autoradiographic exposure, 62 cells contained grains associated with a distinct chromosome. Background from autoradiography was low as grains were rarely seen when nuclei or chromosomes were absent. No grains were associated with nuclei or chromosomes on the control slides which were not exposed to [^{3}H]Act D. This is attributed to the effective removal of Act D from the glass with ethanol while preserving drug bound to the chromosomes.

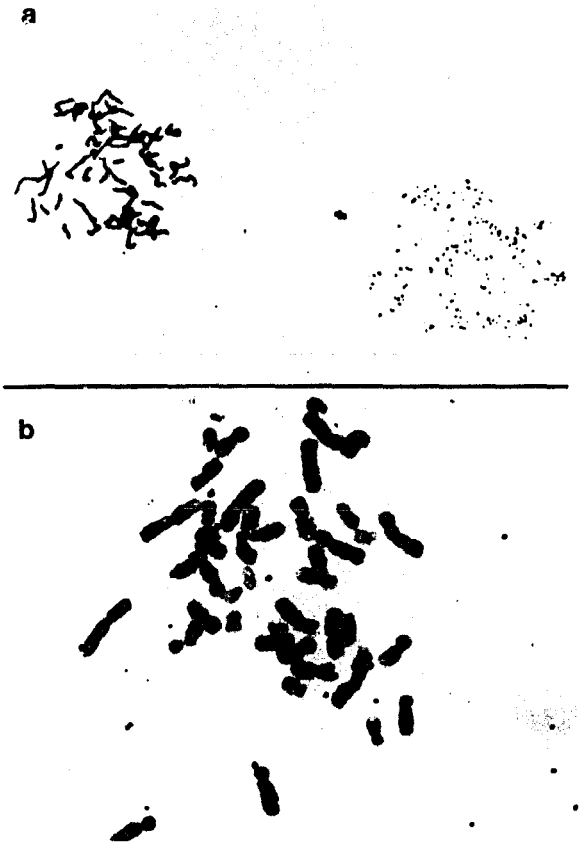

Figure 1-1. Effects of Potassium Chromium Sulfate on Autoradiographs. Human metaphase chromosomes were labeled with 1.0 μg/ml [^{3}H]Act D and exposed for autoradiography for 1 day. a) Fracture and slippage of the autoradiographic emulsion is observed when Formvar is used but the potassium chromium sulfate is omitted in the autoradiographic procedure. The outline of the chromosomal distribution in grains can be observed displaced to the right of the metaphase. Magnification: 100x. b) When potassium chromium sulfate is included in the autoradiographic procedure, grains are detectable over the G-banded chromosomes with no slippage of the emulsion. Magnification: 630x.

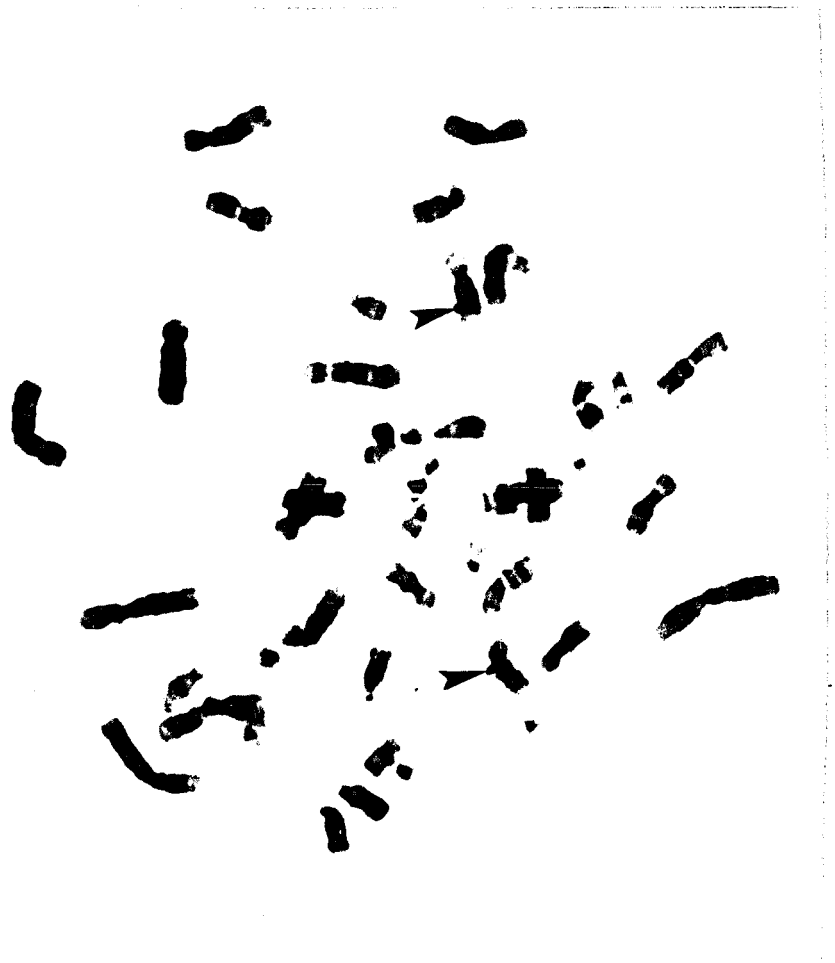

Figure 1-2. G-Banded Human Metaphase Chromosomes After 1-Day Autoradiographic Exposure. This example is one of 120 metaphase spreads observed (see text) to localize bound [^{3}H]Act D at 0.1 µg/ml. The Formvar and potassium chromium sulfate techniques allow simultaneous chromosome identification by G-banding and autoradiographic grains are observed (arrows) associated with chromosomes 8 and 10. Magnification: 1000x.

The pooled data for the 62 cells containing autoradiographic grains associated with chromosomes can be seen in Fig. 1-3. Note that of 125 grains scored, only 4 grains were associated with a ribosomal cistron (chromosome 15p). The rDNA sites previously mapped to the short arms of chromosomes 13, 14, 21, and 22 showed no autoradiographic grains.

When DNA content of the individual human chromosomes (Mendelsohn, 1973; Altman and Katz, 1915) is plotted against total grains observed, a curve is generated which has a correlation coefficient of 0.836 (Fig. 1-4). With the exception of chromosome 13, the other D and G group chromosomes fall below the upper 99% confidence limits of the line. In Fig. 1-3, however, it can be seen that autoradiographic grains associated with chromosome 13 are all on the long arm of the chromosome and are thus not located over the ribosomal cistrons of the chromosome.

Slides exposed for longer periods of time or to greater than 0.1 μg/ml [^{3}H]Act D had autoradiographic grains over the chromosomes that made G-banding a useless technique for individual chromosome identification. For this reason, slides exposed for longer times and those exposed to greater than 0.1 μg/ml [^{3}H]Act D were analyzed without G-banding. Chromosomes were examined for grain density and any observable clustering of autoradiographic grains (Fig. 1-5). When concentrations of [^{3}H]Act D of 0.06 μg/ml or less were used, too few autoradiographic grains for analysis were associated with chromosomes until a 30-day autoradiographic exposure time was employed. Fig. 1-6

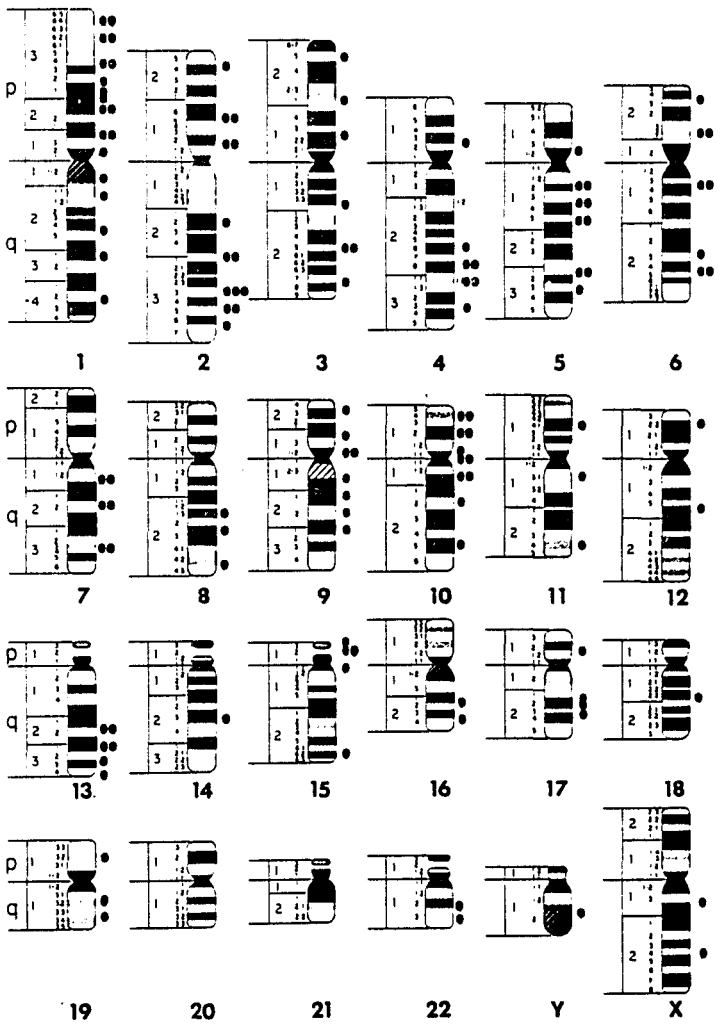

Figure 1-3. Distribution of Act D Binding to Human Chromosomes. When 0.1 µg/ml [^{3}H]Act D is exposed to human cells, G-banded and autoradiographed with a 24-hr exposure time, grains were localized to particular chromosomal regions. rDNA is located on the short arms of chromosomes 13, 14, 15, 21 and 22. Of 120 cells observed, the 125 autoradiographic grains are indicated to the right of the respective chromosomal region; only four grains are associated with ribosomal cistrons. (Schematic adapted from Yunis and Chandler, 1979).

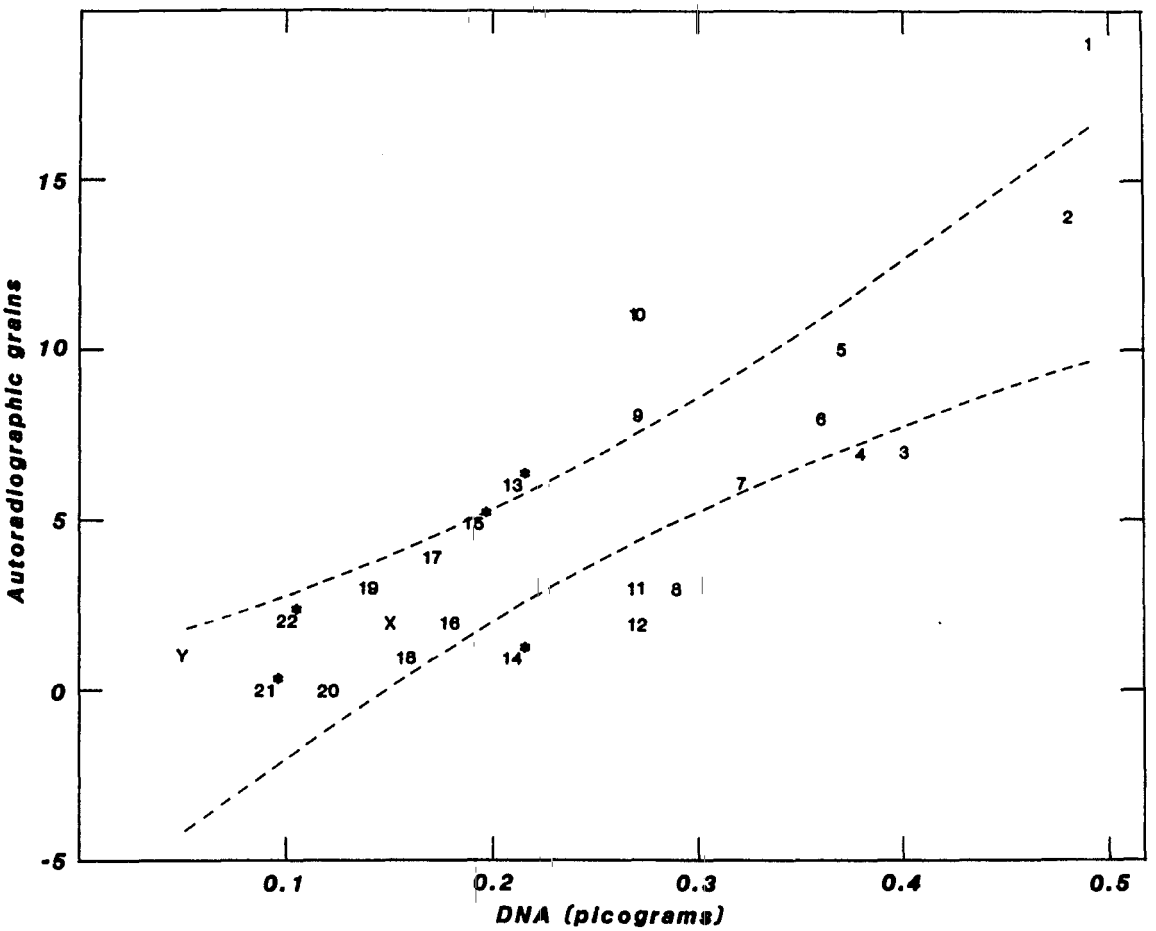

Figure 1-4. Grains Associated With Total DNA Content of Each Human Chromosome After 1-Day Autoradiographic Exposure. Picograms DNA per chromosome (2N) was calculated from the mean value of 5.72 total pg DNA per nucleus (Altman and Katz, 1975) and percent DNA content per chromosome as described by Mendelsohn et al. (1973). Numbers on the graph indicate positions of respective chromosomes with DNA vs. autoradiographic grains observed. Chromosomes denoted by the (*) contain ribosomal cistrons. Dotted lines define the 99% confidence interval of the line representing all the chromosomes plotted by regression analysis. Correlation coefficient, r=0.836.

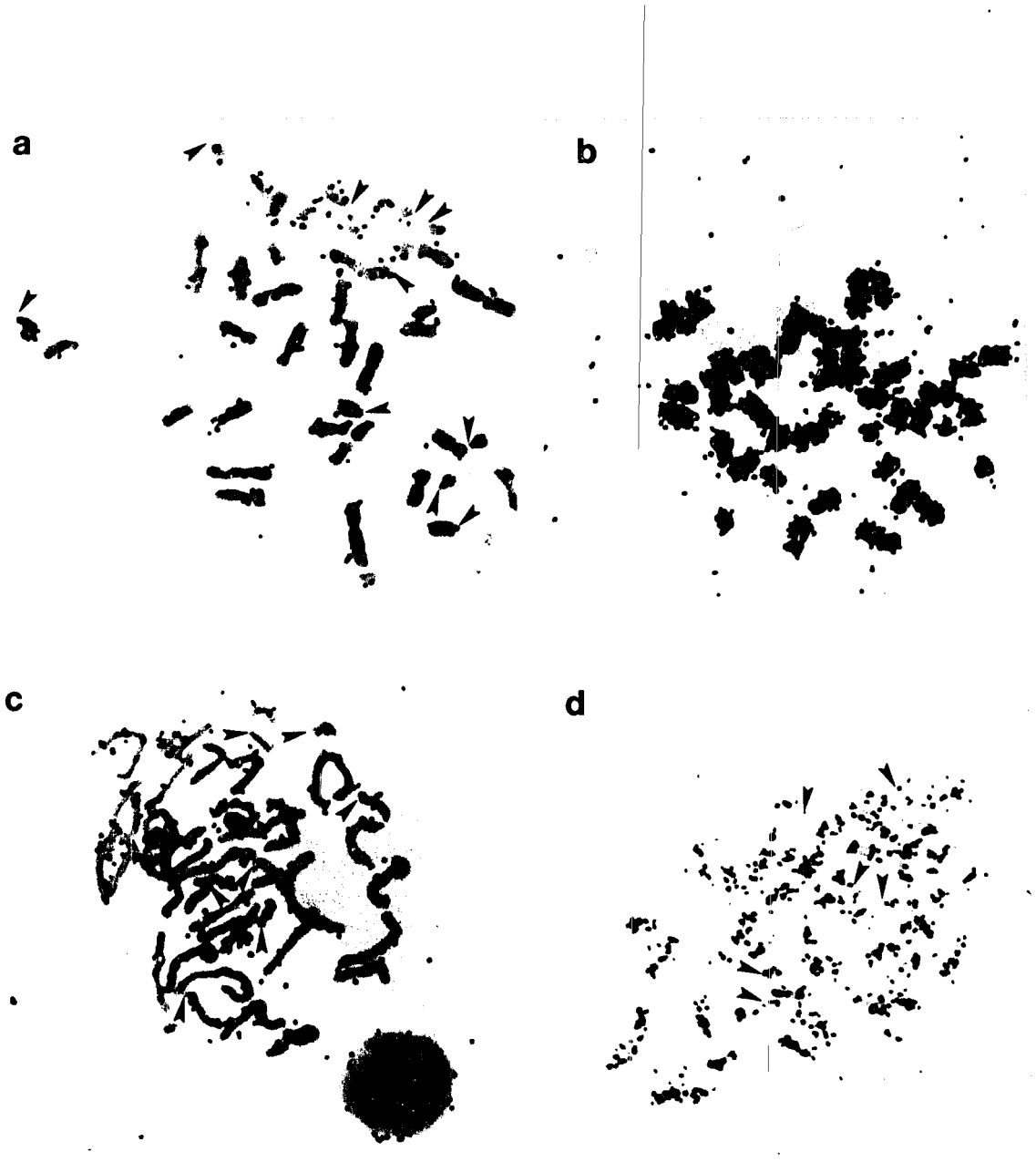

Figure 1-5. Representative Metaphase Spreads With Bound Tritiated Act D After Long-Term Autoradiographic Exposure. (a) 0.06 µg/ml [^{3}H] Act D exposed for 30 days. (b) 0.13 µg/ml [^{3}H]Act D exposed for 30 days. (c) and (d) 0.1 µg/ml [^{3}H]Act D exposed for 7 days. In each case, grain distribution appears to be associated with chromosomal length. No clustering is observed at the sites of rDNA, some of which are indicated by arrows. Magnification: 1000x.

shows the positive correlation of increasing concentration (r=0.985) of [^{3}H]Act D with the number of autoradiographic grains observed after a 30-day exposure. Fig. 1-5 (a and b) shows representative metaphases at two of these concentrations. As observed in the other metaphases examined, even at a concentration of [^{3}H]Act D that selectively inhibits rRNA synthesis, there is no increased amount of autoradiographic grains over the short arms of the D or G group chromosomes; these acrocentrics can be identified without G-banding. It appears (Fig. 1-5, c and d) that there is an association similar to that seen in Fig. 1-4, where the number of autoradiographic grains correlates to chromosomal length. When chromosomal length (as measured from the photograph in Fig. 1-5c) is plotted against the number of autoradiographic grains observed after the 30-day exposure, a curve is generated with a correlation coefficient of 0.837 (Fig. 1-1). Thus, there is a similar relationship of 30-day exposures and 1-day exposures as plotted in Fig. 1-4.

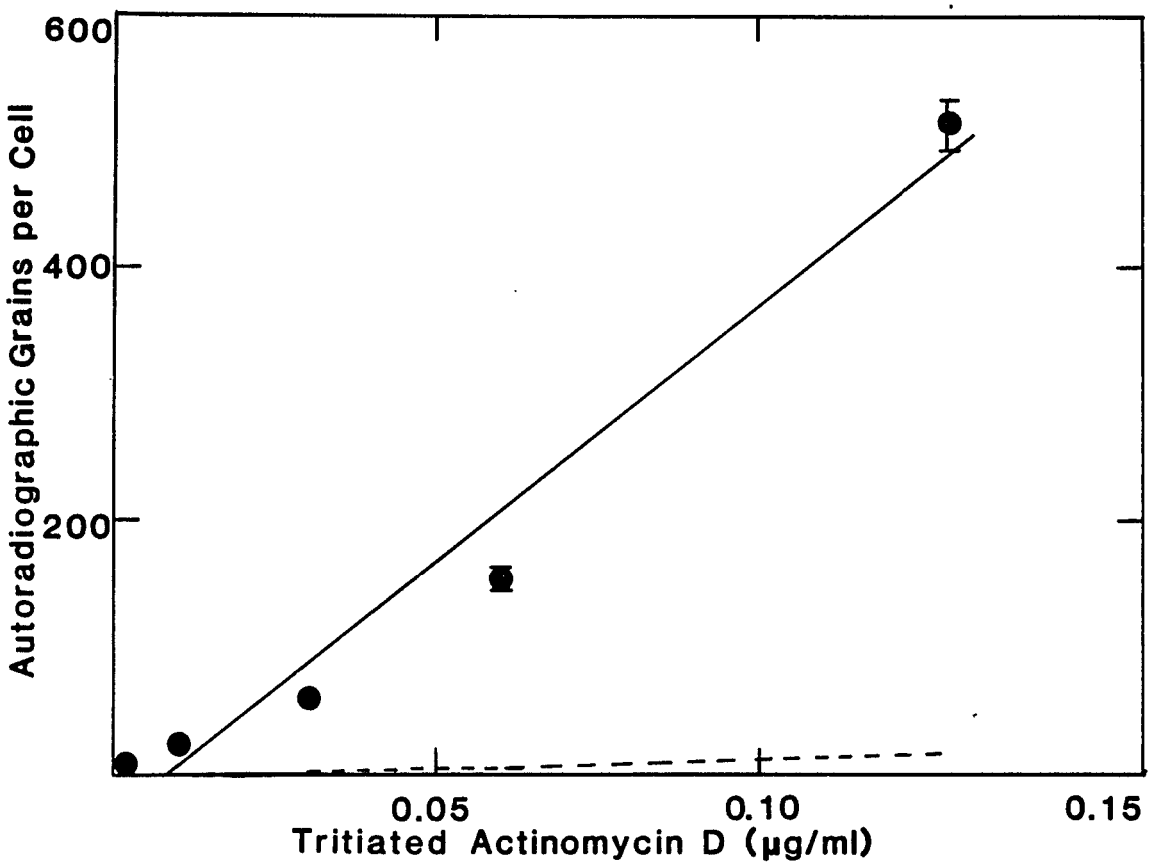

Figure 1-6. Number of Autoradiographic Grains Observed Per Metaphase Cell at Several Low Concentrations of [^{3}H]Act D. As [^{3}H]Act D Concentration increases, there is a positive correlation with increasing number of autoradiographic grains/cell observed (r=0.985). These data are from slides exposed for autoradiography for 30 days. Standard error bars are indicated unless they fall within the symbol. The dotted line represents background grains on the slides examined.

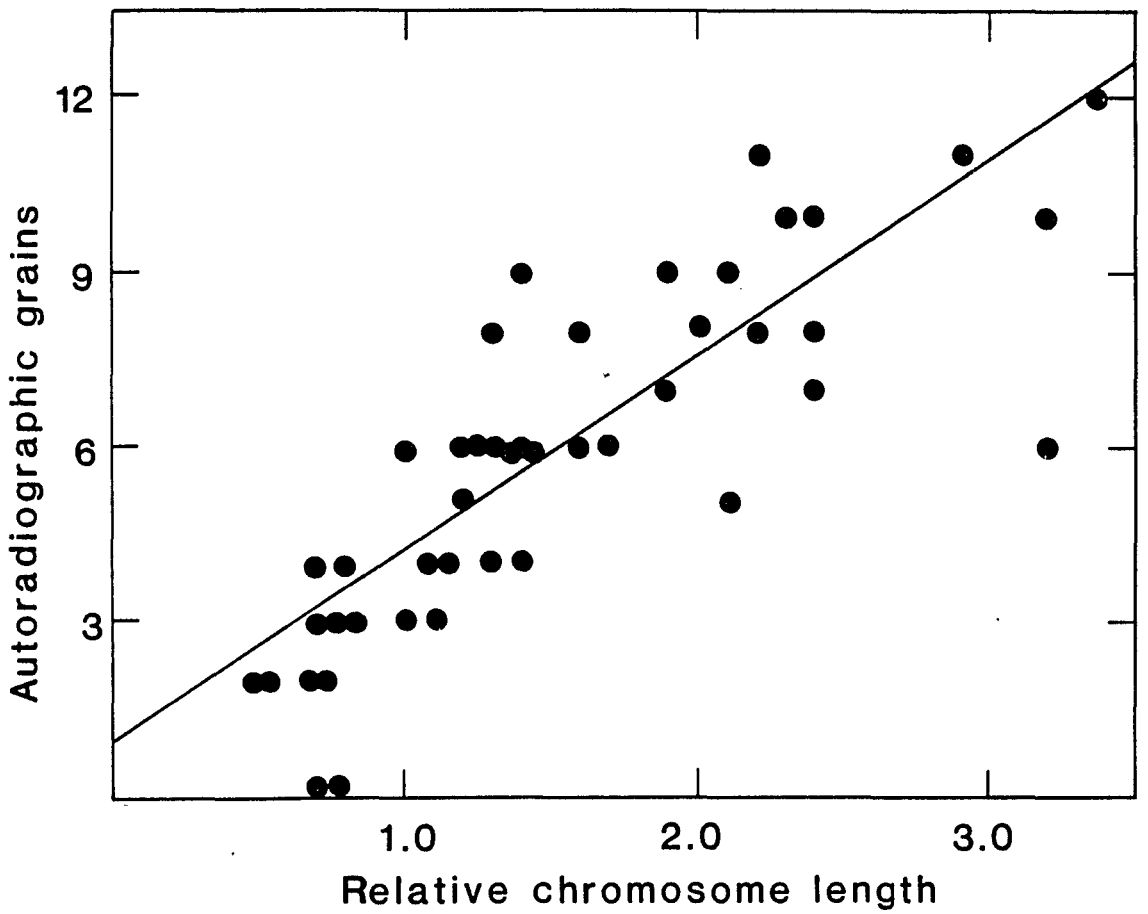

Figure 1-7. Grains Associated With Chromosomal Length After 7-Day Autoradiographic Exposure. Relative chromosomal length was measured directly from the photograph of Fig. 1-5c and associated autoradiographic grains were plotted. Correlation coefficient, r=0.837.

Discussion

The observations presented in this chapter fall into two categories: 1) the binding of 0.1 µg/ml [^{3}H]Act D to G-banded chromosomes after a 24-hr autoradiographic exposure, and 2) the binding of 0.004 - 0.1 µg/ml [^{3}H]Act D to nonbanded chromosomes after 7- or 30-day autoradiographic exposure. Both categories point to the same conclusion; i.e., Act D randomly binds to chromatin in proportion to chromosomal length.

For the specific identification of many mammalian chromosomes, G-banding has become a routine and accepted technique. The analysis of banded metaphase chromosomes with simultaneous detection of autoradiographic grains makes convenient the precise localization of [^{3}H]Act D. In the preliminary experiments, it was determined that an interaction between the Geimsa-stained chromosome and the nuclear track emulsion NTB-3 made silver grain detection over identifiable G-bands difficult. Because of this undesirable interaction, most investigators prefer to G-band after autoradiography (Szabo et al. 1978; Martin-DeLeon, 1980). Because the stain must penetrate the emulsion, timing of this process is difficult to optimize. The difficulty of obtaining nicely banded chromosomes after autoradiography has also recently been reported by Huttner et al. (1981). One procedure which gave intriguing results for post-autoradiographic G-banding using Wright stain was discussed by Chandler and Yunis (1978), and Harper et al. (1981). This method has a disadvantage since it involves destaining and restaining of the chromosomes requiring many manipulations. Since reproducibility was poor using Wright stain, a more simple and consistent technique was sought.

The original procedure described by Rutledge (1979), which described the application of a Formvar coating of G-banded chromosomes prior to autoradiography initially appeared acceptable. The presence of this thin plastic coating prevented distortion of the chromosome banding by the emulsion. However, it was observed that the emulsion fractured and slipped over the Formvar during development, making localization of autoradiographic grains to specific chromosomes impossible. An example of this can be seen in Fig. 1-1a, where autoradiographic grains are observed to be displaced to the right of the metaphase spread. While varying degrees of displacement were observed, any shift in grains relative to the chromosomes could lead to anomalous results. To overcome the problem of slippage, the Formvar coated chromosomes were treated with chromium potassium sulfate prior to application of the emulsion. Subsequent development of the emulsion produced high quality G-banded chromosomes with superimposed autoradiographic grains (Figs. 1-1b and 2).

One common problem in all the previous studies of Act D binding to chromosomes was the inability to identify individual chromosomes. The use of the G-banding - Formvar - potassium chromium sulfate technique gave consistent results, and the individual chromosome identification was as easy as with any G-banded preparation (see Fig. 1-2).

Since Act D has an unusually high affinity for glass, a previous study employed a pretreatment of the slides with Act D to reduce the binding of the labeled drug to glass (Ebstein, 1969). Jones (1976) has shown that Act D bound to DNA remains bound in ethanol. Duffy and Lindell (in preparation) have demonstrated that ethanol removes Act D

bound to glass and plastic surfaces while maintaining the complex of the drug with DNA. For this reason, it was anticipated that ethanol washing would effectively decrease autoradiographic background when Act D is bound to chromosomes. As evidenced in Fig. 1-2, no background autoradiographic grains are seen in this particular 1-day exposure. This was typical for the 1-day autoradiographic exposures at 0.1 μg/ml [^{3}H]Act D. After 30 days autoradiographic exposure, however, the background level increases at this concentration of the drug (Fig. 1-6). This is likely due to the fact that after longer exposure, fewer molecules of Act D are necessary to produce autoradiographic grains. One should note that even after these long autoradiographic exposures, background is only approximately 2% of the grains observed associated with chromosomes (Fig. 1-6). The decrease in nonspecific Act D binding can be attributed to the ethanol washes. Washing the [^{3}H]Act D labeled slides in ethanol therefore eliminates the need for any pretreatment of the slides with unlabeled drug.

After a 1-day autoradiographic exposure, data from the 120 metaphase cells examined after binding 0.1 μg/ml [^{3}H]Act D and G-banding was pooled for evaluation in Fig. 1-3. It appears from this figure that [^{3}H]Act D is not selectively bound to the ribosomal cistrons. Instead, it can be seen from Fig. 1-4 that this binding is proportional to DNA content. Except for 13q none of the rDNA-containing chromosomes had a greater amount of associated autoradiographic grains than the upper limits of 99% confidence of the line showing grains observed per picogram chromosomal DNA. It should once again be mentioned, as seen in

Fig. 1-3, that the autoradiographic grains associated with chromosome 13 are all on the long arm of the chromosome and are, therefore, not associated with the ribosomal cistrons of that chromosome.

These observations indicate that at 0.1 µg/ml, Act D binding at the ribosomal cistrons is not greater than at other chromosomal sites, despite the fact that the D and G group chromosomes contain many copies of ribosomal cistrons. This observation supports the hypothesis that Act D at the low concentrations which selectively inhibit rRNA synthesis in intact cells, is not binding preferentially to the ribosomal cistrons.

Fig. 1-3 also indicates that some chromosomes contain regions where more grains are associated. When presented in Fig. 1-4, chromosomes 1, 9, and 10 have significantly more grains than would be expected if binding were totally random since they fall above the upper 99% confidence limits of the line. Evidence is insufficient, however, to claim that the drug is localized to these regions and acts at a specific locus for the inhibition of rRNA synthesis by the extranucleolar mechanism proposed (Tsurugi et al. 1972; Lindell, 1976; Lindell et al. 1978).

Fig. 1-4 shows the correlation of autoradiographic grains to DNA content. A question could be raised as to evidence that the [^{3}H]Act D observed is bound to DNA rather than chromosomal proteins. This question was first investigated by Ebstein (1967) who concluded that since DNAase treatment of chromosomes prior to Act D labeling eliminates any drug binding, the drug is specifically bound to the DNA. This problem is further addressed in Chapter 3.

The number of autoradiographic grains observed can be directly used in calculation of the amount of drug present after the sensitivity of the system used is determined. Ron and Prescott (1970) have reported that 15 autoradiographic grains are observed in NTB-3 emulsion for every 100 disintegrations of tritium label. This was tested for the system used in the following manner. Whole fixed cells were labeled with 0.1 µg/ml [^{3}H]Act D. Half of these cells were exposed for autoradiography while half were counted by liquid scintillation counting. For every 100 disintegrations of [^{3}H]Act D, it was calculated that 13.8 ± 1 grains were observed. Since this was in agreement with the value previously reported, calculation of the number of molecules of [^{3}H]Act D per autoradiographic grain per time could be considered accurate.

With the specific activity of 15 Ci/mM [^{3}H]Act D, it was determined that in a 24-hr exposure time for autoradiography, approximately 90,000 molecules of bound Act D would be necessary to observe one silver grain in NTB-3. Gaubatz, Prashad, and Cutler (1976) have reported that 30 -60 repeats of the human ribosomal cistron are present per chromosomal location, and Higuchi et al. (1981) have shown that the length of the human rRNA gene is 43 Kb. One can thus determine that the average length of rDNA on each acrocentric chromosome is from 1290-2580 Kb (mean value of 1935 Kb).

Duffy and Lindell (manuscript in preparation) have described a unique binding isotherm in the concentration range of drug that selectively inhibits rRNA synthesis.

Act D was shown to bind at 0.1 μg/ml and saturate the high affinity type of binding site for the drug. At this concentration, one Act D molecule binds at approximately every 300-400 base pairs (J. Duffy, personal communication). Assuming that the average length of rDNA on each acrocentric chromosome is 1935 Kb (shown above), this would suggest, that if the rDNA cistrons were part of this "high affinity site," that 4800-6500 Act D molecules would bind at each short arm of the acrocentric chromosomes (1935 Kb divided by 300 to 400 base pairs). Since the 1-day autoradiographic exposure statistically only shows one silver grain per 90,000 Act D molecules, it becomes obvious that many more than the 120 cells observed would be necessary to statistically show that Act D was not bound to any great extent at the sites of rDNA. A longer exposure time will increase the number of autoradiographic grains observed, and therefore decrease the number of Act D molecules per grain (one grain/90,000 molecules Act D/one day x 30 days = one grain/3000 molecules ActD/30 days). This number fits the 4800-6500 Act D molecules "expected" as described above. For this reason, autoradiographic exposures of 7 and 30 days were analyzed.

Fig. 1-5 shows that, as expected, an increased number of autoradiographic grains are observed with longer exposure times. Even after these longer exposures, no clustering of grains are observed at the sites of the ribosomal cistrons. It also appears, as evidenced in Fig. 1-6, that there is a linear relationship between the concentration of Act D applied and the number of autoradiographic grains observed per metaphase cell after 30-day exposure. This suggests that Act D is binding

in a random fashion, and the greater the drug concentration, the more the drug binds. Fig. 1-7 also shows that there is a positive correlation of autoradiographic grains to chromosomal length. Since these chromosomes are not G-banded, it is not possible to identify all of the chromosomes. It should be noted, however, that because the rDNA-containing chromosomes are acrocentrics, they can usually be identified without G-bands. No clustering of autoradiographic grains is observed at these sites in Fig. 1-5 or on any of the other metaphase spreads examined. Even though all individual chromosomes could not be identified, relative chromosomal length can be determined directly from the photograph, and grains per chromosome counted. A plot of relative chromosomal length versus number of grains observed (Fig. 1-7) shows that there is again a positive correlation ($r=0.837$). This correlation indicates that Act D is bound in proportion to chromosome length. This was also shown by the G-banding - Formvar - potassium chromium sulfate technique for the individual chromosomes after short term autoradiographic exposure (Fig. 1-4). One can thus conclude that at 0.1 μg/ml Act D is bound throughout the human genome, without selective binding at the ribosomal cistrons.

Some other statements can be made regarding autoradiographic detection of Act D on human chromosomes. Since the drug binding is random through the genome and proportional to DNA content, there appears to be no preferential binding of Act D to heterochromatin as suggested by Simard (1967). There is also no preferential drug binding to euchromatin, as suggested by Berlowitz et al. (1969). The data

presented concur with the report of Sieger et al. (1971) who claimed that any differences in Act D binding observed are due mainly to the amount of DNA present.

These findings indicate that there is no characteristic pattern of Act D binding to human chromosomes observed with autoradiography. The longer the exposure time, the more grains appear over the entire chromosomal lengths. Since background also increases with longer exposure times, it becomes more and more difficult to distinguish areas where Act D may be preferentially binding. With the problem of low sensitivity (many molecules/grain) when short exposure times are employed, and increasing background during longer exposures, it becomes evident that autoradiography may not be the best method for detection for drug bound to chromosomes. Act D with a higher specific activity would partially solve the problem; however, 14 Ci/mM (which was used in these studies) is the highest specific activity commercially available for [^{3}H]Act D. It was therefore decided that the preparation of an antibody specific for Act D might facilitate a better means of detecting drug binding to chromosomes. Chapter 2 details production and characterization of such an antibody, and the use of this antibody, as a sensitive and convenient method for the detection of Act D bound to chromosomes is described in Chapter 3.

Chapter Summary

Data presented in this chapter show the characterization of labeled actinomycin D bound to human metaphase chromosomes as detected by autoradiography. A new technique was developed whereby G-banded

chromosomes could easily be identified with simultaneous autoradiographic analysis. Act D was shown to bind proportionally to chromosomal length and DNA content. Strong evidence is presented that counters previous suggestions that Act D at low concentrations selectively binds to the ribosomal cistrons. The autoradiographic data presented supports the notion of extranucleolar regulation of rRNA synthesis. However, a more sensitive detection method of Act D bound to chromosomes is preferred. The following chapters describe the production of an anti-Act D antibody, its characterization, and its use as a sensitive and quick method of detection of drug bound to chromosomes.

CHAPTER 2

THE DEVELOPMENT AND CHARACTERIZATION OF AN ANTI-ACTINOMYCIN D ANTIBODY

Introduction

From the autoradiographic data in Chapter 1, it is evident that a more sensitive and simpler technique for the detection of actinomycin D (Act D) bound to chromosomes would prove advantageous. The development of an antibody, specific for Act D would provide the potential to increase sensitivity for measurement of the drug binding and simplify the methods of detection. Furthermore, an antibody to Act D could be used as a probe for the drug with additional applications besides detection of Act D binding to chromosomes. For example, the development of a radioimmunoassay for Act D would permit the quantitation of drug in biological fluids and tissues. This can be helpful in studies employing the use of Act D in the research laboratory, and also in quantification of drug levels in patients receiving Act D as a chemotherapeutic agent. This chapter describes production of such an antibody in rabbits (Brothman et al. 1982), its characterization, purification, and application to the field of cancer research.

As an antitumor agent, Act D is clinically effective in the treatment of certain human malignancies (Farber et al. 1956). Dosing of the drug for clinical efficacy is aimed at achieving those concentrations of the drug within body fluids that are similar to those that

selectively inhibit rRNA synthesis in cells in culture (0.001 - 0.1 µg/ml, Perry and Kelley, 1970). While Act D is effective in the treatment of certain human malignancies, it is possible that the drug is not readily accessible to certain tumor sites despite apparent widespread distribution (Tattersall, 1975). Therefore, an understanding of the pharmacokinetic distribution of Act D in the body fluids and tissues may aid in increasing the effective use of the drug for treatment. Previous pharmacokinetic studies of Act D required the use of tritiated drug (Galbraith and Mellet, 1975; Tattersall et al. 1975) thus using radioactivity as an indirect measure of the drug itself.

Because selectivity, specificity, and enhanced drug detection are necessary for good pharmacokinetic elucidation, the preparation of an antibody to the drug would provide a useful tool in the development of a radioimmunoassay to measure levels of the drug. Initial application of the assay to measure Act D levels in a patient and a dog demonstrates its utility for the assessment of basic pharmacokinetic distribution.

Materials and Methods

Coupling Reaction for Hapten. Seventy-five mg Act D (Merck) was combined with tracer amounts (1.8 µCi) of [^{3}H]Act D (Amersham) and 25 mg bovine serum albumin (BSA) (Sigma), in a final volume of 1 ml. The mixture was maintained at 0° in ice-slush with constant stirring. This temperature was necessary to maintain Act D in solution (Meinhoffer, 1974). At 1-hr intervals for 8 hr, 10 mg 1 ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) (BioRad) was added as a

coupling reagent. The pH of the solution was maintained at 4.5 by the addition of 0.1 M HCl as needed. Several trials revealed that the optimum coupling ratio was 3 parts Act D to 1 part BSA (w/w). The mixture was stirred for an additional 10 hrs at 4^{o}. The reaction mixture was then transferred to a dialysis bag and dialyzed against 500 ml volumes of 1 M NaCl containing 1 mM β-mercaptoethanol (Sigma) until the deep yellow color of Act D was no longer seen in the dialysate. The insoluble protein in the dialysis bag was yellow-orange and additional removal of non-covalently bound drug was achieved by extensive dialysis against 8 M urea containing 1 mM β-mercaptoethanol. When the contents of the bag were no longer yellow, stepwise dialyses against lower concentrations of urea were performed with a final dialysis against a 0.9% NaCl solution. Remaining material in the dialysis bag was white and only partially soluble. Tritium tracer was used to quantitate the amount of Act D bound. The amount of BSA in the final complex was determined by the Folin-Lowry assay (Lowry et al. 1951) using BSA as a standard.

Antibody Production in Rabbits. Aliquots containing either 100 µg or 250 µg of the Act D-BSA complex (4.4 µg and 11 µg Act D, respectively) were emulsified with an equal volume of Freund's complete adjuvant (Difco) and two male New Zealand rabbits were given 10 subcutaneous injections over sites along both sides of their backs. A control animal received BSA in Freund's complete adjuvant. After three weeks, the rabbits were boosted with the same amount of emulsified complex (or BSA for the control) in complete adjuvant. One week later,

each rabbit was bled from the ear artery, using a 21 gauge butterfly needle. Blood was collected directly into a 10 ml disposable centrifuge tube (Falcon Plastics). Serum was separated by centrifugation at 500 x g and analyzed as described in the following section. Blood was collected from the rabbits for the following two weeks until potency of the antibody began to decrease. A boosting schedule with Act D-BSA in incomplete adjuvant (Difco) was used for continued antisera collection. Antibody production peaked approximately two weeks after each boost. Serum samples containing antibody were stored at -20° until use.

Antibody detection by gel filtration. The rabbit serum (25 µL) was mixed with an equal volume of 0.1 µg/ml [^{3}H]Act D and allowed to incubate on ice for one hr. The Act D and serum were then passed over a Biogel P-10 column (15 cm x 1.5 cm) (BioRad) and protein content (absorbance at 280 nm) and Act D content (dpm) were determined for each fraction.

Radioimmunoassay for Actinomycin D. It has been observed in this laboratory that Act D in aqueous solution binds to plastics and glassware with significant loss of the drug. Therefore, all material which would come into contact with Act D was first treated with silicone (Sigmacote, Sigma). To perform the radioimmunoassay for Act D, undiluted serum (25 µL) was mixed with an equal volume of 0.1 µg/ml [^{3}H]Act D (0.025 µCi) in a 1.5 ml microfuge tube and incubated on ice. After one hr, 100 µL of 0.01 M Tris buffer pH 7.3 was added to increase the working volume. One 2.0 mg dextran-coated charcoal pellet (Westchem Products, San Diego, CA) was added to the tube and mixed. After

five min, samples were centrifuged at 12,000 x g in a microfuge (Brinkman, Westbury, NY) for 30 sec and 75 µL of the supernatant was transferred to 3 MM filters (Whatman), dried, and counted in a toluene-based fluor containing 2.5% NCS (Amersham) and 4.4% Omnifluor (New England Nuclear) in a Searle Analytic 81 liquid scintillation counter. To develop a standard curve, increasing amounts of unlabeled Act D were added to 25 µL of 0.1 µg/ml [^{3}H]Act D in the same volume. The mixture was combined with antisera and assayed as previously described. Triplicate samples were analyzed at each concentration of Act D, resulting in a standard radioimmunoassay curve. Characterization of antibody specificity was performed by addition of actinomine (gift of Dr. T. R. Krugh, University of Rochester), actinomycin V (Sigma), or doxorubicin (Adria Labs, Wilmington, DE).

Pharmacokinetic analysis of Actinomycin D. The human subject was a 59-year-old male patient who was receiving treatment for metastatic melanoma at the University of Arizona Health Sciences Center. The patient received Act D (Cosmegen®; Merck, Sharpe and Dohme) by intravenous push at 1.50 mg Act D/m^2. Serial blood samples were removed via an intravenous catheter from the opposite arm from which the drug was administered. Ten ml of blood drawn prior to dosing (time 0) and 3 ml each at 1, 5, 10, 30, 45, and 60 min were immediately transferred into silicone-coated tubes (Sherwood Medical, St. Louis, MO). After the blood samples clotted, each sample was centrifuged at 550 x g for 15 min, the serum was separated, and stored in siliconized tubes at -20^o. Analysis of blood levels of Act D was performed by adding a

constant amount of [^{3}H]Act D (0.10 μCi) to 100 μL of serum sample. Aliquots of 10 μL were taken from each sample for determination of input tritium counts so all samples could accurately be compared with each other. A standard aliquot of 25 μL of rabbit anti-Act D was then added to an equal volume of sample serum, allowed to incubate on ice for 1 hr and assayed as described above in the "Radioimmunoassay for Act D." The unknown amount of Act D present in the serum was quantitated by interpolation on the linearized standard curve (log-logit plot) described below.

A female mongrel dog, mildly sedated with 6 mg acepromizine, received Act D (Cosmegen, Merck) by intravenous push at 2.0 mg Act D/m^2. Serial blood samples were removed via an intravenous catheter from the opposite leg from which the drug was administered. Blood collection and serum analysis was the same as described previously for the human subject.

For both the patient and dog, standard curves were performed with serum obtained prior to drug administration by the addition of known amounts of Act D. Serum half lives of drug were calculated using nonlinear regression analysis with a computer program (courtesy, Dr. William Banner).

Purification of the IgG fraction from serum. 6.0 ml of immune rabbit serum was passed over a Pharmacia Protein A CL-4B-Sepharose column (1.5 cm x 6 cm) and washed with 0.02 M Tris-HCl pH 7.3 with 0.15 M NaCl. When the absorbance at 280 nm was down to background (0.010), the IgG fraction was eluted with 0.1 M acetic acid. The peak

fractions eluted with 0.1 M acetic acid were then dialyzed against 0.01 M Tris pH 7.3 and 0.9% NaCl overnight. The IgG fraction was then stored at -20°. After each use, the CL-4B-Sepharose column was washed with 1 ml of 1.0 M acetic acid and stored at 4° in 0.02 M Tris-HCl pH 7.3 with 0.15 M NaCl and 0.06% sodium azide.

Results

Actinomycin D was coupled to BSA with the intention of producing a specific anti-Act D antibody. The 2-amino group of the heterocyclic chromophore (Fig. 2-1) provides a suitable means for coupling the drug to free carboxyl groups of proteins. The EDC catalyzed coupling reaction is illustrated in Fig. 2-2, resulting in an Act D-BSA complex. After the coupling reaction reached completion (as evidenced by no further increase in pH) and following extensive dialysis, it was determined that 2.4 moles Act D bound per mole of BSA.

Four weeks after the initial inoculation with Act D-BSA complex, antibodies were detected in two out of two rabbits. Initial demonstration of Act D bound to antisera was obtained by reacting tritiated drug with antisera and performing gel filtration. It can be seen in Fig. 2-3a that bound and free drug are well-separated. Antisera from a control rabbit presented with BSA in adjuvant revealed no Act D binding to the void volume protein (counts were only observed in the included peak), indicating that Act D does not bind to serum proteins in a nonspecific fashion (Fig. 2-3b). Using gel filtration as a criteria for binding, antiserum obtained from the rabbit initially injected with the highest

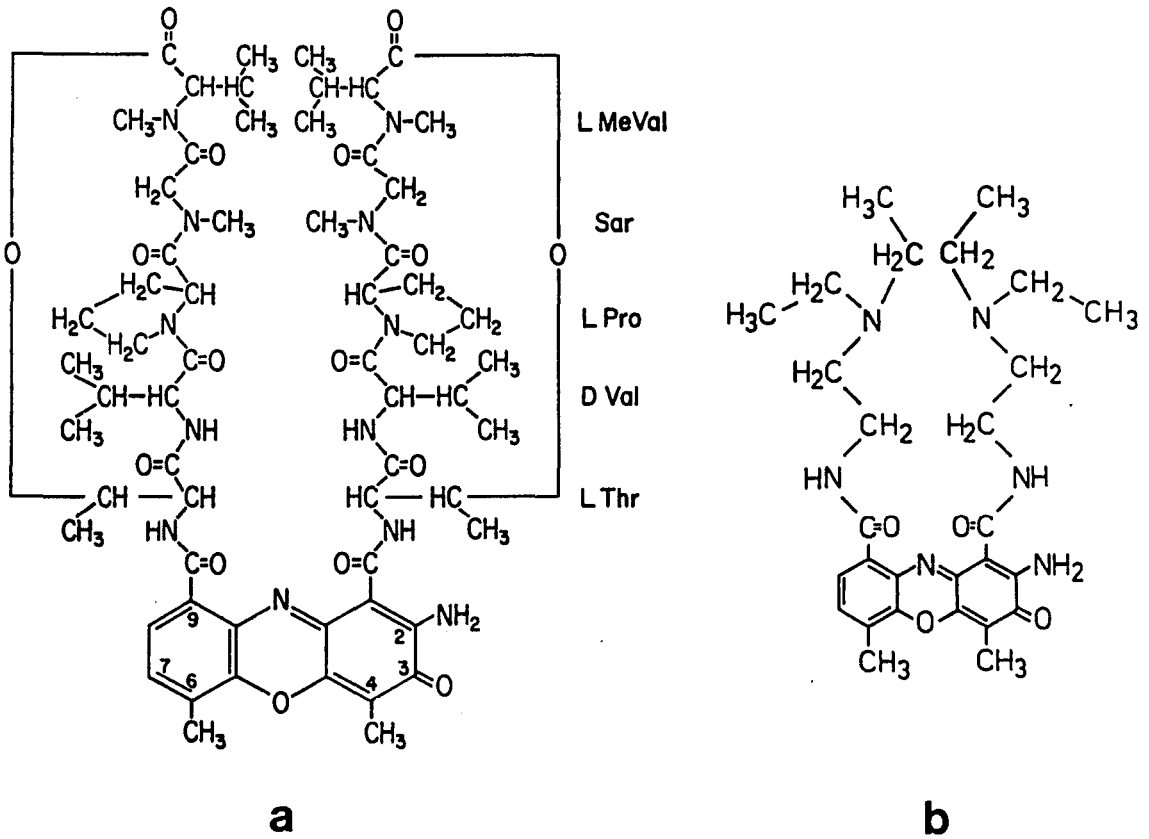

Figure 2-1. The Chemical Structures of (a) Actinomycin D (MW:1255) and (b) Actinomine (MW:524).

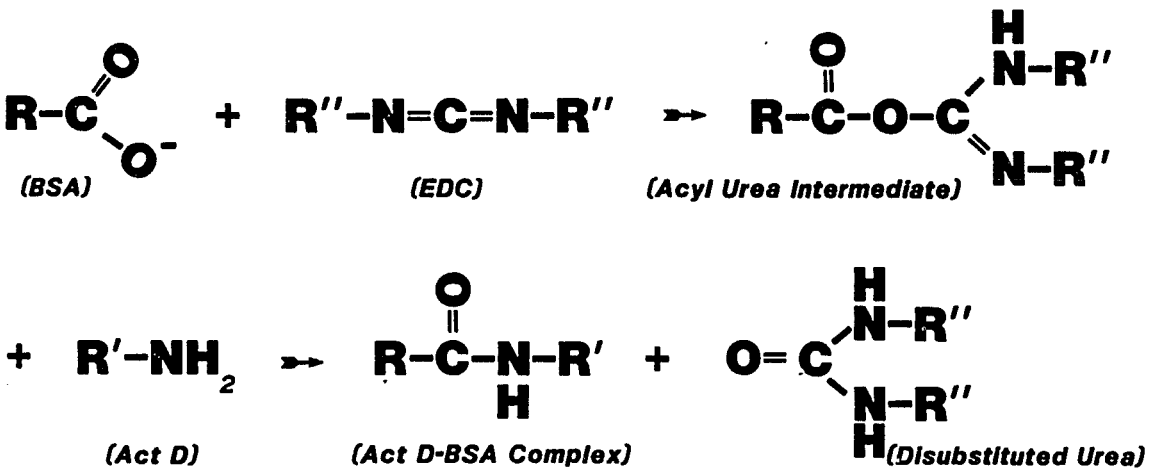

Figure 2-2. The Chemical Coupling of Bovine Serum Albumin (BSA) to Actinomycin D using 1 ethyl-3-(3-dimethyl-aminoprophyl) Carbodiimide (EDC) as a Catalyst.

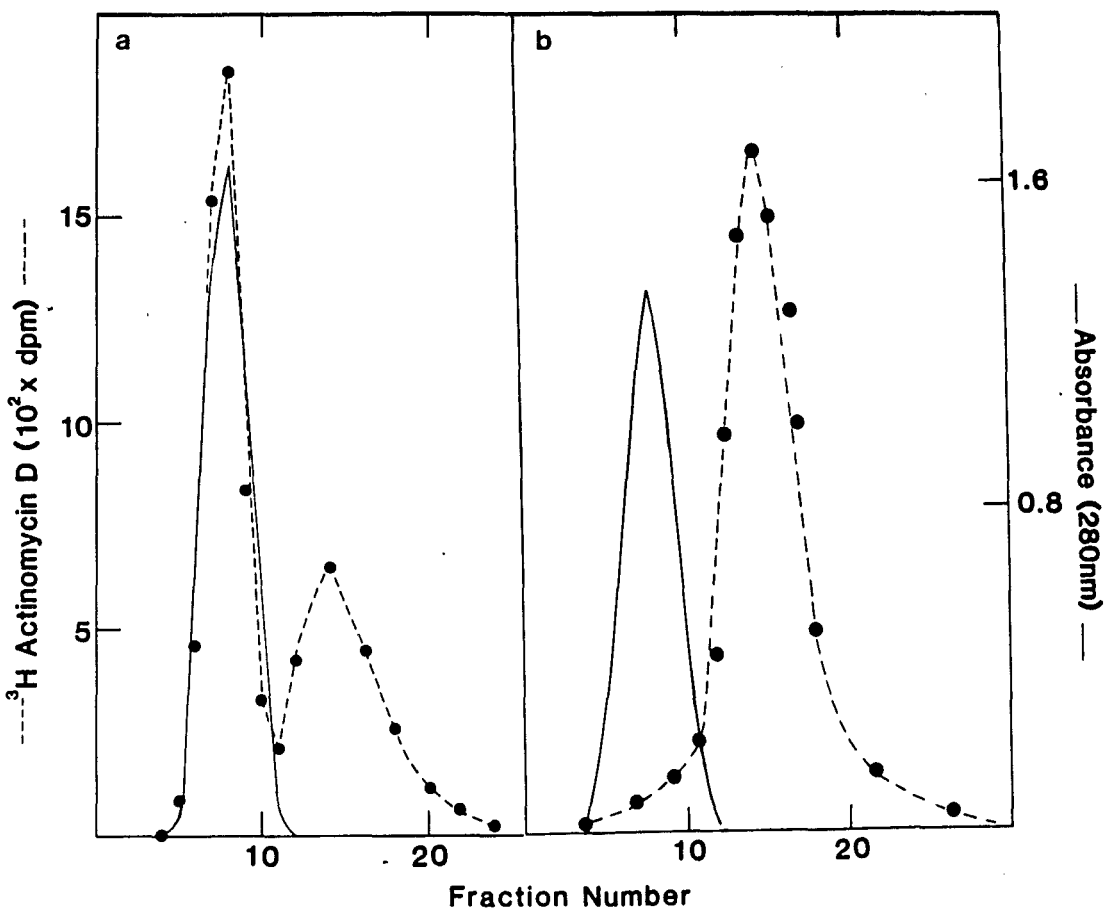

Figure 2-3. Separation of Act D Bound to Antibody From Free Act D by Gel Filtration. a) Tritiated drug was mixed with immune serum and passed over a Biogel P-10 column, as described in Materials and Methods. The drug (dotted line) is observed in the excluded volume. b) When nonimmune serum is used, Act D is observed only in the included volume.

amount of Act D-BSA complex (250 μg) consistently demonstrated higher Act D binding than the antiserum from the rabbit receiving the smaller initial amount of antigen (100 μg).

To determine the optimum time of maximum antibody activity, blood was drawn from each rabbit at various intervals after boosting with the Act D-BSA complex. The serum was incubated with [^{3}H]Act D and passed over the Biogel P-10 column described in Fig. 2-3. The results (Fig. 2-4) show that the rabbit initially injected with 250 μg Act D-BSA complex showed a peak in antibody activity 18 days after boost, while the rabbit initially injected with 100 μg Act D-BSA complex showed a peak in antibody activity 11 days after boost. The control rabbit showed no change, and the radioactivity detected was considered to be nonspecific binding.

Due to the time-consuming nature of the gel filtration procedure, a more rapid assay procedure was investigated. It has previously been demonstrated in this laboratory that charcoal is effective for removal of free Act D from solution. No significant loss of Act D antibody due to the presence of charcoal in serum has been observed. The charcoal assay was highly sensitive, allowed for the analysis of small volumes of serum and drug, exhibited low background due to nonspecific binding, and required a minimum amount of time. Serum obtained from rabbits at various times after boosting revealed different amounts of Act D binding, and this method proved to be an excellent rapid technique for determining antibody activity.

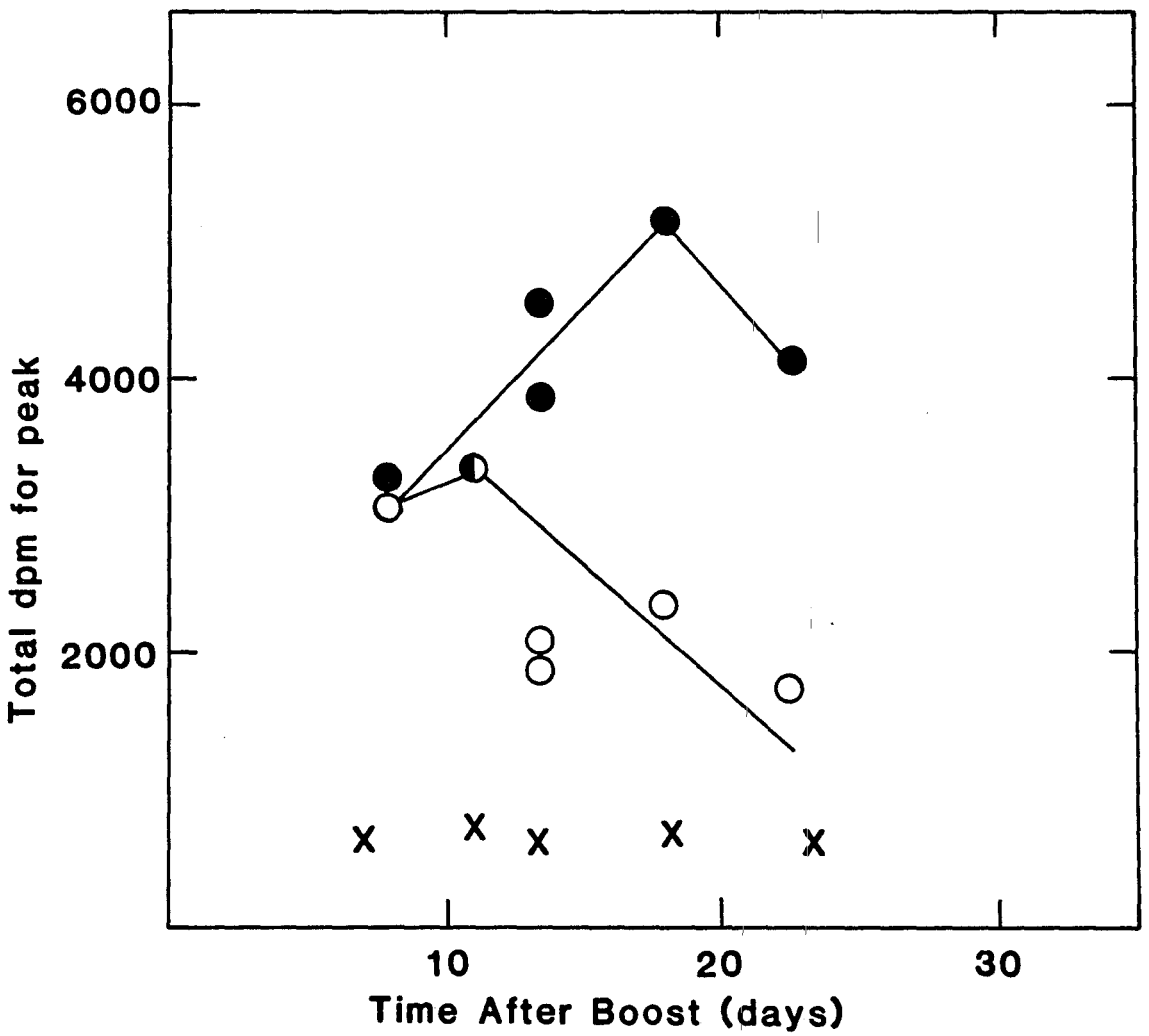

Figure 2-4. Production of Anti-Act D Antibody After Boost as Detected by Gel Filtration. Closed circles represent analysis of serum from the rabbit initially injected with 250 µg Act D-BSA complex. Open circles represent analysis of serum from the rabbit initially injected with 100 µg Act D-BSA complex. The "Xs" represent analysis of the control rabbit, initially injected only with BSA.

The charcoal assay was then used in the development of a radioimmunoassay for Act D. It can be seen in Fig. 2-5 that increasing concentrations of unlabeled Act D produced a characteristic standard curve indicative of the competition of unlabeled drug with labeled drug as classically demonstrated in other radioimmunoassays. When plotted on logit-log paper (Fig. 2-6), a linearized standard curve was obtained with a coefficient of correlation of -0.980. This linear curve permitted quantitation of Act D accurately down to 0.1 picomoles (0.005 μg/ml).

Since a large number of Act D analogs have been identified (Meinhoffer, 1974; Meinhoffer and Atherton, 1977), characterization of the specific part of the molecule which contains the antigenic determinant was attempted. Since it was anticipated that the pentapeptide portion of the drug might be antigenic, an analog was tested with a substantial synthetic modification at that part of the molecule. Actinomine contains the same chromophore as Act D, but lacks the pentapeptide lactone ring (Fig. 2-1). When actinomine was substituted for unlabeled Act D in the radioimmunoassay, it can be seen from Fig. 2-5 that very little competition with Act D was observed. Actinomycin V, which has oxoproline substituted for proline in the pentapeptide loop of the molecule produced identical results to that of Act D. Doxorubicin, which also has a heterocyclic ring system and intercalates into DNA, produced no displacement in this assay.

The utility of the radioimmunoassay can be seen in Fig. 2-7 where serum levels were quantitated after Act D administration to a human and a dog. Since the human subject had previously received one

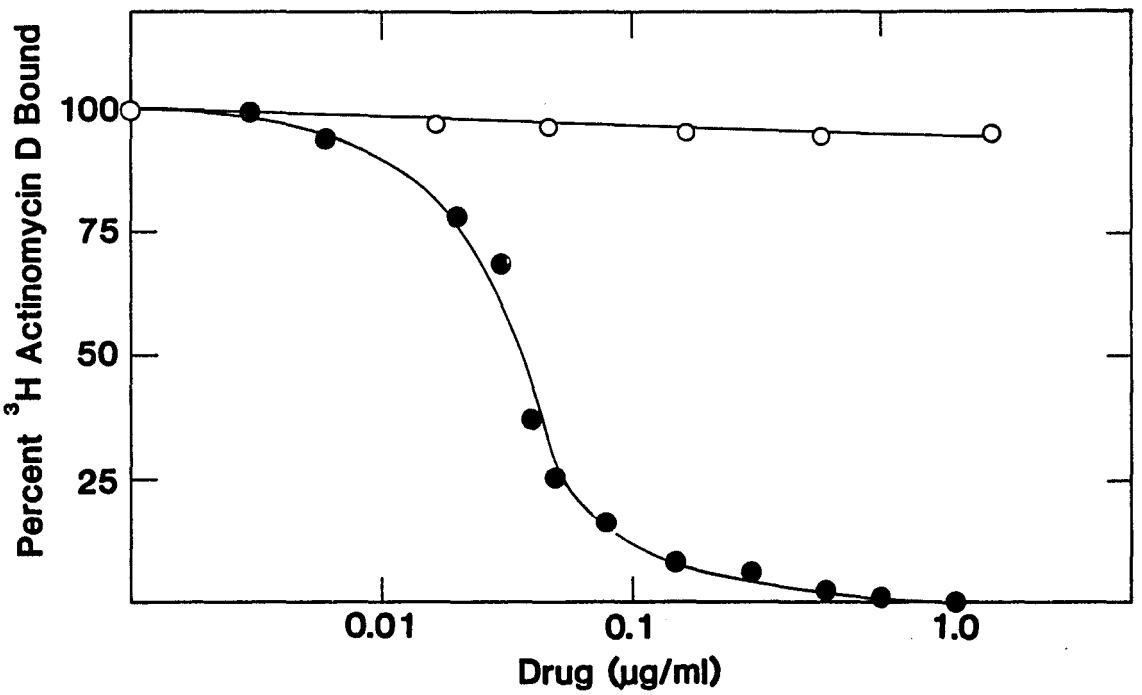

Figure 2-5. Competition of Act D and Actinomine for Specific Act D Antibody. Closed circles represent Act D; open circles represent actinomine. Increasing amounts of the respective compounds were added to [^{3}H]Act D, the mixture was combined with antibody, and assayed as described in Materials and Methods. Percent [^{3}H]Act D bound to the antibody at the various concentrations was calculated. 100% = 20,000 dpm; background = 500 dpm. The abscissa range is 10^{-13} to 10^{-10} moles for Act D, and 10^{-11} to 10^{-9} moles for actinomine. Data represent the average of triplicate samples with the maximum standard error of 2.7%.

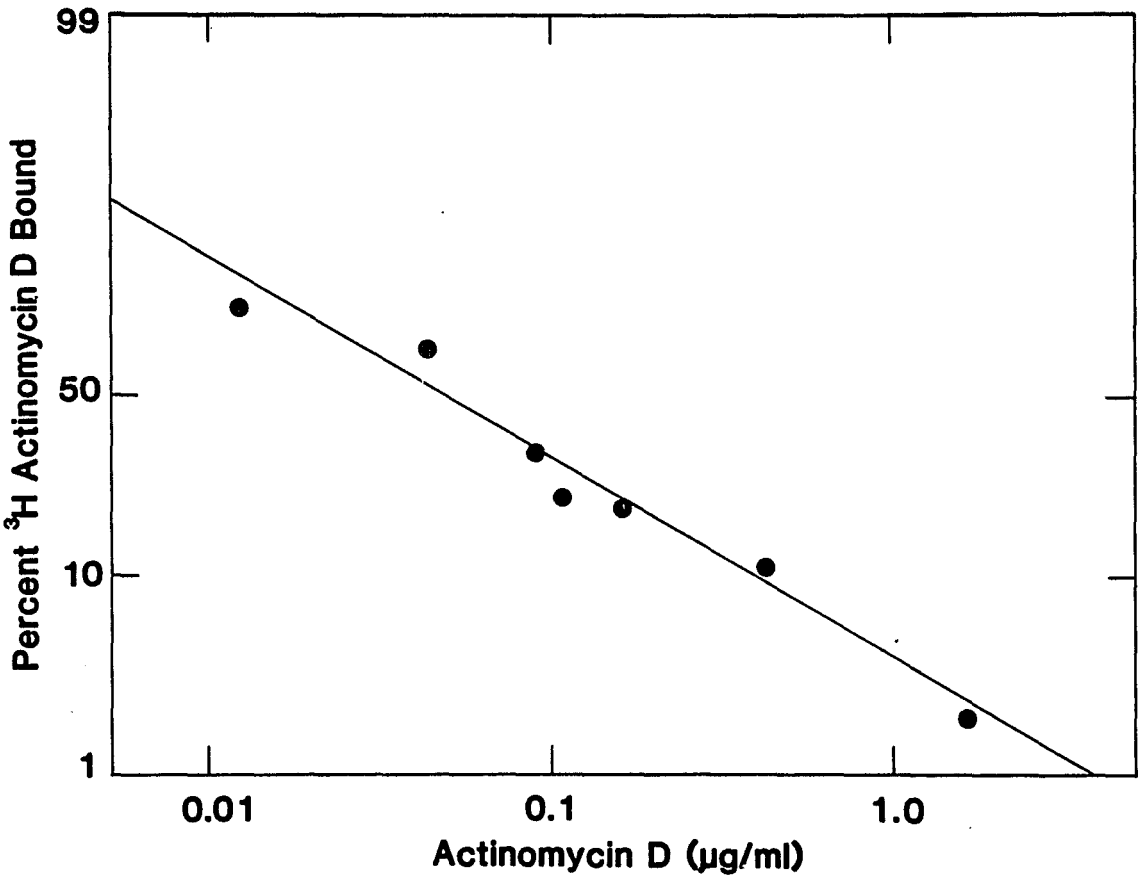

Figure 2-6. Log-Logit Plot of the Act D Radioimmunoassay. (See Legend, Fig. 2-5.)

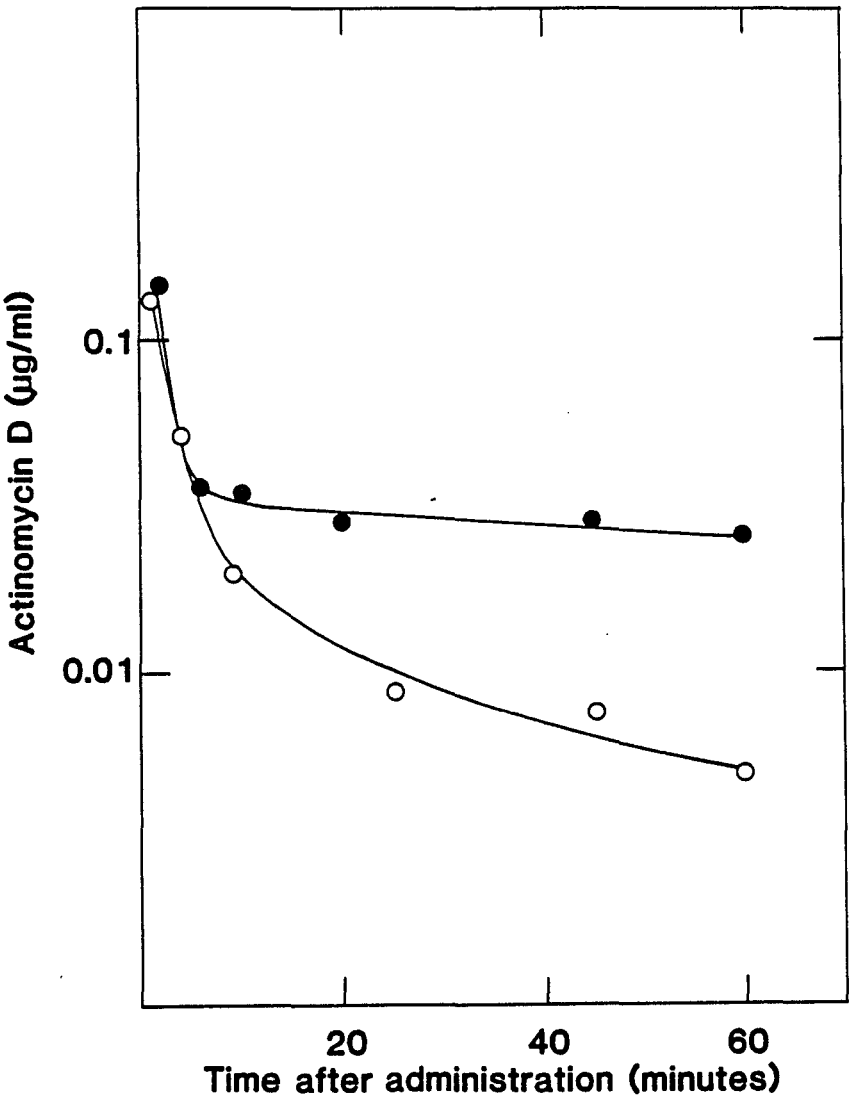

Figure 2-7. Serum Concentrations of Actinomycin D With Time After Initial Dose. Serum samples obtained at various times after Actinomycin D injection were assayed using the radioimmunoassay described in Materials and Methods. Open circles represent data from serum of a human; closed circles represent that from dog serum. The human serum $\alpha t_{\frac{1}{2}}$=1.78 min, and $\beta t_{\frac{1}{2}}$= 34 min. The dog serum $\alpha t_{\frac{1}{2}}$= 0.78 min, and $\beta t_{\frac{1}{2}}$= 208 min. Data represent the average of triplicate samples with the mean coefficient of variation for all human points of 9.4% and the mean coefficient of variation for all points on the dog curve of 5.1%

identical course of therapy three weeks prior to this dose, it was necessary to determine if antibodies to Act D were present in the serum. This was done by comparison of the subject's zero time serum with that of the nonimmune rabbit control. No Act D antibody was detected. Reproducibility (i.e., % coefficient of variation) of the assay for both human and dog samples was determined to be 9.5% and 5.1%, respectively. Serum levels revealed a biphasic pharmacokinetic pattern within one hr after administration. The human alpha phase serum half-life was 1.78 min while the beta phase was 34 min. The alpha serum half-life in the dog was 0.78 min and beta phase was 208 min. Knowing the sensitivity of the assay to be at least 0.005 μg/ml, it would appear that additional values beyond the one-hr time point are possible to obtain.

For determination of the anti-Act D antibody class, the IgG fraction was easily purified from the serum by affinity chromatography using the Protein A C1-4B Sepharose column. Fig. 2-8 is representative of the type of separation of proteins observed. Approximately 5 mg of IgG was separated from each ml of serum applied to the column.

Discussion

Since Act D is known to negatively effect the immune system (Pratt and Ruddon, 1979) and the drug is a relatively small molecule, it was coupled to the larger BSA molecule to enhance the immune response. It was anticipated that the Act D-BSA complex would serve a dual purpose since the heterocyclic chromophore portion of the molecule is that which intercalates into the DNA helix (Muller and Crothers, 1968). The chromophore attached directly to BSA (Figs. 2-1 and 2-2)

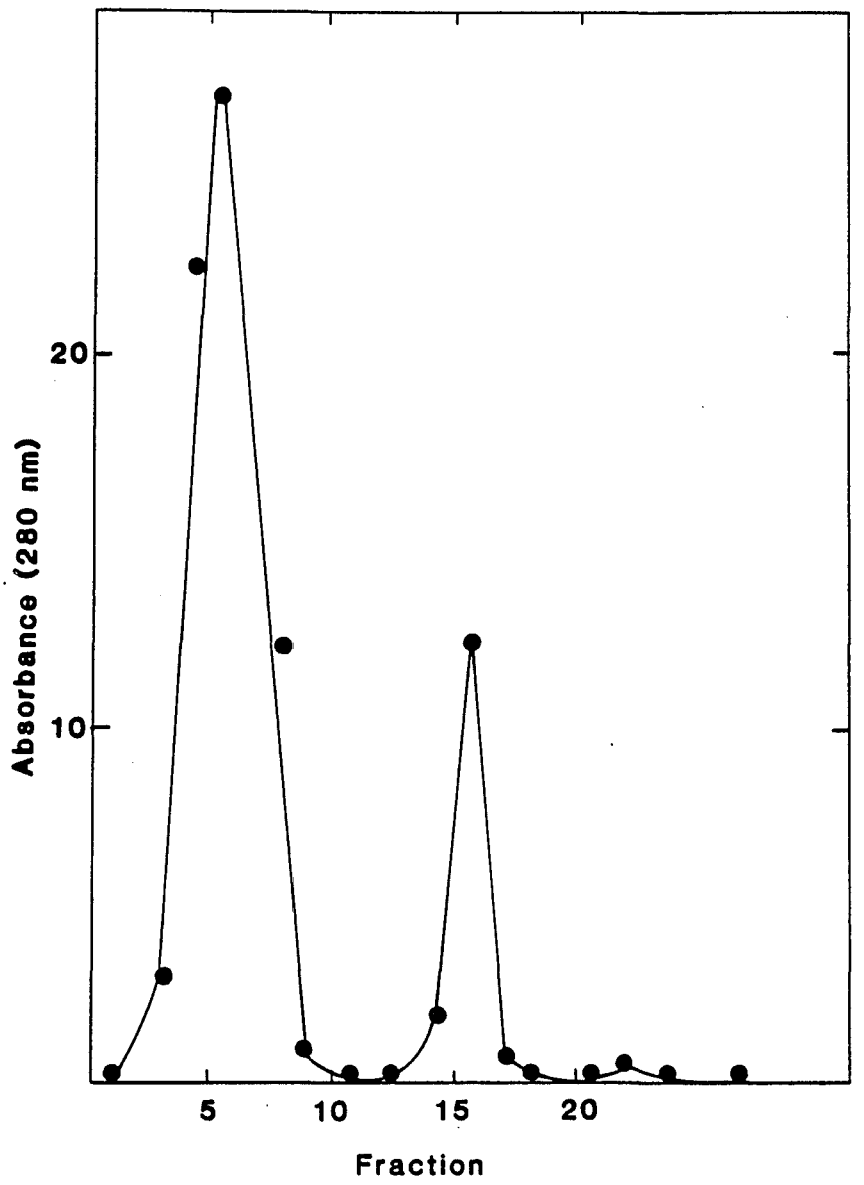

Figure 2-8. Separation of IgG From Serum by Affinity Chromatography. When rabbit serum is passed over the protein A C1-Sepharose 4B column described in the text (and Appendix C), serum proteins are seen in the flow-through peak (first) and the IgG fraction is eluted with 0.1 M acetic acid (second peak).

would be expected to: 1) not suppress the immune response as the free drug does, and 2) generate antibodies to the pentapeptide lactone portion of the molecule, enabling these antibodies to potentially detect Act D that is already intercalated into DNA.

The EDC catalyzed coupling reaction (Fig. 2-2) was a simple procedure, but optimum coupling occurred when pH was carefully maintained at 4.5, and the ratio (w/w) of 3 Act D:1 BSA was used. The resulting 2.4 moles Act D bound per mole of BSA was the best coupling observed. When the ratio of reagents deviated from the optimum 3 Act D:1 BSA (w/w), the coupling ratio generated was less satisfactory. For example, when the reagent ratio was reversed, [1 Act D;3 BSA (w/w)], a coupling ratio of only 0.07 moles Act D per mole of BSA was generated.

Four weeks after the initial inoculation, anti-Act D antibody was detected in serum of both rabbits injected with the Act D-BSA complex. Fig. 2-4 shows the production of antibody after boost as detected by gel filtration. The rabbit initially injected with 250 μg Act D-BSA complex showed a peak in antibody production 18 days after boost. The rabbit initially injected with 100 μg Act D-BSA complex demonstrated maximum antibody production 11 days after boost. These times were important so future bleeding of rabbits could be scheduled accordingly after boost. Since the rabbit initially injected with the highest amount of Act D-BSA complex (250 μg) consistently demonstrated higher Act D binding by gel filtration than did the rabbit initially injected with the smaller dose (100 μg), serum from this higher anti-Act D antibody activity rabbit was used in all subsequent experiments. The use

of gel filtration was adequate in detecting antibody activity (Fig. 2-3a) and also showed that there was no nonspecific serum protein interactions with Act D (Fig. 2-3b). Gel filtration was, however, a time-consuming and cumbersome assay for anti-Act D antibody, so a more efficient assay system was investigated for multiple sample analysis.

The double antibody-technique using goat anti-rabbit IgG (Parker, 1976) was attempted and proved useful in antibody detection, but was unacceptable because of high nonspecific background binding. The charcoal assay, on the other hand, proved to be extremely efficient in removing free Act D and reducing the background. In the 5-min incubation period with charcoal, greater than 99% of the free Act D was bound to the charcoal, leaving only Act D-antibody complex present in solution; hence, labeled drug in the supernatant after centrifugation was a good measure of [^{3}H]Act D bound to antibody. The charcoal assay is rapid, with an initial Act D-antiserum incubation period of only one hr. The charcoal assay was an essential step in developing a radioimmunoassay for Act D. This radioimmunoassay (Figs. 2-5 and 2-6) allowed detection of Act D down to 0.005 μg/ml. Linearization of the curve on log-logit axes (Fig. 2-6) was necessary for interpolation of samples with unknown Act D concentration.

Utility of the Act D antibody has been demonstrated by using the radioimmunoassay for quantitation of serum levels of the drug in a patient and a dog. The differences in serum half-lives for the human and the dog (Fig. 2-7) are not unexpected. Galbraith and Mellett (1975) show species differences in Act D serum clearance. More complete

pharmacokinetic studies are necessary before any conclusions can be drawn about actual half-lives and species variation. Due to the simplicity of the assay described, complete pharmacokinetic analysis could be easily performed.

Pharmacokinetic studies of Act D in humans and animals have previously been reported (Galbraith and Mellet, 1975; Tattersall et al. 1975) using labeled drug. The radioimmunoassay developed eliminates the introduction of unnecessary radioactivity into the patient. Furthermore, since tritiated drug was used in the previous study, the possibility exists that some tritium may exchange with body water, and the amount of drug detected in later time samples could be due to ^3H_2O since actual drug was not measured. Using a radioimmunoassay specific for the drug eliminates this possible artifact.

While two phases of serum levels of Act D have previously been observed in humans (Tattersall et al. 1975), the half-life of the alpha phase has not been accurately reported. By examining early time points, it was determined that this value was 1.78 min, which indicates the rapid redistribution of the drug from serum to tissue. It is likely that the half-life of the beta phase will be more accurately determined upon examination of serum samples beyond one hr.

A preliminary characterization of an antibody to Act D was previously presented by Raso (1977), with sensitivity reported to 1 pmol. The work described in this chapter details the methodology for Act D antibody preparation, and presents a simplified radioimmunoassay procedure which has a ten-fold increase in assay sensitivity as compared

to the Raso procedure. These studies also demonstrate a clinical application of this radioimmunoassay by detection of Act D serum levels. It is possible that the sensitivity for Act D detection could be increased by varying volumes of antiserum used and amount of [^{3}H]Act D in the assay. Sensitivity of the assay might also be increased using protein A-purified IgG instead of whole serum in the radioimmunoassay; however, if this is done, the protein content must be increased (e.g. with BSA) to a concentration similar to that of serum to prevent nonspecific binding of the IgG. It appears from Fig. 2-7 that for detection of blood levels of Act D, whole rabbit serum is of sufficient sensitivity indicating that IgG purification is not necessary for radioimmunoassay.

Chemotherapeutic use of Act D is known to suppress the immune system (Pratt and Ruddon, 1979). However, the present study demonstrated that Act D can elicit an antibody response when the drug is bound to BSA. Apparently, the drug-BSA complex did not inhibit an immune response. Further, the data suggest that the antigenic determinant resides in the pentapeptide lactone portion of the molecule (Fig. 2-5). Because Act D binds to DNA by intercalation of the heterocyclic chromophore moiety (Muller and Crothers, 1968), it should be possible to ultimately examine drug binding to DNA at the molecular level at concentrations which are known to inhibit rRNA synthesis in vivo. Since a unique binding isotherm for Act D has been found at this concentration range (Duffy and Lindell, in preparation), the antibody preparation described may be useful in further defining this site.

The protein A column was used in separating the IgG fraction from serum since protein A has been shown to be highly specific for the Fc portion of the IgG molecule (Kronvall and Frommel, 1970). This purification step was not necessary for detecting Act D by radioimmunoassay; however, use of the IgG fraction may increase assay sensitivity as described earlier. As evidenced in Chapter 3, the IgG purification was necessary for the detection of Act D bound to chromosomes.

Steps in antibody purification are described in Appendix C. Pure anti-Act D IgG was not obtainable, presumably due to an extremely high affinity of the antibody for the drug. When a specific Act D affinity column was constructed (Appendix C), the specific antibody was easily removed from both serum and the IgG fraction. This investigator was unable, however, to elute the tightly bound antibody from the affinity column to yield an active specific IgG molecule, even when the most harsh conditions of elution were attempted. Preparation of Actinomycin D acid, which has an open peptide ring structure (Brockmann and Manegold, 1967) was shown to have a weaker affinity for the antibody. The affinity purification (Appendix C), when attempted with this molecule, also proved unsuccessful.

The need for pure antibody was believed to be necessary for recognition of chromosomal sites where Act D was bound. However, experiments with the IgG fraction indicated that this was not so, and it was eventually determined that the IgG fraction was sufficient for detection of Act D bound to chromosomes (Chapter 3).

Chapter Summary

This chapter presents the methodology for the development and characterization of a specific antibody for Actinomycin D. Application of this antibody for the development of a radioimmunoassay for Act D proved useful for the pharmacokinetic analysis of the drug, describing its clearance from serum. The purification of this antibody for use in detection of Act D bound to chromosomes was described. This application will be discussed in detail in the following chapter.

CHAPTER 3

IMMUNOFLUORESCENT DETECTION OF ACTINOMYCIN D BOUND TO CHROMOSOMES

Introduction

As discussed in Chapter 1, several investigators have examined the binding of Actinomycin D (Act D) to human metaphase chromosomes by autoradiography. For the most part, conclusions about drug binding to chromosomes were inconsistent, and the drug concentration employed was not optimized with regard to inhibition of ribosomal RNA synthesis. Since 0.001 - 0.1 μg/ml Act D is known to selectively inhibit rRNA synthesis in intact cells (Perry, 1962), this was the range of Act D-chromosome binding most closely examined by autoradiography in Chapter 1. Also presented in Chapter 1 was a new and simple technique that utilized Formvar and potassium chromium sulfate prior to autoradiography. This methodology permitted the simultaneous visualization of G-banded chromosomes and autoradiographic grains.

Previous reports suggested that Act D inhibition of rRNA synthesis at 0.001 - 0.1 μg/ml occurs due to selective binding of the drug to the ribosomal cistrons. Data from Chapter 1 does not support this suggestion since, at these low concentrations, autoradiographic grains are associated at numerous chromosomal sites. Very little binding was observed at the ribosomal cistrons that are located on the short arms

of the D and G group chromosomes. Evidence was also presented that was unsupportive of the theories that Act D preferentially binds to heterochromatin (Simard, 1967) or euchromatin (Berlowitz, Pallotta, and Sibley, 1969). The binding observed was randomly associated with chromatin.

Due to the limitations of the specific activity of [^{3}H]Act D available and increased autoradiographic background observed with prolonged exposure times, alternate procedures to study Act D binding to chromosomes were considered. In addition, autoradiography is a time-consuming procedure, requiring days (or weeks) before results (with inherent limitations) can be observed. It was thought that some of these limitations could be overcome if an antibody specific for Act D could be used to detect drug bound to chromosomes. Production of such an antibody and its characterization was described in Chapter 2. This chapter will describe the use of this antibody in detection of Act D bound to human metaphase chromosomes.

Antibodies belong to a group of structurally related glycoprotein molecules known as immunoglobulins. As shown in Chapter 2, since the active portion of the immune rabbit serum that binds Act D can be separated on a Protein A-Sepharose affinity column, it was determined that anti-Act D was of the class immunoglubulin G (IgG). This was not unexpected, since the IgG component of serum comprises 75% of the total serum immunoglobulins (Weir, 1977).

The IgG molecule (Fig. 3-1) is composed of two identical "heavy" amino acid chains (bridged by disulfide bonds) and two identical "light"

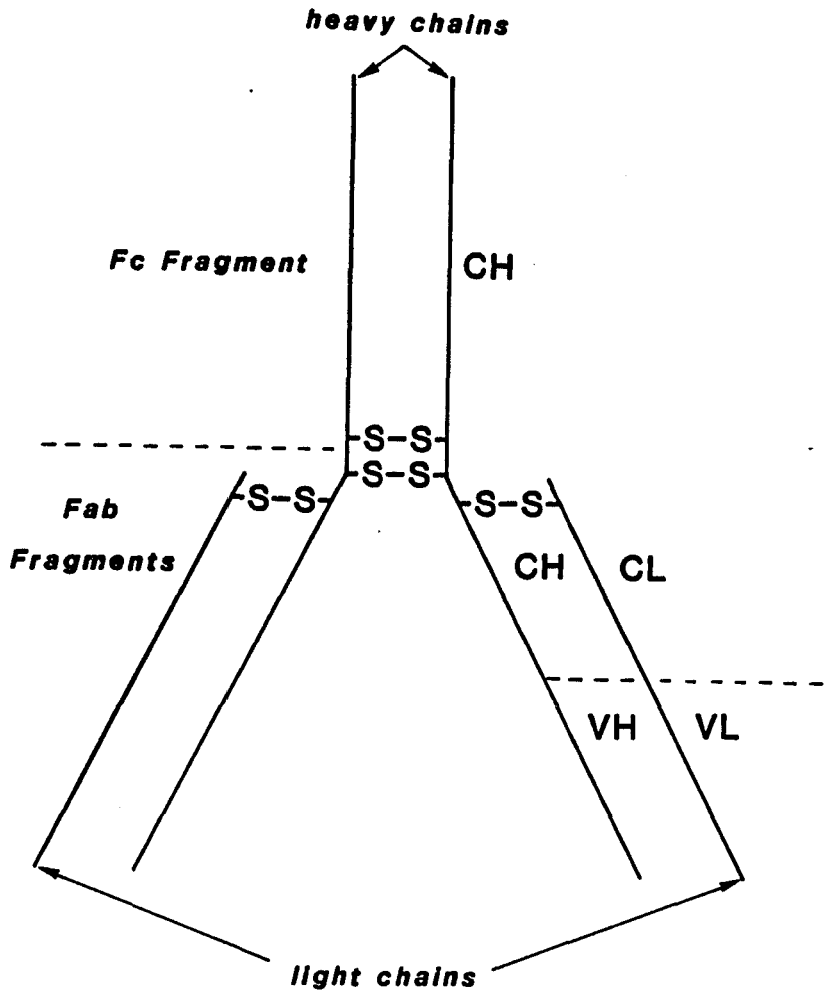

Figure 3-1. The Immunoglubulin G Molecule (IgG). (MW:145,000) This diagrammatic representation of the 446 amino acid chain is divided into constant (C) and variable (V) regions. Two identical heavy chains (H) are connected by disulfide bonds, and each heavy chain is connected to a light chain (L). The CH regions determine the immunoglobulin class, and the V regions contain the antigen-binding site. The Fab fragments are thus necessary for antigen recognition, and it is the Fc fragment that combines with a secondary antibody in immunofluorescence.

amino acid chains, each bridged to a heavy chain with a disulfide bond (Edelman et al. 1969). The Fc fragment of the molecule contains only parts of the heavy chains, and this "constant" amino acid sequence (see Fig. 3-1) determines the class of a specific immunoglobulin. The Fc portion is also the part of the molecule that binds to Protein A (Kronvall and Frommel, 1970). The Fab fragments are composed of both the light chains and parts of the heavy chains, each containing a constant amino acid sequence. The remainder of the Fab fragments contain variable regions on both the heavy and light chains (Edelman et al. 1969). These variable regions contain a unique amino acid sequence, which provides high binding affinity for the antigenic determinant (Sternberger, 1979). Since the pentapeptide lactone portion of the Act D molecule is the antigenic determinant (Chapter 2), it would bind to the variable regions of the Fab portion of the IgG molecule.

There are two methods generally employed to detect antibody bound to biological preparations (Fig. 3-2). Direct immunofluorescence (Fig. 3-2a) is a procedure where the primary antibody (in this case anti-Act D) is covalently bound (tagged) to fluorescein and visualized with a fluorescent microscope. Indirect immunofluorescence (Fig. 3-2b) involves the use of a secondary antibody that is produced in a species different from the primary antibody. If the secondary antibody is coupled to fluorescein, detection of the primary antibody is indirectly observed by binding to the secondary antibody followed by fluorescent microscopy (Sternberger, 1979).

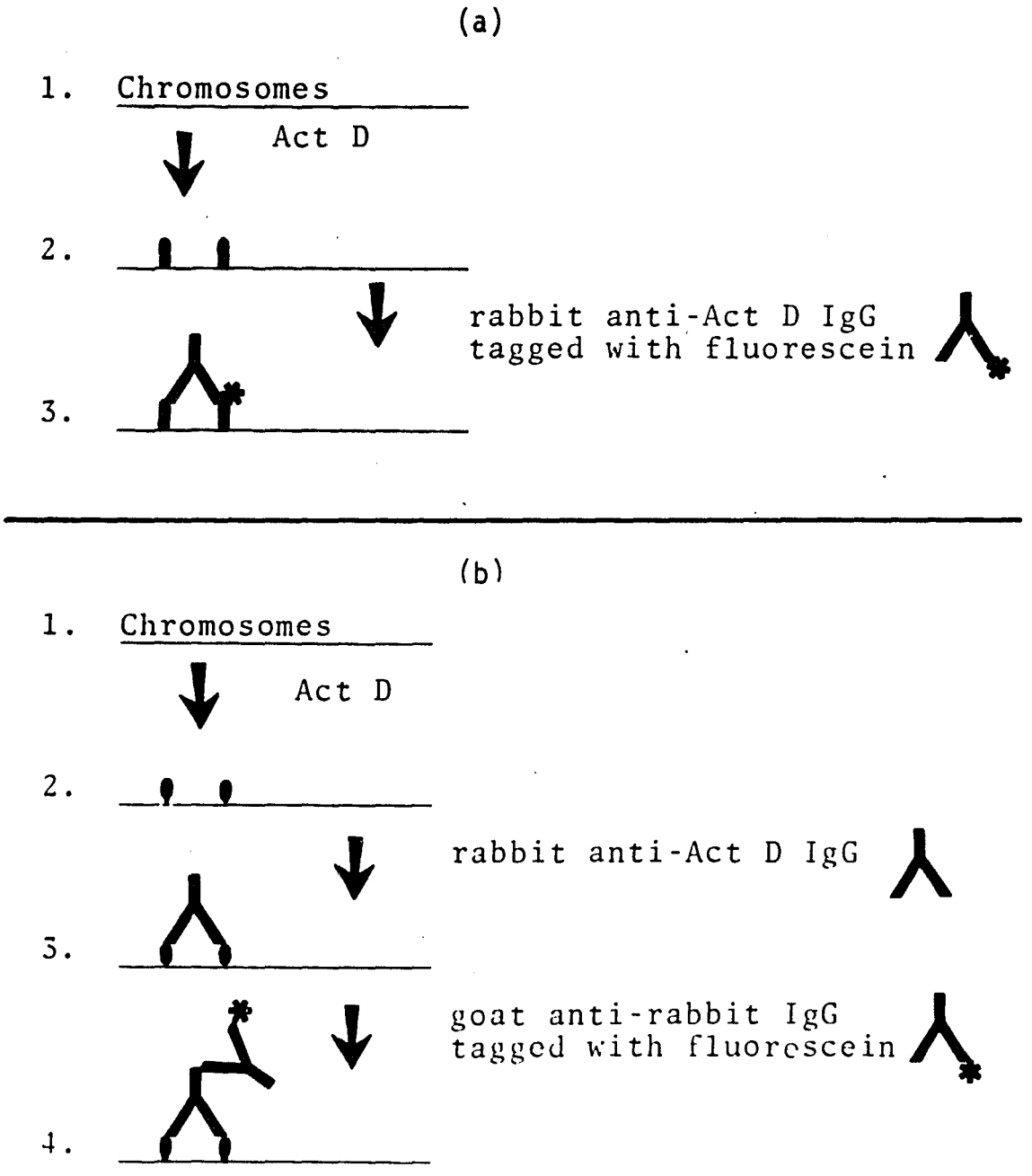

Figure 3-2. Diagrammatic Representation of Immunofluorescence. a) Direct immunofluorescence. b) Indirect immunofluorescence.

The use of secondary antibodies has previously been employed with chromosomes. Schreck et al. (1973) and Schreck, Erlanger, and Miller (1974) demonstrated the use of an antibody, specific for purine and pyrimidine bases, to observe binding of this antibody to fixed metaphase chromosomes by indirect immunofluorescence. Miller et al. (1974) were able to demonstrate specific localization of 5-methylcytosine on fixed metaphase chromosomes with an anti-5-methylcytosine antibody. Immunofluorescent detection of anti-nucleoside antibodies on metaphase chromosomes has also been presented by Morin, Marcollet, and Leng (1979). Other reports of immunofluorescent detection of antibodies bound to chromosomes included an anti-Z-DNA antibody (Nordheim et al. 1981), antibodies specific for mitotic chromosomes (Davis and Rao, 1982), and anti-histone antibodies (Bustin et al. 1976).

All these reports describe localization of an antibody bound directly to chromatin. This chapter describes the first demonstration of an antibody bound to a compound that is not part of the chromosome structure. In Chapter 2, it was shown that the anti-Act D antibody was specific for the free drug. Since the antibody was found to be specific for the pentapeptide portion of Act D, it was thought that the antibody might recognize the drug bound to DNA because the pentapeptide is not that portion of the molecule intercalated (Muller and Crothers, 1968). This chapter shows that the antibody can detect binding of drug already bound to chromatin using indirect immunofluorescence. This chapter also introduces the use of this system as a tool that may be useful in the elucidation of chromosome structure and organization.

Materials and Methods

Chromosome preparation. Human lymphocyte cultures, obtained during the course of these studies from six male and six female volunteers, were grown for 72 hr in RPMI-1640 media (Gibco) supplemented with 25% fetal bovine serum (Gibco), and 120 µg/ml phytohaemagglutinin (Burroughs-Wellcome), with penicillin-streptomycin (Gibco) as a bacteriostatic agent. Cultures were grown in sterile disposable centrifuge tubes (Falcon Plastics) at 37°. Chromosomes were harvested following standard procedures as described by Moorhead et al. (1960), and fixed in 3 parts methanol:1 part acetic acid. (See Appendix A.)

Slides were prepared and only those containing a satisfactory number (averaging one metaphase per field of view using a 16X phase contrast objective) of good quality metaphase chromosomes were used. Slides were used within three days after they were made (see Appendix A). All procedures were performed at room temperature unless otherwise specified.

Labeling chromosomes with Act D. A stock solution of 10 µg/ml Act D (Merck) was prepared in 5% ethanol, and kept at -20° until use. Immediately prior to chromosome labeling, the stock was diluted 1:100 in 5% ethanol in 0.01 M Tris-HCl, pH 7.3. Prior to initial characterization of the antibody system, [^{3}H]Act D (Amersham) was used to determine exact concentrations of drug by liquid scintillation counting. Slides were layered with 300 µL of the appropriate concentration of Act D and incubated with a coverslip in the horizontal position for 15 min in a

humid chamber. Drug that was not bound to chromosomes was removed by washing the slide three times in 95% ethanol (50 ml/wash) and slides were air-dried.

Direct immunofluorescence. The Protein A purified IgG containing anti-Act D IgG was conjugated with fluorescein isothiocyanate (FITC, Sigma) as described by Johnson and Dorling (1981) (see Appendix D). Slides were pretreated with 300 µL of bovine serum albumin (10 mg/ml), coverslipped, incubated in a humid chamber for 20 min, and then rinsed with 50 ml phosphate buffered saline (PBS, Appendix D). Slides were layered with 300 µL of the appropriately diluted FITC conjugated anti-Act D IgG, coverslipped, incubated in a humid chamber for 20 min, and washed in 50 ml PBS and immediately coverslipped with Gelvatol (Monsanto) as a mounting medium.

Indirect immunofluorescence. Slides were pretreated with 300 µL of human IgG (10 mg/ml), coverslipped, and incubated in a humid chamber for 20 min, washed in 50 ml PBS, and 300 µL of anti-Act D IgG (10 mg/ml) was applied. Slides were coverslipped, incubated 20 min in a humid chamber, and rinsed in 50 ml PBS. Slides were then layered with 300 µL of the appropriately diluted goat anti-rabbit IgG (Cappel, 1:20 dilution), incubated under a coverslip for 20 min in a humid chamber, and washed in 50 ml PBS. Slides were immediately coverslipped with Gelvatol as a mounting medium.

Proteolytic enzyme treatment. Prior to Act D labeling, slides were dipped into a 1:20 dilution of Viokase (Gibco, 37^{o}) for the length of time necessary to produce good quality G-bands (generally 3 sec),

or dipped in a 1:20 dilution of trypsin (Gibco, 37°). Some slides were then partially stained with Geimsa (Gurr) to determine if adequate banding was observed. Act D was then bound to non-stained slides or Geimsa-stained slides after removal of the stain with 3 washes of 95% ethanol (50 ml/wash).

DNAase I treatment. Prior to Act D labeling, 300 µL of 100 units/ml DNAase I (Worthington) in Sorenson's buffer, pH 7.6 containing 5 mM $MgCl_2$ was layered on the slides, which were then coverslipped and incubated at 37° in a humid chamber for 2 hr (Schweizer, 1977). Slides were then rinsed in 50 ml distilled water.

HCl treatment. Slides were layered with 300 µL of 0.2 N HCl, coverslipped and incubated in a humid chamber at room temperature for 4 hr (Comings and Avelino, 1974).

Constitutive heterochromatin staining (C-banding). Slides were dipped in 0.2 N HCl for 30 min, rinsed, placed in a saturated solution of $Ba(OH)_2$ for 20 min, rinsed in tap water, and incubated in 2X SSC for 2 hr at 60° (see Appendix A). Some slides were partially stained with Geimsa to determine the quality of the C-bands.

Determination of Act D dissociation from chromosomes. Slides were labeled with 0.1 µg/ml [^{3}H]Act D as described above and in Appendix B. Slides were then layered with 300 µL of PBS, coverslipped, and incubated at room temperature for 0, 15, 30, 45, or 60 min. Slides were rinsed in distilled water and prepared for autoradiography as described in Appendix B, exposed for 7 days, and developed.

Incubation with anti-native DNA antibody. Human antisera specific for native double stranded DNA was obtained from the Department of Pathology, Arizona Health Sciences Center (courtesy, Dr. R. Nagle). This was appropriately diluted in PBS (1:2) and 300 µL was layered on fresh slides containing metaphase spreads. Slides were coverslipped, incubated in a humid chamber for 20 min, and rinsed in 50 ml PBS. Slides were then layered with a 1:20 dilution of fluorescein conjugated goat anti-human IgG (Cappel), coverslipped, and incubated in a humid chamber for 20 min. Slides were rinsed in 50 ml PBS and coverslipped with Gelvatol as a mounting medium.

Photomicroscopy. Chromosomes containing fluorescent primary or secondary antibody were photographed on a Zeiss photomicroscope with an M-35 camera using a Zeiss Fluorescein filter and a Planapo 63x objective/1.4 oel. A mercury arc high vacuum lamp was used as a light source and chromosomes were photographed on Ilford Hp5 ASA 400 film. Autoradiographs were photographed on a Zeiss Photomicroscope II equipped with a Planapo 100x objective/1.3 oel, using Kodak Technical Pan Film 2415.

Results

Actinomycin D is not irreversibly bound to deproteinized DNA in an aqueous medium. Since the antibody preparation must be suspended in an aqueous buffer, it was important to examine the stability of Act D bound to chromosomes. The dissociation of Act D from chromatin was therefore examined by autoradiography. Fig. 3-3 shows that after 60 min aqueous exposure, there is a very slight decrease in the number of autoradiographic grains associated with each metaphase cell ($t_{\frac{1}{2}}$=4.5 hr).

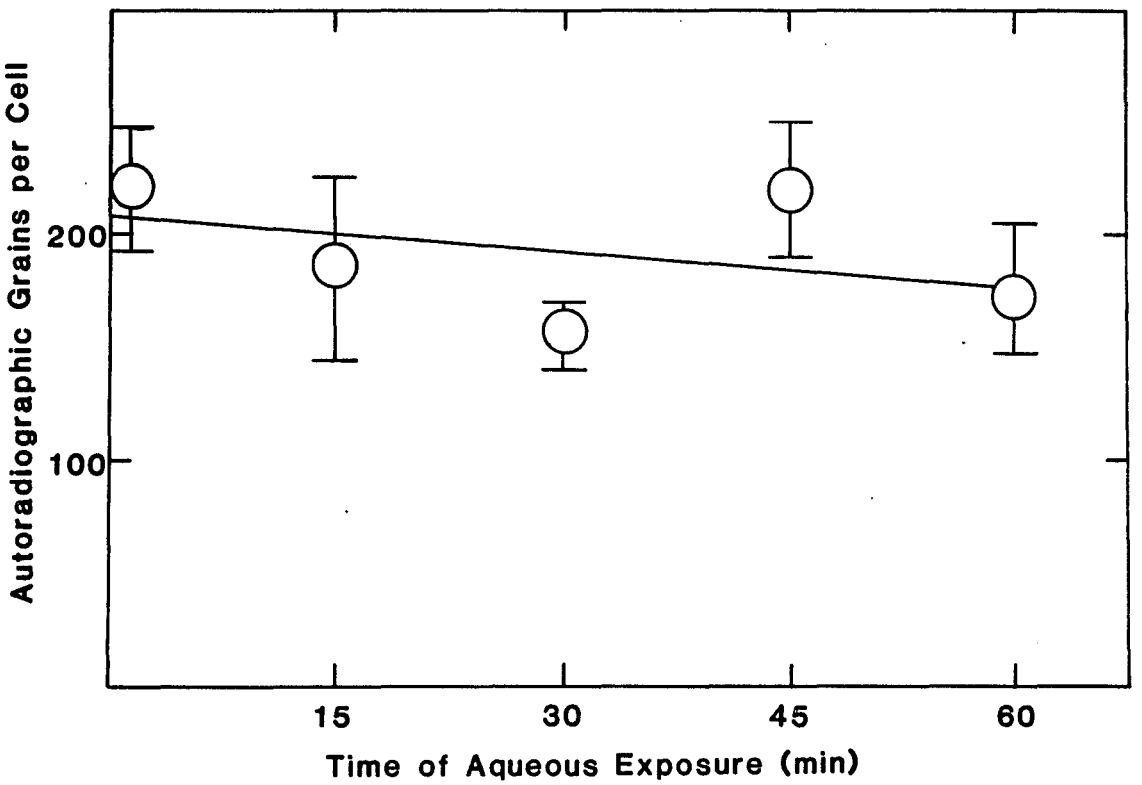

Figure 3-3. Dissociation of Act D From Chromosomes in Aqueous Solution. 0.1 µg/ml [^{3}H]Act D was bound to human metaphase chromosomes, and then slides were incubated with phosphate buffered saline for 0, 15, 30, 45, or 60 min. Slides were then exposed for autoradiography for 7 days and metaphase cells scored for autoradiographis grains. Bars indicate standard error of the 12 metaphases counted per timepoint. $t_{1/2}$ of Act D dissociation = 4.5 hr.

Since the maximum aqueous exposure to chromosomes after the binding of Act D was 60 min, it was determined that most drug detected by antibody would still be bound to chromatin. Furthermore, when anti-Act D IgG was used in the aqueous incubation (10 mg/ml), no difference was observed in the number of autoradiographic grains when compared to incubation only in PBS (data not shown). This indicates that the antibody neither increases nor decreases the rate of dissociation of Act D from chromosomes.

In the initial characterization of antibody detection of Act D bound to chromosomes, the results of several procedures investigated are shown in Fig. 3-4. Direct immunofluorescence (Fig. 3-4a) produced faint chromosome fluorescence with a considerable amount of fluorescent background. Indirect immunofluorescence using anti-Act D serum (Fig. 3-4b) also shows faint chromosomal fluorescence. When the anti-Act D IgG fraction is used in indirect immunofluorescence (Fig. 3-4c), the chromosomes appear brighter, but noticeable fluorescent background is still present. However, if human IgG is used as a preincubation to the anti-Act D IgG localization by indirect immunofluorescence, the chromosomes are bright, distinctly fluorescent, and background is extremely low (Fig. 3-4d). These observations, along with those of the negative controls are summarized in Table 3-1. It can be seen (Table 3-1) that when antigen (Act D) or primary antibody (anti-Act D) are missing, chromosomal and nuclear fluorescence are absent. If a different primary antibody (nonimmune rabbit IgG) or a different secondary antibody (goat anti-human IgG) are used, the chromosomes or nuclei also do not fluoresce.

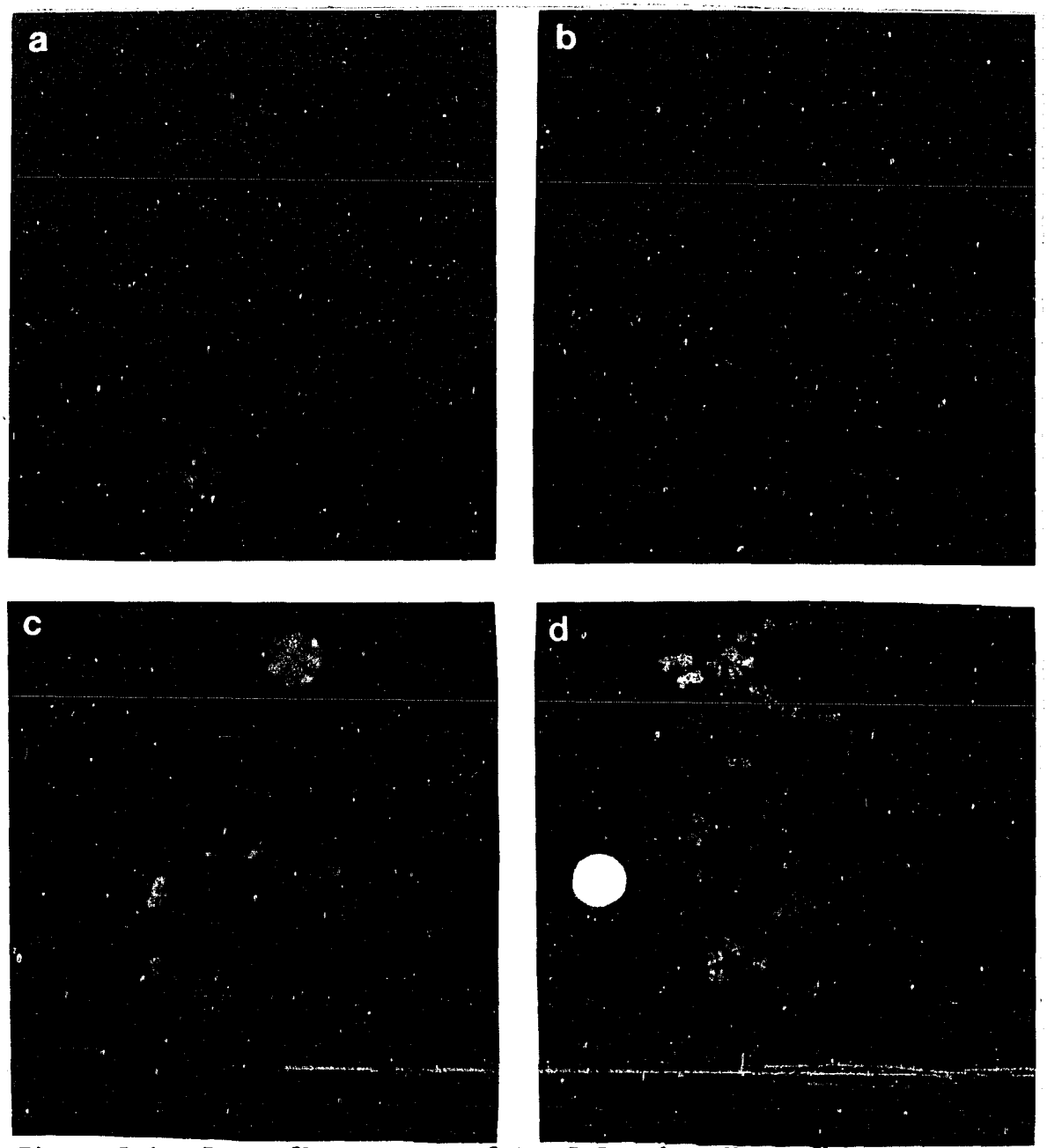

Figure 3-4. Immunofluorescence of Act D Bound to Human Chromosomes. Photographs of human metaphase chromosomes were taken after binding of 0.1 µg/ml Act D and various antibody procedures. a) Direct immunofluorescence using FITC-conjugated anti-Act D IgG. b) Indirect immunofluorescence after preincubation with human IgG, followed by whole rabbit anti-Act D serum and FITC-conjugated goat anti-rabbit IgG. c) Indirect immunofluorescence using rabbit anti-Act D IgG and FITC-conjugated goat anti-rabbit IgG (no human IgG preincubation). d) Indirect immunofluorescence after preincubation with human IgG followed by rabbit anti-Act D IgG and FITC-conjugated goat anti-rabbit IgG. Magnification: 630x.

Table 3-1. Observations After Various Antibody Treatments.

	Treatment	Observation	Interpretation
1.	0.1 µg/ml Act D, FITC-conjugated anti-Act D IgG	Chromosomes and nuclei faintly fluoresced. Background fairly high. (Fig. 3-3a.)	Insufficient fluorescein signal for Act D detection.
2.	0.1 µg/ml Act D, anti-Act D serum, goat anti-rabbit IgG*	Chromosomes and nuclei faintly fluoresced. (Fig. 3-3b.)	Insufficient Act D detection due to presence of serum proteins.
3.	0.1 µg/ml Act D, (no human IgG) anti-Act D IgG, goat anti-rabbit IgG*	Chromosomes and nuclei had considerable fluorescence. Background high. (Fig. 3-3c.)	Increased background due to nonspecific rabbit IgG binding to Act D.
4.	0.1 µg/ml Act D, human IgG, anti-Act D IgG, goat anti-rabbit IgG*	Bright fluorescence of chromosomes and nuclei. Background fluorescence low. (Fig. 3-3d.)	Ideal conditions for detection of Act D bound to chromosomes.
5.	(No Act D) Human IgG, anti-Act D IgG, goat anti-rabbit IgG*	No fluorescence of chromosomes or nuclei.	Confirmation that the primary antibody (anti-Act D) system is functional.
6.	0.1 µg/ml Act D, human IgG, non-immune IgG (from control serum), goat anti-rabbit IgG*	Extremely weak fluorescence of chromosomes and nuclei.	Primary antibody is specific for Act D only.
7.	0.1 µg/ml Act D, anti-Act D IgG, goat anti-human IgG*	No fluorescence of chromosomes or nuclei.	Secondary antibody system (goat anti-rabbit IgG) is functional.
8.	0.1 µg/ml Act D, human IgG, (no anti-Act D IgG), goat anti-rabbit IgG*	No fluorescence of chromosomes or nuclei.	Act D itself does not cause the fluorescence observed.

*Molecule labeled with fluorescein.

It should also be mentioned that when concentrations below 0.1 μg/ml Act D were used and localization was attempted with indirect immunofluorescence, the fluorescence became more faint, and the drug was not detectable when less than 0.01 μg/ml Act D was used.

As a positive control for determining the presence of DNA, the human anti-native DNA antibody was used (Fig. 3-5). The positive control with no pretreatment (Fig. 3-5a) shows fluorescent chromosomes. After a 3-sec Viokase treatment (Fig. 3-5b), DNA is still observed by fluorescence, although the morphology appears to be somewhat distorted. When chromosomes are pretreated with DNAase I, very little DNA appears to be present since the fluorescence is greatly decreased (Fig. 3-5c). After the C-banding procedure, fluorescence is also greatly decreased (Fig. 3-5d).

Act D binding to chromosomes can also be observed before and after Viokase treatment in Fig. 3-6. As seen with indirect immunofluorescence, before Viokase treatment (Fig. 3-6a), the chromosomes have a distinct morphology and bright fluorescence. After a 3-sec Viokase treatment (Fig. 3-6b), the fluorescence remains but the chromosomes appear ragged along the edges. This can be compared to autoradiographic detection of [^{3}H]Act D binding. Similar grain counts are observed on chromosomes labeled with [^{3}H]Act D before (Fig. 3-6c) and after Viokase treatment (Fig. 3-6d).

When Act D was bound after DNAase I treatment of chromosomes, the chromosomes or nuclei did not fluoresce after the indirect antibody procedure. This indicates that the Act D observed in the non-DNAase

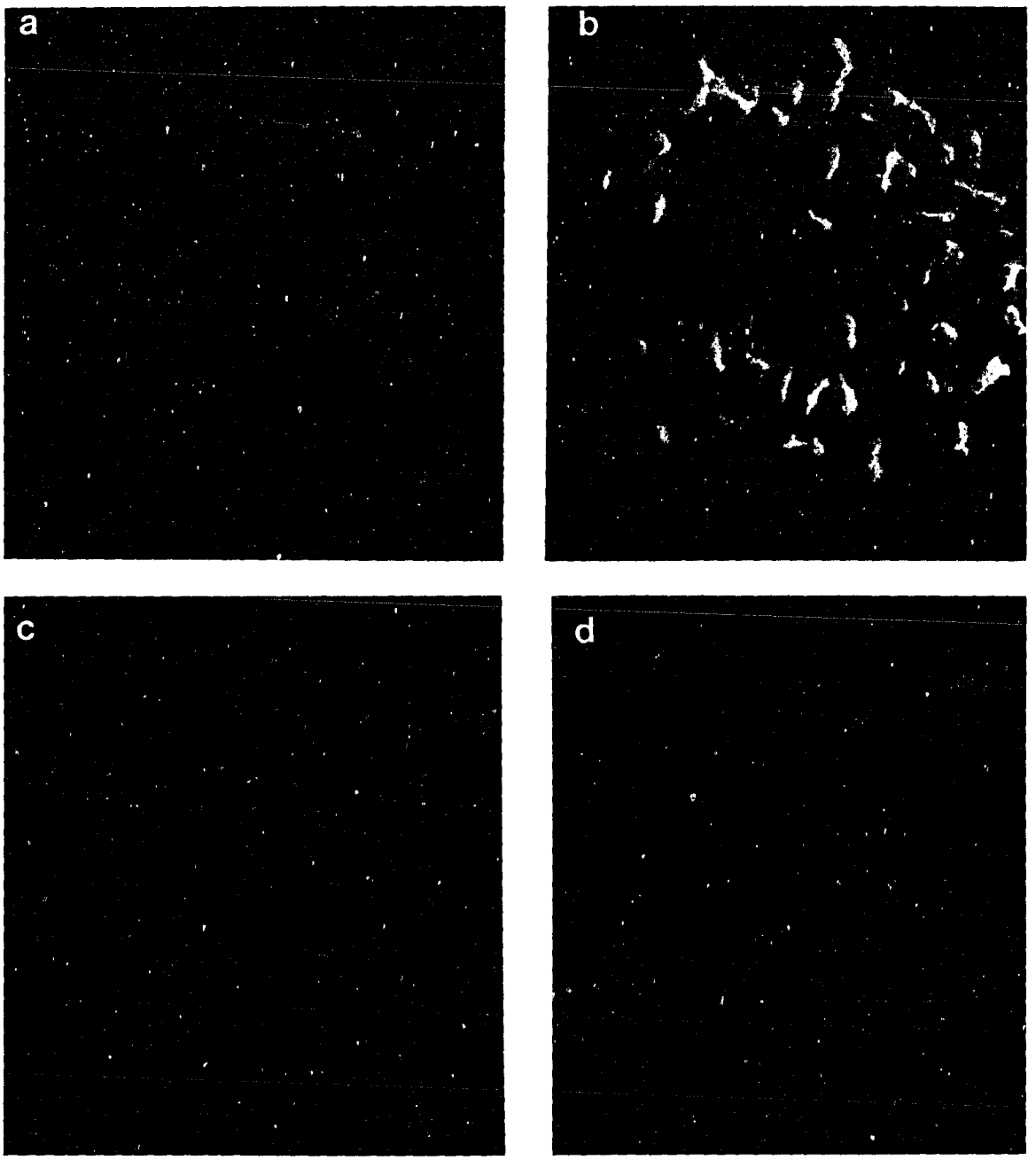

Figure 3-5. Immunofluorescence of DNA. Photographs of human metaphase chromosomes were taken after various pretreatments followed by indirect immunofluorescence using human anti-native DNA serum and goat anti-human IgG. a) No pretreatment. b) Pretreated with Viokase for 3 sec. c) Pretreatment with DNAase I. d) Pretreated with the C-banding procedure. Magnification: 630x.

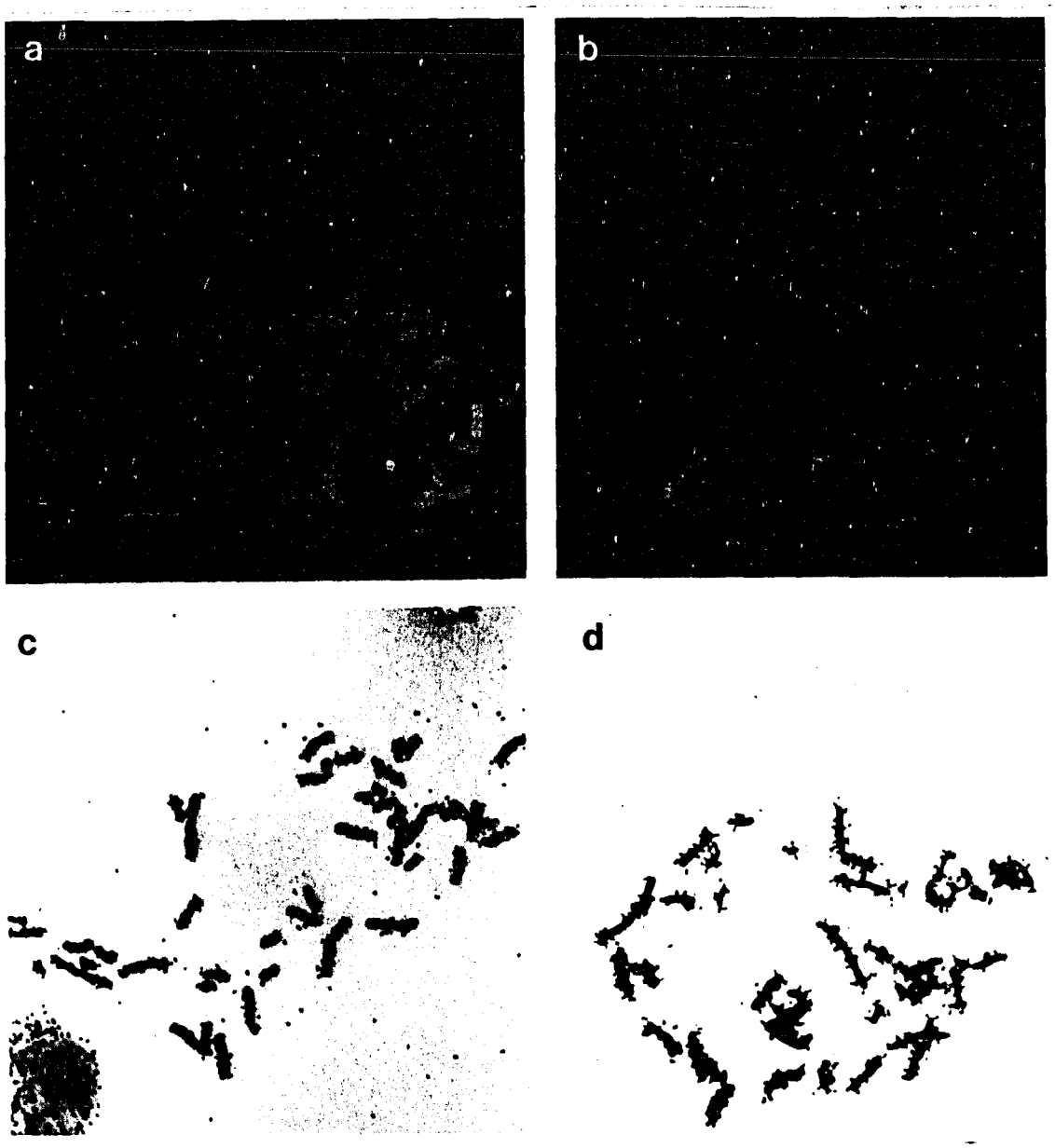

Figure 3-6. Act D Binding After Viokase Treatment. Photographs of 0.1 µg/ml [^{3}H]Act D binding to human metaphase chromosomes were taken after no pretreatment (a and c) compared to a 3-sec Viokase pretreatment (b and d). Act D is detected in a and b by indirect immunofluorescence, and in c and d after 7-day autoradiographic exposure. Magnification: 630x.

treated preparation is bound to the DNA. This result was also observed by autoradiographic analysis of [^{3}H]Act D bound after DNAase I treatment since no autoradiographic grains were observed.

Some unusual results of 0.1 μg/ml Act D binding as detected by indirect immunofluorescence can be seen in Fig. 3-7. The prevalent result (Fig. 3-7a) once again shows the bright fluorescent chromosomes. After C-band treatment (Fig. 3-7b), the fluorescence is generally decreased over the entire chromosome. In a small percentage (about 10%) of the metaphase spreads pretreated with Viokase before Act D binding, a decreased degree of fluorescence was observed, and the chromosome periphery was much brighter than the center of the chromosome itself (Fig. 3-7c). In approximately 5% of all the metaphase spreads observed that were not pretreated in any way, a banding pattern of 0.1 μg/ml Act D binding was seen (Fig. 3-7d) with indirect immunofluorescence. This observation is analyzed further in the Discussion and in Fig. 3-8.

Discussion

The technique of indirect immunofluorescence has been applied for the detection of Act D bound to fixed human metaphase chromosomes. The results confirm the conclusions reached in Chapter 1. At a concentration that Act D maximally inhibits rRNA synthesis (0.1 μg/ml), binding of the drug occurs in a random fashion throughout the genome, with no selective binding at the ribosomal cistrons. Furthermore, the results indicate that the immunofluorescent detection of Act D may be applied to the analysis of eukaryotic chromosome structure.

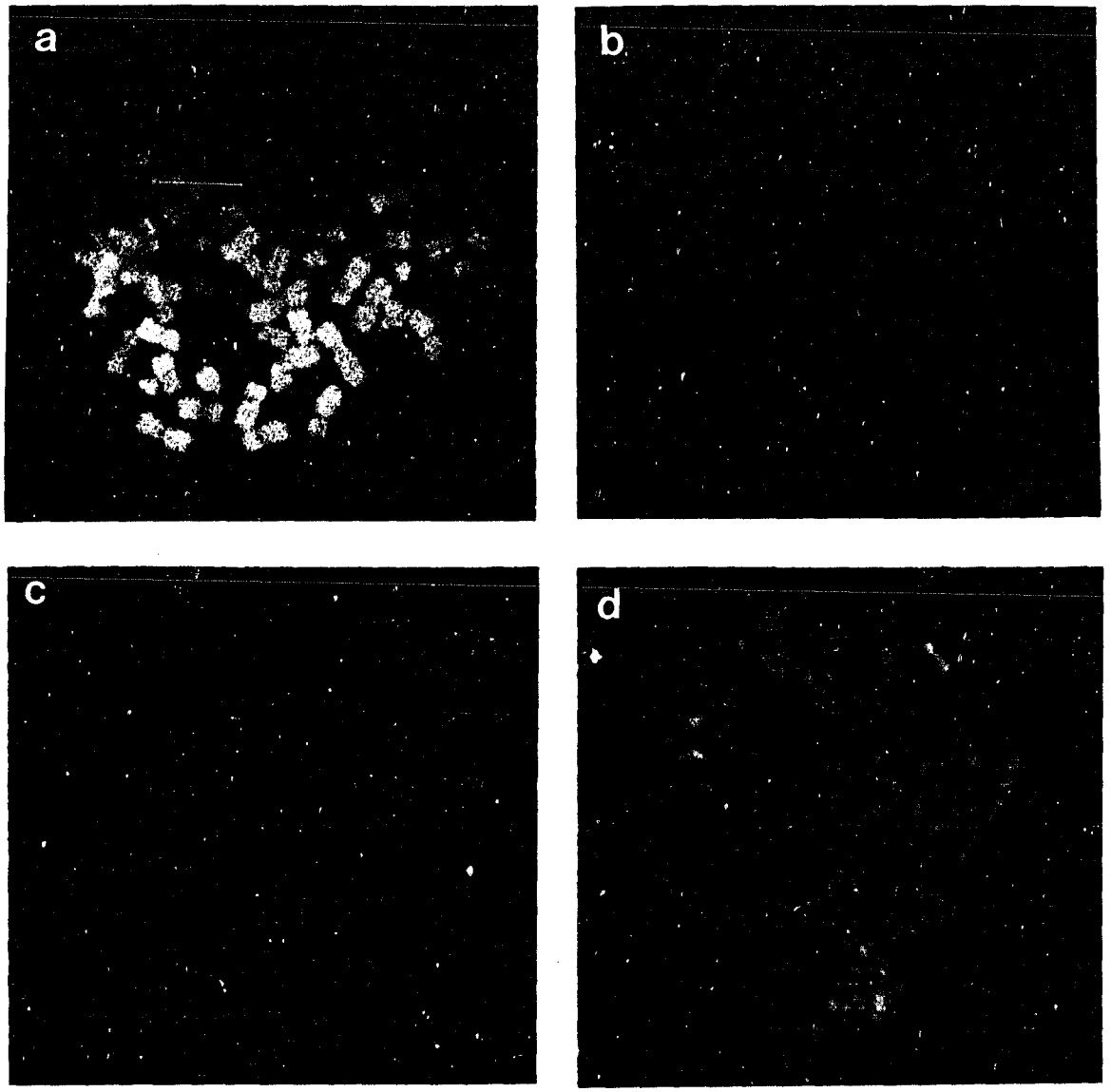

Figure 3-7. Immunofluorescence of Act D Binding Under Various Conditions. Photographs of 0.1 μg/ml Act D binding to human metaphase chromosomes were taken after indirect immunofluorescence. a) No pretreatment. b) Pretreated with the C-banding procedure. c) An unusual observation following 3-sec Viokase pretreatment. d) A banding pattern is observed in a few of the metaphases of the non-pretreated slides (see text). Magnification: 630x.

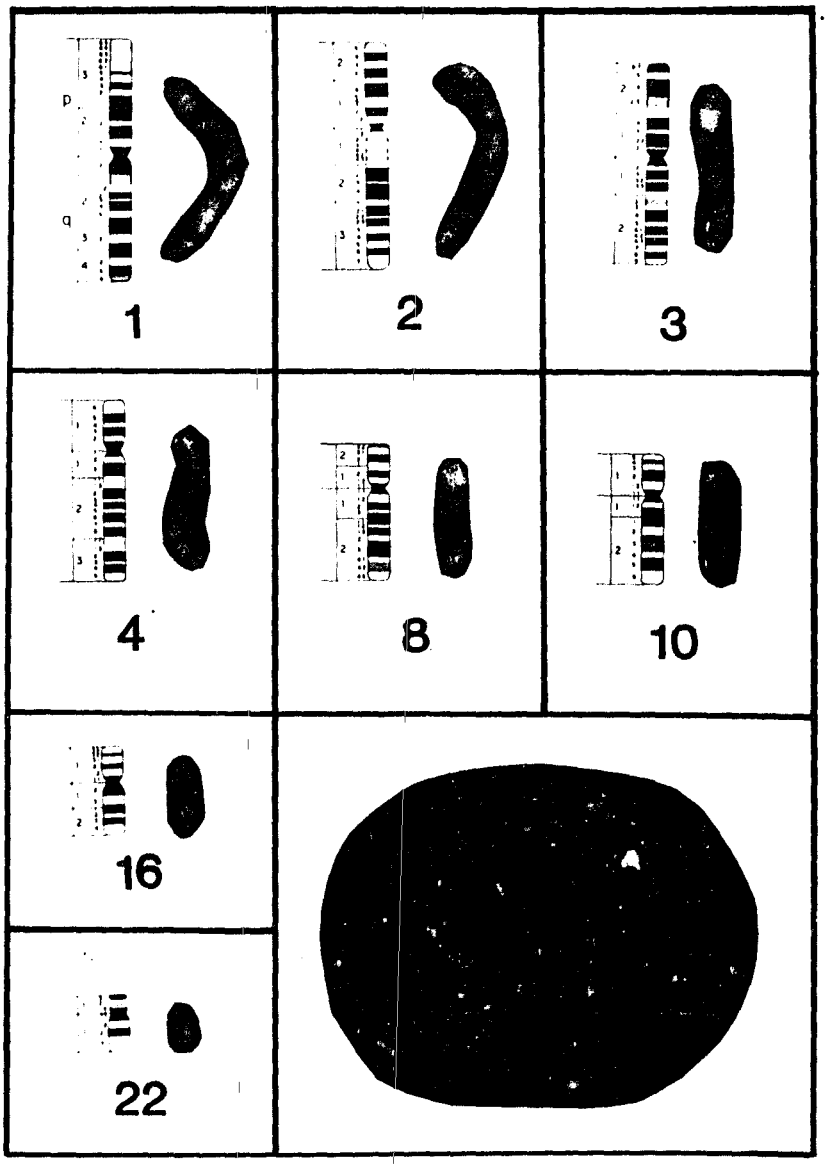

Figure 3-8. Partial Karyotype of an Act D Banded Human Metaphase Spread. Chromosomes (to the right of their respective diagrams) are identified by their R-type banding pattern after labeling with 0.1 µg/ml Act D and indirect immunofluorescence. R-bands are interbands on the diagram. Inset: the complete metaphase. (Diagrams adapted from Yunis and Chandler, 1979.)

Prior to characterization of the indirect immunofluorescent detection of Act D bound to chromatin, it was necessary to determine whether the fluorescence observed was due to antibody binding to free drug or to drug bound to chromatin. It had been previously determined (Lindell and Duffy, in preparation) that in aqueous solution, Act D is not stably bound to deproteinized DNA. Act D dissociates from DNA at room temperature in the absence of free Act D, with a $t_{\frac{1}{2}}$ of 18 min. There was no evidence that Act D would behave the same way in binding to chromatin. With the knowledge that Act D dissociates from DNA relatively rapidly, and the need for a prolonged antibody incubation (in aqueous buffer for 60 min), an autoradiographic dissociation experiment was performed. Fig. 3-3 shows the results of that experiment. The $t_{\frac{1}{2}}$ of Act D bound to chromatin at room temperature was calculated to be 4.5 hr. It was previously demonstrated by Ringertz et al. (1969) that aqueous incubation of unlabeled Act D with human lymphocytes containing bound [^{3}H]Act D for periods of up to 4 hr did not result in any marked loss of [^{3}H]Act D. Since it is evident that nearly all the Act D remains bound to chromatin in the 60 min needed for antibody incubations, it can safely be concluded that Act D observed in that time remains bound to the chromosomes. The presence of chromosomal proteins and the highly compact state of DNA in the metaphase chromosome very likely contribute to this different rate of dissociation of Act D from chromosomes as compared to deproteinized DNA. Also, since the use of antibody in the aqueous solution after chromosomes were labeled with

[^{3}H]Act D does not increase or decrease the rate of dissociation, one can conclude that the antibody does not affect the binding of Act D to chromatin.

The general scheme of both the direct and indirect methods for detection of antibody by fluorescence are diagrammed in Fig. 3-2. In theory, both these systems should work. Each has advantages and disadvantages. Direct immunofluorescence involves only one antibody incubation, taking less time and eliminating all the background problems that may be encountered with a secondary antibody. The main disadvantage is the need for FITC-conjugation to each batch of freshly prepared anti-Act D IgG. Indirect immunofluorescence offers the advantage of possible amplification of the Act D "signal" since more than one secondary antibody may attach to the combining site of the primary antibody (Sternberger, 1979). This is a possible reason that the indirect method proved to be the superior method in detecting Act D (Fig. 3-4). A minor disadvantage of the indirect method is the need for many controls (Table 3-1) to verify that both the primary and secondary antibody reactions are specific.

Besides showing the relative fluorescence of the direct versus the indirect methods of immunofluorescence, Fig. 3-4 shows that Protein A purified IgG is more efficient in detecting Act D bound to chromosomes by the indirect method than is anti-Act D serum (Fig. 3-4a and d). A likely explanation for this is the presence of many other proteins in serum. In addition, some DNAase may be present in serum that could partially degrade the DNA and/or dissociate the Act D. Other serum

proteins could possibly block the recognition of the antibody for Act D when bound to chromatin. Fig. 3-4c shows that there is a significant amount of nonspecific rabbit IgG binding to chromatin and the surrounding area, since the fluorescein-labeled secondary antibody is specific for rabbit IgG. It was initially believed that purification of the specific anti-Act D IgG would eliminate any nonspecific IgG binding to chromatin. This purification, however, was unsuccessful due to an extremely high affinity of the antibody for Act D (Chapter 2, Appendix C). Human IgG does not cross react with anti-rabbit IgG (verified in Table 3-1) and was predicted to block these nonspecific binding sites from the rabbit IgG. As evidenced by Fig. 3-4d, the background was eliminated when human IgG was used in a preincubation step before anti-Act D IgG. All subsequent experiments utilized human IgG to block these nonspecific sites.

Table 3-1 outlines the observations seen in Fig. 3-4, and also presents the conclusive evidence that observed fluorescence (as seen in Fig. 3-4d) represents Act D bound to the chromosomes. Items 5 through 8 are essential controls that are needed to determine a specific antibody reaction in indirect immunofluorescence. Item 5 shows that when the antigen is left out, no fluorescence is observed on chromosomes or nuclei. Since human IgG was added to block any nonspecific IgG binding sites, the absence of Act D (the antigen) eliminates any binding of the anti-Act D antibody. Therefore there is no antigen for the secondary antibody to combine with and no fluorescence is observed. Item 6 (Table 3-1) shows that the IgG purified from nonimmune rabbit serum

does not significantly react with Act D bound to chromatin. It is possible that when Act D is present, a small amount of nonspecific binding of rabbit IgG occurs that was not blocked by human IgG. This is not surprising because there is a minor structural difference between rabbit and human IgG (Weir, 1977). This may account for the extremely weak fluorescence of chromosomes and nuclei observed. Item 7 shows that the secondary antibody response (goat anti-rabbit) is functional, since goat anti-human IgG should not react with the rabbit (anti-Act D) IgG. It should be noted that the human IgG preincubation was omitted in this control since it would cause a positive reaction with goat anti-human IgG. Finally, Item 8 shows that the antigen, Act D itself does not cause the fluorescence observed. Chinsky and Turpin (1977) have shown that Act D complexed to DNA fluoresces. This was not observed, however, when the low concentrations of drug used were complexed to chromatin.

The use of the anti-native DNA antibody proved to be a helpful positive control (Fig. 3-5). Besides its use in determining the presence of DNA, this antibody was useful in establishing the indirect immunofluorescent technique (Fig. 3-5a). The use of an anti-native DNA antibody has been reported to detect human chromosomal DNA by immunofluorescence by Kanayama et al. (1976). Viokase treatment (Fig. 3-5b) resulted in no loss of DNA as evidenced by fluorescence; however, the morphology of the chromosomes becomes distorted. This is probably due to proteolytic removal of some of the chromosomal proteins, resulting in a destabilization of chromatin. This is believed to be one of the factors involved in the generation of G-bands (McKay, 1973).

Incubation with DNAase I, as expected, caused a marked decrease in fluorescence with the anti-native DNA antibody (Fig. 3-5c). This is presumably due to removal of the DNA from the chromosomes. It is interesting to note that basic chromosome morphology was retained after DNAase treatment. It should also be mentioned here that all DNAase I treatments were performed after incubation of the chromosomes with 0.2 N HCl, which is known to remove residual histones (Comings and Avelino, 1974), allowing DNAase I accessibility to nucleosomal DNA. Treatment with HCl alone had no effect on anti-DNA or anti-Act D antibody binding to Act D on chromosomes.

Fluorescence is decreased, as expected, after the C-banding procedure when anti-native DNA antibody is used. Comings et al. (1977) have shown that an average of 65% of the DNA is removed during the C-banding procedure. Pathak and Arrighi (1973) demonstrated C-banding removes euchromatic DNA, as analyzed by [^{3}H]thymidine labeling of DNA and autoradiography, but does not remove constitutive heterochromatin. If constitutive heterochromatin (mostly centromeric) is indeed present after the C-banding described here, it was not detected by the anti-native DNA antibody.

The use of Viokase has a marked effect on the binding of Act D to chromosomes (Fig. 3-6). Since it is used in a reproducible procedure for obtaining G-bands in this laboratory (Appendix A), Viokase was a logical choice for studying a possible mechanism of G-banding while also characterizing Act D binding with indirect immunofluorescence. Most studies that examine the mechanism of G-banding involve the use of

trypsin to induce G-bands (Seabright, 1971). While one experiment was performed showing similar Act D binding results on trypsinized versus Viokase-treated chromosomes, the basic assumption must be made that Viokase treatment affects chromosomes the same way as trypsin treatment. Viokase contains trypsin, and Viokase-generated G-bands are identical to those obtained with trypsin (Muller and Rosenkranz, 1972).

Fig. 3-6 shows the effect of Act D binding to chromosomes after a 3-sec Viokase treatment. The degree of fluorescence is similar to the untreated chromosomes (Fig. 3-6a) but binding is indicative of a change in chromosome morphology after Viokase treatment (Fig. 3-6b). This is also shown by autoradiography where the approximate same number of autoradiographic grains are associated with the Viokase-treated (Fig. 3-6d) compared to the untreated chromosomes (Fig. 3-6c). Fig. 3-6 shows a comparison of autoradiographic detection of Act D and immunofluorescent detection of the drug. The only information generated from the 7-day autoradiographs is quantitative: the amount of Act D binding to Viokase treated versus untreated chromosomes is no different (silver grain number is the same). After only 1 hr. incubation, the immunofluorescent detection of Act D also gives information regarding drug distribution, since all chromosomes are fluorescent. The morphology of the chromosomes is different after Viokase treatment, in that the edges appear more ragged (Fig. 3-6b). This is likely due to changes induced by proteolytic enzymes, and the DNA conformation itself, as indicated by Act D binding, may be altered. Since Viokase does not remove DNA, as evidenced by Fig. 3-5b, the presence of Act D bound to the Viokase-treated chromosomes is good support that Act D is bound to the DNA.

Comings et al. (1973) have shown that trypsin removes 13.2% of the chromosomal proteins, and less than 1% of the chromosomal DNA. Kato and Moriwaki (1972) postulate that the G-banding procedure removes some "condensing protein." Burkholder (1974, 1975) has shown that trypsin causes a dispersion of the chromatin when preparations are examined by electron microscopy. Since the removal of all histones with HCl had no effect on Act D binding, Viokase treatment most likely affects non-histone chromosomal proteins. This is also suggested by Comings (1973, 1977) and is further supported by SDS gel electrophoresis studies (Burkholder and Duczek, 1980). These "scaffolding proteins" as described by Adolph, Cheng, and Laemmli (1977) are believed to maintain the DNA superstructure in a highly folded conformation in metaphase chromosomes.

Actinomycin D binding to chromosomes after DNAase I treatment showed no fluorescence of the chromosomes or nuclei after indirect immunofluorescence. This is again evidence that Act D is bound to the DNA, since, when the DNA is removed, no Act D binding is observed.

Fig. 3-7 compares some other observations to the prevalent result of 0.1 µg/ml Act D binding as detected by indirect immunofluorescence (Fig. 3-7a). After C-banding treatment, the fluorescence is decreased over the entire chromosome (Fig. 3-7b). Since constitutive heterochromatin is not removed during C-banding (Kato and Moriwaki, 1972), one can interpret the observation in Fig. 3-7b in two ways since one would expect to see constitutive heterochromatin fluorescence due to the presence of Act D: 1) Under any conditions Act D does not bind

as well to constitutive heterochromatin, or 2) Act D does bind to constitutive heterochromatin but C-banding treatment somehow decreases accessibility of this Act D binding to constitutive heterochromatin. The first interpretation is unlikely because untreated constitutive heterochromatin regions (centromeric) show Act D binding (Fig. 3-7a). The second interpretation is most likely, since this apparent Act D binding to constitutive heterochromatin is eliminated after C-banding treatment. Also, when the anti-native DNA antibody is applied to C-banded chromosomes, constitutive heterochromatic regions do not fluoresce by indirect immunofluorescence. If the heterochromatin is present, it may be altered in a way by the C-banding procedure that prohibits even the anti-DNA antibody from binding.

Approximately 90% of the metaphase spreads treated with Viokase showed a general distortion of Act D binding, very much like Fig. 3-6b. Fig. 3-7c shows an unusual exception to this Viokase pretreatment followed by Act D binding to chromosomes. It is possible, under certain conditions not yet understood, that the removal of the nonhistone proteins by Viokase could occasionally alter chromatin to the point that Act D no longer can bind (Fig. 3-7c). An observation with a similar "peripheral fluorescence" was reported by Matsukuma and Utakoji (1976) in that the protein specific fluorescent dye, dansyl chloride, produced a fluorescent contour "brighter than the chromatids" on trypsin-treated chromosomes. They suggest that the chromatin could simply be more condensed around the edges. Burkholder (1975), using electron microscopy, observed chromosomes after trypsin treatment. Besides the observation

that the chromatin became much less compact, he observed a "halo" of electron dense material and postulated that the peripheral chromatin was more resistant to trypsin treatment since the edges of the chromosomes are more firmly attached to the glass. This is a likely explanation in the Viokase-pretreated, Act D labeled chromosomes as shown in Fig. 3-7c. The dispersed chromatin could have aggregated to the periphery, where the chromosome edges were most tightly affixed to the slide.

Fig. 3-7d shows another unusual observation. In approximately 5% of the untreated chromosomes labeled with 0.1 µg/ml Act D, a banding pattern was observed with indirect immunofluorescence of the drug. Unfortunately, because the number of metaphases observed with this pattern were so low, a detailed inter-metaphase analysis of the bands was not possible. Of the metaphases containing these bands, it was determined that the bands are definitely not G-bands. A partial karyotype is shown in Fig. 3-8, with chromosomes along the side of their respective G-band schematic. The G-negative bands are the inter-bands in the schematic, and also represent R-positive bands, or reverse G-bands as described by Dutrillaux and Lejeune (1971). It is this investigator's conclusion that the "Act D banding" observed here is a type of R-banding.

Miller et al. (1973) and Screck et al. (1973) used anti-nucleoside antibodies to demonstrate that the R-bands of human chromosomes are GC rich. This was confirmed by Comings (1978). Since Act D is proposed to intercalate at guanine and cytosine base pairs (Muller

and Crothers, 1968), one would expect to observe Act D in greater amounts in GC rich DNA. Schweizer (1976) used Act D as a counter-stain for the AT specific dye 4-6-diamidino-2-phenylindole (DAPI). When the nonfluorescent Act D was used as a counter-stain for the fluorescent DAPI, Schweizer observed fluorescent G-bands with DAPI binding to AT rich DNA. Schweizer suggests that Act D binds to the GC rich DNA, or the R bands. Even though the number of banded metaphases with Act D and the indirect immunofluorescent procedure is low (ten banded metaphases were observed), it is probably more than coincidental that these bands are in the GC rich areas of DNA. Since the metaphases observed all have patterns like the spread in Fig. 3-8, one can conclude that Act D is preferentially bound to GC rich DNA in these metaphases. It is possible that the brightness of fluorescence in the "non-banded" preparations, which are by far in the majority of metaphases seen, hides the R-banding pattern. For this reason, lower concentrations of Act D were used (down to 0.01 µg/ml), but as mentioned earlier, only a decrease in overall fluorescence was seen; no bands were observed with these lower concentrations. With alterations in antibody dilutions and incubation times, it is possible that this banding pattern can be made more reproducible.

Some conclusions can now be made regarding localization of Act D bound to chromosomes at 0.1 µg/ml. In the majority of the cases observed (e.g. Figs. 3-4d, 3-6a, and 3-7a), all chromosomes fluoresced with equal brightness; there were no distinctive bright spots indicating Act D localization. The remaining metaphases analyzed had the banding

pattern previously described (Figs. 3-7d and 3-8). It appears that the bands are in the GC rich DNA, and dispersed randomly throughout the human genome. Under no circumstances did the ribosomal cistrons fluoresce more brightly than other regions of the genome. This information supports the extranucleolar regulatory model for ribosomal transcription described in Chapter 1. These conclusions also confirm those in Chapter 1; at the concentrations of Act D that specifically inhibit rRNA synthesis, the drug does not selectively bind to rDNA.

Although the immunofluorescent detection of Act D proved to be an efficient and simple method of localizing drug bound to chromosomes, there are some disadvantages to the technique. Besides the need for a fluorescent microscope for analysis and photography, the preparations are not permanent. Fluorescein fades over time, and after a one-min photographic exposure, most of the fluorescence has faded. The simplest way to overcome the problem of fading would be to use a rhodamine tagged secondary antibody, since the fluorescence is brighter and fading would occur at a lower rate (Sternberger, 1979). Another option would be the use of a tertiary antibody and the peroxidase-anti-peroxidase procedure first described by Nakane and Pierce (1967). The immunoperoxidase technique was used by Lubit et al. (1975) and Schreck, Erlanger, and Miller (1974) in localization of 5-methylcytosine on human chromosomes. The immunoperoxidase localization of anti-nucleoside antibodies is comparable to immunofluorescent detection as described by Morin, Marcollet, and Leng (1979). However, immunoperoxidase techniques require more controls, since a tertiary antibody is involved, but localization by

diaminobenzidine-4HCl eliminates the need for fluorescence, and its fading after illumination. If one were to undertake the immunoperoxidase localization of Act D bound to chromosomes, it would be important to use an anti-native DNA antibody as a positive control as described by Kanayama et al. (1976).

Compared to autoradiography, immunofluorescent detection of Act D bound to chromosomes was superior. Localization of Act D binding with the antibody was determined in 60 min instead of the 7- or 30-day autoradiographic exposure. Both autoradiography and immunofluorescence generated the same conclusion: at low concentrations, Act D binds in a random fashion to fixed chromosomes. It should be emphasized that these studies examined Act binding only to fixed chromatin.

Previous observations (Chapter 1, Table 1) indicated that acetic-methanol fixation of chromatin increased the amount of Act D binding. One must therefore consider the possibility that binding of Act D to fixed chromosomes, whether detected by autoradiography or immunofluorescence, could be different from what would be observed in whole cells. It has been shown that histones are removed with acetic-methanol fixation (Bustin et al. 1976). These authors also show that 1% glutaraldehyde fixation yields suitable but fragile chromosomes for analysis without removal of histones. Future analysis of Act D-chromosome binding should include this glutaraldehyde fixation of chromosomes, and also Act D binding to non-fixed chromatin should be examined.

While actual levels of Act D binding can be quantitated by autoradiography (Chapter 1), quantitation of drug bound becomes more difficult with an immunofluorescent system. Differential fluorescence

patterns in quinacrine-stained chromosomes have been reported by Capsersson, Lomakka, and Zech (1976). This system involves photoelectric recording of photographs of the chromosomes. This could potentially be used in the analysis of the Act D "R-bands" described earlier. If the Act D bands could be further characterized, this technique may be a good means of following GC-rich DNA through various treatments.

Chapter Summary

The binding of Act D at 0.1 µg/ml to human metaphase chromosomes was observed by detection with a specific anti-Act D antibody and indirect immunofluorescence. The Act D was shown to be bound to chromosomal DNA and distributed throughout the human genome. These observations concur with the observations of low concentration Act D binding to chromosomes after detection by autoradiography. Both immunofluorescent and autoradiographic detection of drug showed no selective binding to the ribosomal DNA cistrons. This observation supports a model of extranucleolar control of rRNA synthesis. The Act D is believed to be selective for GC rich DNA as evidenced by the appearance of an Act D-R-banding effect occasionally observed. This is the first procedure utilizing immunofluorescence in the detection of a drug bound to chromosomes, and it is suggested that this system will be useful as a tool for the study of chromosomal structure and further understanding the mechanism of chromosome banding. With the use of a tertiary antibody and immunocytochemical techniques, the sensitivity of drug detection can potentially be increased, enabling the localization of 0.01 µg/ml Act D and less bound to chromatin.

APPENDIX A

CHROMOSOME PREPARATION

Biologicals and Reagents:

1. Growth medium (assemble under sterile conditions)

   a. RPMI-1640 (Grand Island Biological Co.) 100 ml

   b. Fetal bovine serum (Gibco) 25 ml

   c. Penicillin-streptomycin (Gibco), 500 units of each/ml in frozen aliquots 2 ml

   d. Phytohaemagglutinin (Burroughs-Wellcome), lyophilized. Resuspend in 5 ml medium or distilled water; may be stored frozen after hydrating. 1.65 ml

   Under sterile conditions add other ingredients to bottle of RPMI. Store in refrigerator or aliquot 5 ml amounts into sterile disposable centrifuge tubes (Falcon Plastics) and freeze. Warm to 37^{o} before using.

2. Colcemid solution (Gibco)

   Make up according to manufacturer's instructions.

3. Hypotonic solution 0.075 M KCl

   Store at room temperature; warm to 37^{o} before using.

4. Fixative

   Freshly prepared 3 parts absolute methanol to 1 part glacial acetic acid. Keep chilled in freezer.

5. Geimsa stain for routine staining

   Use commercial stock solution (Biomedical Specialties) and dilute 1:50 with distilled water before each use.

6. Hanks' balanced salt solution (Gibco) without calcium and magnesium.

7. Viokase (Pancreatin) (Gibco)

Protocol 1: Peripheral Blood for Chromosome Analysis (modified from Moorhead et al. 1960).

Methods for Blood Collection:

1. Venipuncture. Aseptically collect 3 ml blood into sodium heparin Vacutainer tube. Invert several times to prevent coagulation. Keep tube at room temperature until ready to set up blood culture (maximum of two days). 0.5 ml of blood will be added to a growth medium tube.

2. Skin lancet puncture. Cleanse skin at chosen site with alcohol wipe, let site dry, and puncture with lancet. Have ready a tube of medium and a heparin-rinsed sterile Pasteur pipet with bulb. Pull enough blood into pipet to approximately 8 drops and put into tube of medium. Invert tube to prevent clotting.

Cultivation:

1. Mix blood sample and add 7-8 drops into a plastic centrifuge tube with medium. Invert tube 2-3 times to mix, and loosen cap if CO_2 incubator is used (or tightly capped, if not CO_2). Incubate at 37°C with tube slightly inclined for approximately 72 hrs.
2. 1½-2 hrs before harvesting, add .05 ml of colcemid (10 µg/ml) to the culture tube, invert to mix, and return tube to incubator.
3. At end of incubation time, resuspend cells, cap, and centrifugate tube 5 min at approximately 1000 x g.
4. Pour off supernatant, resuspend pellet with a lab mixer, and add 5 ml of 0.075 M KCl solution (prewarmed to 37°C). Mix again if necessary and incubate tube at 37°C for 10 min. Time may be adjusted for future cultures according to how satisfactorily metaphases are spreading.
5. Centrifugate tube for 5 min after hypotonic incubation, pour off all supernatant, and resuspend cells with mixer, making sure there is no pellet left in bottom of the tube.
6. Slowly add 2-3 ml of fresh cold fixative (0°) to the tube. Mix as the fixative is added, and with Vortex mixer afterward.
7. Chill tube in freezer at least 15 min.
8. Centrifugate, decant, and add 2-3 ml of fresh fixative. Shake tube to mix. (At this point, tube can be capped and stored in freezer until it is convenient to continue.)

9. Centrifugate and replace supernatant fixative. Shake tube to mix. Repeat this step two more times. After the third wash, 1-3 ml fixative is added to suspend cells for slide making.

Slide Making:

1. Place new, clean-glass slides (preferably frosted on one end for labeling) in a Coplin jar with distilled water.
2. Bubble air into cell suspension in fresh fixative (0^{o}) with a clean, Pasteur pipet. Do not draw suspension into pipet as chromosomes will be lost along the inside glass.
3. Draw up 5-10 drops of the suspension into the pipet, and drop from about 2 inches onto a slide that was soaking in distilled water.
4. Wipe dry the back of the slide and gently blow on the slide for about 30 sec, then drain remaining solution onto absorbent paper.
5. Examine the slide at 160x magnification using phase contrast microscopy.
6. Concentrations of chromosome spreads can be controlled by varying the amount of fixative added to the final pellet.
7. If no banding is necessary, immerse slide vertically into Geimsa stain for routine staining for 4 min.*
8. Rinse slide with tap water and dry; slide is now ready for subsequent treatment.*

---

*Omit these steps for antibody labeling.

Protocol 2: Geimsa-Trypsin Stain Modification for Banding (Muller and Rosenkranz, 1972).

Reagents:

1. Viokase solution 2.5% (10X), Gibco

   A natural mixture of pancreatic enzymes. Available in 25 ml lyophilized or 100 ml liquid units. May be aliquoted into 2 ml portions in sterile disposable tubes and frozen until needed.

2. Gurr's buffer tablets, Searle Diagnostic

   A convenient and reliable source of buffer. Dilute 1 tablet in 100 ml tap water, giving final pH of 6.8. Prepare fresh. Any buffer system can be substituted.

3. Geimsa stock solution, Gurr's

4. Hanks' BSS, Gibco

   Balanced salt solution. Store at room temperature.

Procedure:

It is preferable to use freshly prepared slides within one week of harvest for all enzyme-banding procedures. However, slides may be stored in a cool, dry area for longer periods of time and still obtain a degree of successful staining.

1. Dilute stock Viokase solution 1:10 with Hanks' BSS (2 ml Viokase plus 18 ml Hanks' in plastic slide mailer). Stabilize temperature to 35-37^{o}C in water bath.
2. Immerse slide vertically into mixture for 3 sec. Times may vary and test slides should be examined.
3. Rinse slide with tap water.

4. Stain slide in freshly prepared Geimsa working solution (1 ml Geimsa stock solution in 50 ml Gurr's buffer 6.8) for 3 min.*
5. Slide is rinsed in tap water and dried; slide is now ready for subsequent treatment.*

Protocol 3: "C" Constitutive Heterochromatin Stain (Cytogenetics Lab, AHSC).

Reagents:

1. 0.2 N HCl (37% HCl = 12 N HCl. Use 1.67 ml 12 N HCl and H_2O to make 100 ml of 0.2 N HCl).
2. Barium Hydroxide
   a. Add 2-3 grams $Ba(OH)_2$ to Coplin jar - add distilled H_2O to full level.
   b. Mix until saturated. Let stand.
   c. Pour off supernatant into clean Coplin jar for use above.
3. 2X SSC

   Sodium chloride 1.753 g (0.3 M) in 100 ml distilled H_2O.

   Trisodium citrate 0.882 g (0.03 M) in 100 ml distilled H_2O.

   Mix the two together (1:1) for final working solution.

Procedure:

1. Prescreen slide for good metaphase spreads.
2. Pretreat slide in 0.2 N HCl for 30 min.
3. Rinse in running tap H_2O.
4. Place slide in saturated solution of $Ba(OH)_2$ for approximately 20 min. (Various factors can influence incubation time. Three slides can be used at incubation times of 15, 20, and 25 min.)

---

*Omit these steps for antibody labeling.

5. Rinse thoroughly in running tap H_2O.
6. Incubate in preheated 2X SSC for 2 hrs in 60°C H_2O bath.
7. Rinse in tap water.
8. Stain in 4% Geimsa for 10 min. (Check stain quality at end of 10 min and stain longer if pale or rinse if too dark.)*
9. Rinse in tap water and dry; slide is now ready for subsequent treatment.*

*Omit these steps for antibody labeling.

APPENDIX B

AUTORADIOGRAPHIC TECHNIQUES

Reagents:

1. [^{3}H]Act D 14 Ci/mM (Amersham)
2. NTB-3 Emulsion (Eastman Kodak)
3. Formvar in 1.0% ethylene dichloride (E. F. Fullam, Inc.)
4. Subbing Solution: Freshly prepare 0.01% chromium potassium sulfate and 1.0% gelatin in boiling distilled water. Filter and cool to room temperature before use.
5. TWEEN 80 (Polyoxyethylenesorbitan) (Sigma)
6. Drierite (Hammond Drierite Co.)

Protocol 1: Preparation of G-banded chromosomes for autoradiography

1. Use slides with freshly G-banded chromosomes (see Appendix A). Examine with 40 X objective to verify that banding quality is satisfactory.
2. If examined with oil, dip slide in xylene to clean.
3. Label slide with specified concentration of [^{3}H]Act D, wash 3 x in 50 ml 95% ethanol.
4. Dry with forced air - slide must be clean.
5. In 1% Formvar solution dissolved in dichloroethane, dip slide and drain excess solution on absorbent paper (Rutledge, 1979).
6. Place slide (face up) in saturated atmosphere of 1,2 dichloroethane for 5 min to allow uniform film formation on slide.

7. Dip slide in freshly prepared and filtered subbing solution.
8. Drain excess solution on absorbent paper and allow slide to dry in dust-free place.
9. Slide is now prepared for autoradiography. The Formvar protects cells from interaction with the nuclear track emulsion.

Protocol 2: Autoradiography

NOTE: Preparation of slides and development should be done in total darkness. Care should be taken to avoid any light leaks. (Even static electricity will greatly increase the number of autoradiographic background grains.)

Coating of Emulsion:

1. Warm a fresh batch of Kodak NTB-3 emulsion to 42^{o}.
2. Warm 60 ml of 1% Tween 80 detergent to 42^{o}.
3. In the dark, open the warmed emulsion (inside three lightproof boxes) and with a Kodak safelight #2, pour off half the bottle (60 ml) into another bottle to be "light-proofed" in the same manner and stored at 4^{o} until ready for dilution.
4. Add the 60 ml of 1% Tween 80 and mix. Then pour off 30 ml of the diluted emulsion into a small jar with a volume sufficient to stain one microscope slide at a time.
5. Carefully dip each slide for 10 sec and hold upright on absorbent paper in the darkroom to drain excess.
6. Allow slide to air dry (in the dark) for 30 min.
7. Place slides in black slide box containing Drierite (Hammond Drierite Co.).

8. Carefully tape seam of box with black electrician's tape.
9. Wrap entire box in aluminum foil.
10. Place box in foil in a large black plastic bag and tape bag shut.
11. Slides are now to be stored at -70° for entire exposure time.

Development:

Plan to feel locations of solutions in sink since development is in the dark. Use Kodak D-19 developer and fixer prepared to manufacturer's specifications. Cool to 17° (400 ml/ea).

1. In darkroom, carefully open slide box. Slides should be warmed to room temperature before development. Place slides in a metal slide rack so all slides can be developed at once.
2. Pour cooled D-19 into glass staining dish and place slide rack in this for 3 min.
3. During these 3 min, prepare another staining dish with distilled water (17°) and pour fixative (17°) into a third staining dish.
4. Dip slide rack in distilled water for 10 sec.
5. Dip slide rack in fixative for 3 min.
6. Let slides sit in large 17° water bath for 15 min (lights may be turned on at this point).
7. Let slides dry.

NOTE: Formvar-coated G-banded chromosome slides are ready for examination.

APPENDIX C

TECHNIQUES OF ANTIBODY PURIFICATION

A. Purification of the IgG Fraction From Serum

Protein A is a single polypeptide chain with molecular weight of 42,000 (Sjoquist, Meloun, and Hjelm, 1972) isolated from Staphylococcus aureus (Forsgren and Sjoquist, 1966). Protein A, when immobilized on a matrix is ideal for isolating IgG from various species, as it specifically binds to the Fc portion of the IgG molecule (Kronvall and Frommel, 1970). IgG can be eluted from the protein A bound matrix under acidic conditions (Chenais et al. 1977). A good review of the use of Staphylococcal protein A as an immunological reagent is presented by Goding (1978).

Protein A immobilized on Sepharose, described below, proved to be an excellent and reproducible system for separation of rabbit IgG containing active anti-Act D antibody. If cared for and stored as suggested, the column will last indefinitely.

The affinity column is packed with protein A Cl-4B-Sepharose (Pharmacia) according to the manufacturer's specifications, and washed in 0.02 M Tris-HCl pH 7.3 with 0.15 M NaCl (the "running buffer"). A column 1.5 cm x 6 cm is both economical and convenient.

1. After passing 4 ml of running buffer through the column, and calibrating fraction sizes, 5 ml of immune rabbit serum is

passed over the column with a flow rate of approximately 15 drops per min, collecting 1 ml fractions.

2. Protein is monitored by absorbance at 280 nm, and the running buffer is continuously passed through until protein levels are at background (absorbance of about 0.010).
3. Elution of IgG is accomplished by passing 0.1 M acetic acid over the column, again reading absorbance at 280 nm for each fraction. (The absorbance of 1.0 mg IgG/ml = 1.38 OD at 280 nm.) If the column was not overloaded (the IgG concentration of the serum used was not too great, or no more than 5 ml of serum was applied), the IgG peak should be sharp, with almost all the IgG eluted in 2 or 3 fractions (see Fig. 2-8).
4. 3 ml of 1.0 M acetic acid is now passed over the column to remove any nonspecifically bound proteins.
5. Running buffer containing 0.06% sodium azide is used to wash the column of any acid, and the column is then stored in running buffer containing sodium azide at 4^{o}.
6. The IgG fractions are dialyzed overnight against 0.01 M Tris pH 7.3 and 0.9% NaCl.
7. The IgG can now be frozen (-20^{o}) until use in the charcoal Act D assay (Chapter 2) or as the primary antibody in indirect immunofluorescence.

B. Purification of Specific Antibody

It should be noted that this section contains basic references to methods of antibody purification by specific affinity chromatography.

Specific anti-Act D antibody was never successfully isolated, as explained in Chapter 2, presumably due to an extremely high Act D-antibody affinity. The techniques, however, may be helpful in purification of a different antibody, or modifications or alterations thereof, may eventually prove successful in obtaining purified anti-Act D antibody. It should also be noted, however, that pure anti-Act D antibody is not necessary for immunofluorescent detection of Act D bound to chromosomes, as described in Chapter 3.

The method of specific antibody purification by affinity chromatography is similar to that of IgG purification described in Section A. The antigen (Act D) is immobilized to a matrix so that it can bind antibody, and then this antigen-antibody interaction must be gently disrupted so as to elute active antibody off the column. A good outline of this technique is given by Pardue et al. (1981).

Act D (with tracer [^{3}H]Act D) was coupled to CH-Sepharose 4B (Pharmacia) with 1 ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (BioRad) as a catalyst. (This is the same reaction as outlined in Fig. 2-2, except CH-Sepharose 4B would be substituted for BSA.) The Act D-Sepharose was washed according to manufacturer's specifications, and a 1 ml affinity column (in a 1 ml plastic syringe) was packed with running buffer as described in Section A. (Tritium tracer was used for confirmation that covalent coupling of Act D to Sepharose occurred.)

When immune serum or IgG was passed over the Act D-CH-Sepharose-4B column, all antibody activity was removed, as no antibody activity was detectable in the flow-through material when used in the charcoal

assay described in Chapter 2. Elution of antibody in the backward direction (Pardue et al. 1981) was attempted with 0.1 M - 1.0 M acetic acid and up to 4.0 M $MgCl_2$ in borate saline buffer (Pardue et al. 1981). No success was achieved in removing antibody as none was detected with absorbance at 280 nm, or with the Act D charcoal assay described in Chapter run in 10 mg/ml BSA (to decrease nonspecific protein absorption). After several runs, it was concluded that specific antibody was extracted by the affinity column from serum or IgG, and this was due to the presence of bound Act D, since noncoupled CH-Sepharose-4B removed no detectable antibody. This antibody, however, was not able to be eluted from the column using the elution conditions described.

Several authors (Kristiansen, 1977; Levison, Kieszenbaum, and Dandliker, 1970; and Dandliker et al. 1967) describe the use of chaotropic ions in disrupting the antigen-antibody complex. The techniques described by Dandliker et al. (1967) were attempted, and a small amount of protein eluted off the Act D affinity column when NaSCN was used. This protein was presumably specific antibody, but was not active.

To further characterize the protein eluted with NaSCN, 1.7 mg of anti-Act D IgG was labeled with ^{125}I by the method of Greenwood, Hunter, and Glover (1963) and this material was passed over the Act D affinity column. With the specific ^{125}I label on IgG, one can now detect relatively small amounts of IgG that may be eluted off. The results of this elution after application of 525 µg ^{125}I IgG are shown

in Fig. C-1. Two separate peaks are seen after elution with a 3.5 M NaSCN. These peaks showed no antibody activity when tested in the charcoal assay described in Chapter 2. When 4 M HCl was applied to the column, one large protein peak came off, also showing no antibody activity. Each peak after point A (Fig. C-1) was individually dialyzed 24 hr against running buffer, and concentrated to 1 mg/ml by surrounding the dialysis bag with dry Sephadex G-75 (Pharmacia). Three changes of dry Sephadex were made in 2 hrs. Each peak was then tested for antibody activity in the charcoal assay. After repeated observations, it was concluded that the specific IgG can be eluted in 3.5 M NaSCN, but becomes inactivated upon elution under these conditions. It was also concluded that yet a tighter antibody-antigen bond exists, and this cannot be disrupted until 4 M HCl is added. The observation of two protein peaks with an addition of 3.5 M NaSCN is presumably due to two species of antibody that bind to the column with different affinities. From Fig. C-1, it can be calculated that 5.2% of all IgG in this immune sample was specific anti-Act D IgG.

When specific antibody cannot be purified with any of the methods mentioned above, an alternative method as described by Murphy et al. (1976) involves a chemical modification of the antigen. As described in Chapter 2, preparation of actinomycin D acid (Brockmann and Manegold, 1967), which has an open peptide ring structure, also did not permit purification of antiAct D antibody. This was one of the most obvious chemical modifications of Act D to attempt decreased antibody affinity since the antigenic portion of the molecule resides in

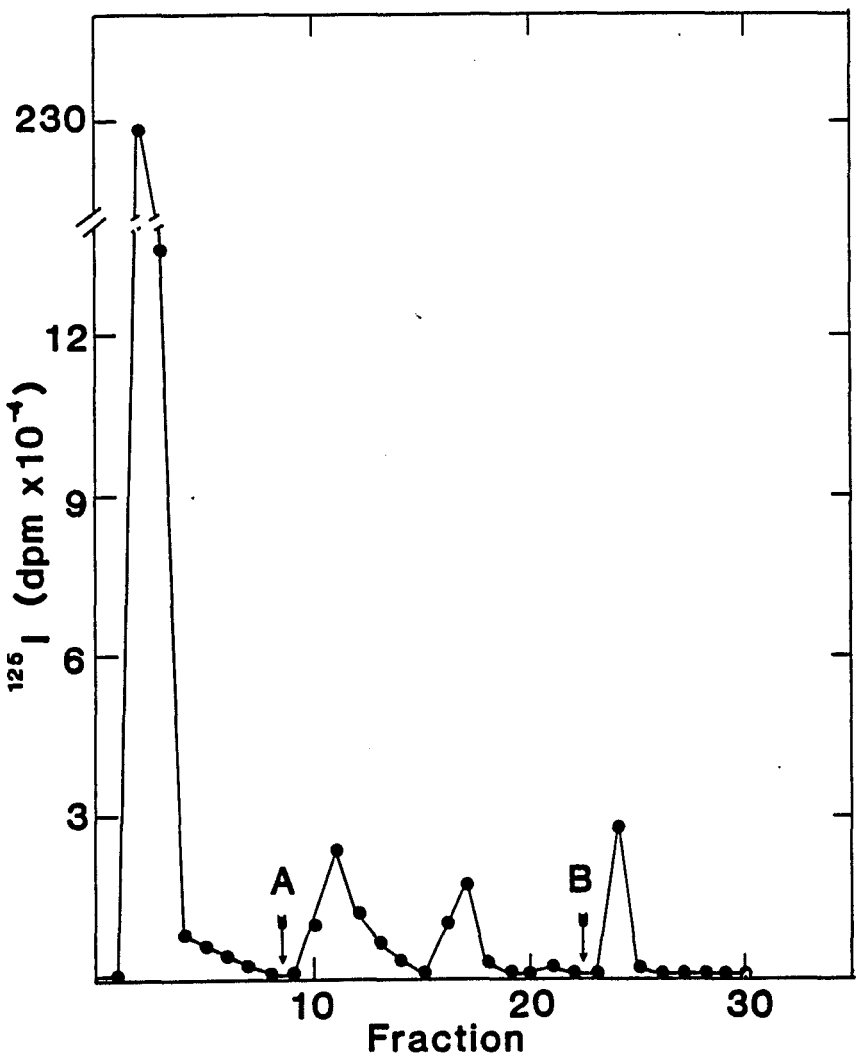

Figure C-1. Affinity Purification of ^{125}I anti-Act D IgG. 525 µg of ^{125}I immune IgG was passed over the Act D-CH-Sepharose-4B affinity column described in the text. Point A represents addition of 3.5 M NaSCN; point B represents addition of 4 M HCl. Each point represents a fraction of 0.5 ml. The three peaks after point A signify specific anti-Act D IgG, which, when added together equal 5.2% of the total IgG applied.

the pentapeptide lactone (Chapter 2). This reaction was also chosen because the use of 4 N sodium methoxide produces 92% actinomycin D acid (Brockmann and Manegold, 1967). Actinomycin D acid, when substituted for Act D in a modified radioimmunoassay (as described in Chapter 2) appeared to have a lesser affinity for the antibody than did Act D (Fig. C-2). Actinomycin D acid was then coupled to Affi-Gel 10 (Biorad) (CH-Sepharose-4B and the carbodiimide coupling reaction would not work with actinomycin D acid since there now are free carboxyl groups present on the molecule with the open peptide ring and actinomycin-actinomycin coupling would occur). Act D was also coupled to Affi-Gel 10 following the manufacturer's specifications. Tritium tracer confirmed that Act D and actinomycin D acid coupled to the Affi-Gel 10. It was concluded that Act D coupled to Affi-Gel 10 could remove active antibody from the IgG fraction, but (as previously seen with Act D-CH-Sepharose-4B) antibody could not be successfully eluted from the affinity column. No detectable antibody was removed from the IgG fraction when passed over the actinomycin D acid-Affi-Gel 10 column. Either the circumstances allowing antibody binding were not adequate in the actinomycin acid-Affi-Gel 10 material, or antibody does not bind actinomycin acid with sufficient affinity to separate it from the IgG fraction.

Fig. C-2 shows that actinomycin D acid has approximately a ten-fold lower affinity for the anti-Act D antibody as does Act D. Since Brockmann and Manegold (1967) claim that the actinomycin D acid is present due to conversion of 92% of the Act D, one must consider as an

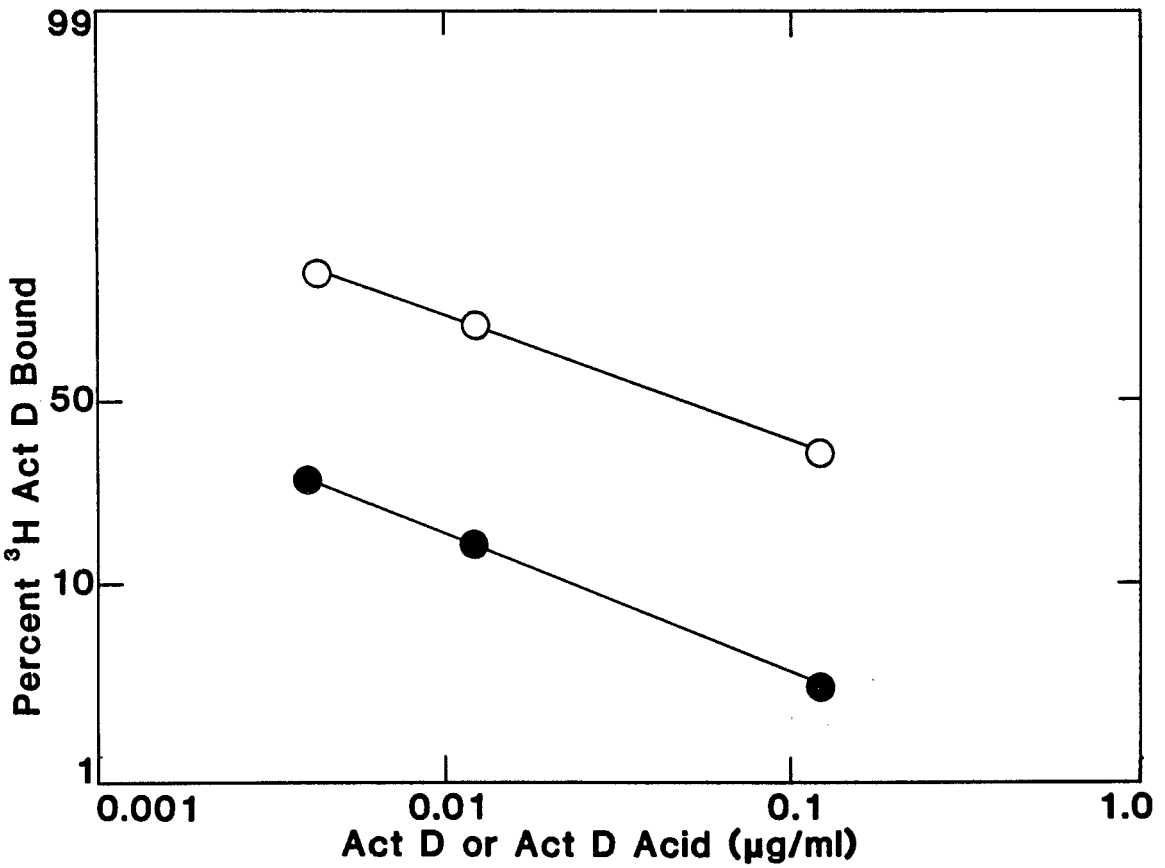

Figure C-2. Radioimmunoassays for Actinomycin D and Actinomycin D Acid. A modified competitive radioimmunoassay was performed with Act D (open circles) and actinomycin D acid (closed circles). The curve is plotted on log-logit axes.

interpretation for Fig. C-2 that actinomycin D acid has no affinity for the antibody, and the 10% affinity observed is due to unreacted Act D.

At the same time that these experiments were being performed to purify anti-Act D antibody, the indirect immunofluorescent technique (Appendix D) was also being characterized. Once detection of Act D bound to chromosomes was demonstrated employing the IgG fraction alone, preparation of specific anti-Act D antibody was no longer necessary. Therefore, no further attempts to purify the antibody were made.

This section is intended to be of use as an outline for antibody purification. Application of the techniques described may be of use for purification of other antibodies, or, possibly as a starting point for the future purification of anti-Act D antibody.

APPENDIX D

IMMUNOFLUORESCENCE

Reagents: Phosphate Buffered Saline (Schreck, Erlanger, and Miller, (1974).

Stock solutions for making PBS:

1. 6.8 g KH_2PO_4/200 ml distilled water (0.25 M)
2. 35.5 g Na_2HPO_4 (anhydrous)/liter distilled water (0.25 M)

   -or-

   67.02 g $Na_2HPO_4 \cdot 7H_2O$/1,000 ml distilled water

Preparation of 1 liter 10X PBS:

Take 60 ml solution 1, 340 ml of solution 2, and 80 g of NaCl. Add distilled water to make up to one liter in a volumetric flask. Store at room temperature; dilute 1:10 for use.

Mounting medium:

Gelvatol 20-30 (Monsanto Labs, MA)

1. Add 80 ml PBS to 20 g Gelvatol.
2. Heat to 80° while stirring for 16-18 hrs.
3. Add 40 ml Glycerol.

A. Direct Immunofluorescence

The purified antibody (or IgG fraction) is first conjugated with fluorescein isothiocyanate as described by Johnson and Dorling (1981). The antibody should then be dialyzed against phosphate

buffered saline for 24 hr and an F:P (Fluorescein to Protein) ratio should be determined at 495 nm and 280 nm, respectively. Satisfactory labeling ratio is between 0.5 and 1.0.

1. Apply 300 µL of specified concentration of Act D to a slide, coverslip, and lay flat in a humid chamber for 30 min.
2. Wash off coverslip with 95% ethanol, then wash slide 3 times in 50 ml 95% ethanol (up to 5 slides can be washed together in a Coplin jar).
3. Apply 300 µL of 10 mg/ml bovine serum albumin to slide, coverslip, and incubate in humid chamber for 20 min.
4. Wash with 50 ml cold PBS (4^{o}).
5. Apply 300 µL of fluorescinated antibody (diluted appropriately with PBS) to slide and incubate in humid chamber for 20 min.
6. Wash with 50 ml cold PBS, taking care not to let the slide dry out beyond this point.
7. Apply one drop of Gelvatol and immediately coverslip.
8. Slide will be ready for evaluation with U.V. microscopy after about 15 min.
9. Photograph with Ilford HP5 ASA 400 film. Store in dark at 4^{o}.

   NOTE: The direct method proved to be much inferior to the indirect method for the anti-Act D antibody.

B. Indirect Immunofluorescence (Schreck, Erlanger, and Miller, 1974)

1. Apply 300 µL of specified concentration of Act D to slide, coverslip, and lay in a humid chamber for 30 min.

2. Wash off coverslip with 95% ethanol, then wash slide 3 times in 50 ml 95% ethanol. (Up to 5 slides can be washed together in a Coplin jar.)
3. Apply 300 µL 10 mg/ml human IgG, coverslip, and incubate in humid chamber 20 min.
4. Wash off coverslip in PBS and wash slide in 50 ml PBS.
5. Apply 300 µL 10 mg/ml anti-Act D IgG (purified as described in Chapter 2), coverslip, and incubate in humid chamber 20 min. Care should be taken from this point not to let the slide dry out.
6. Same as Step 4.
7. Apply 300 µL of a 1:20 dilution of goat anti-rabbit IgG (Cappel) in PBS, coverslip, and incubate in humid chamber 20 min.
8. Same as Step 4.
9. Apply one drop of Gelvatol and immediately coverslip.
10. After 15 min slide is ready for examination on a U.V. microscope.
11. Photograph with Ilford HP5 ASA 400 film.

Store slides in dark at 4^{o}.

REFERENCES

Ada, G. L., Humphrey, J. H., Askonas, B. A., McDevitt, H. O., Nossal, G. J. V.: Correlation of grain counts with radioactivity (^{125}I and tritium) in autoradiography. Exp. Cell Res. 41:557-572 (1966).

Adolph, K. W., Cheng, S. M., Laemmli, U. K.: Role of nonhistone proteins in metaphase chromosome structure. Cell 12:805-816 (1977).

Altman, P. L., Katz, D. D. (eds.), In: Cell Biology, Vol. 1, p. 368, Bethesda, MD: Federation of American Societies for Experimental Biology, 1975.

Berlowitz, L., Pallotta, D., Sibley, C. H.: Chromatin and histones: binding of tritiated actinomycin D to the heterochromatin in mealy bugs. Science 164:1527-1529 (1969).

Brockmann, H., Manegold, J. H.: Abbau von actinomycin C_1(D) zu actinomycin C_1 - saure und deren recyclisierung zu actinomycin C_1(D). Chem. Ber. 100:3814-3819 (1967).

Brothman, A. R., Davis, T. P., Duffy, J. J., Lindell, T. J.: Development of an antibody to actinomycin D and its application for the detection of serum levels by radioimmunoassay. Cancer Research 42:1184-1187 (1982).

Burkholder, G. D.: Electron microscopic visualization of chromosomes banded with trypsin. Nature 247:292-294 (1974).

_____. The ultrastructure of G- and C-banded chromosomes. Exp. Cell Res. 90:269-278 (1975).

Burkholder, G. D., Duczek, L. L.: Proteins in chromosome banding. I. Effects of G-banding treatments on the proteins of isolated nuclei. Chromosoma 79:29-41 (1980).

Bustin, M., Yamasaki, H., Goldblatt, D., Shani, M., Huberman, E., Sachs, L.: Histone distribution in chromosomes revealed by anti-histone sera. Exp. Cell Res. 97:440-444 (1976).

Camargo, E. P., Plaut, W.: The radioautographic detection of DNA with tritiated actinomycin D. J. Cell Biol. 35:713-716 (1967).

Caspersson, T., Lomakka, G., Zech, L.: The 24 fluorescence patterns of the human metaphase chromosomes - distinguishing characters and variability. Hereditas 67:89-102 (1971).

Chandler, M. E., Yunis, J. J.: A high resolution in situ hybridization technique for the direct visualization of labeled G-banded early metaphase and prophase chromosomes. Cytogenet. Cell Genet. 22:352-356 (1978).

Chenais, F., Virella, G., Patrick, C. C.: Isolation of soluble immune complexes by affinity chromatography using staphylococcal protein A-Sepharose substrate. J. Immunol. Methods 18:183-192 (1977).

Chinsky, L., Turpin, P. Y.: Fluorescence of actinomycin D and its DNA complex. Biochim. Biophys. Acta 475:54-63 (1977).

Cionini, P. G., Avanzi, S.: Pattern of binding of tritiated actinomycin D to Phaseolus coccineus polytene chromosomes. Exp. Cell Res. 75:154-158 (1972).

Comings, D. E., Avelino, E.: Mechanisms of chromosome banding. II. Evidence that histones are not involved. Exp. Cell Res. 86: 202-206 (1974).

Comings, D. E.: Mammalian chromosome structure. Chromosomes Today 6: 19-26 (1977).

_____. Mechanisms of chromosome banding and implications for chromosome structure. Ann. Rev. Genet. 12:25-46 (1978).

Dandliker, W., Alonso, R., deSaussure, V., Kierszenbaum, F., Levison, S., Schapiro, H.: The effect of chaotropic ions on the dissociation of antigen-antibody complexes. Biochemistry 6:1461-1467 (1967).

Darnell, J. E., Philipson, L., Wall, R., Adesnik, M.: Polyadenylic acid sequences: Role in conversion of nuclear RNA into messenger RNA. Science 174:507-510 (1971).

Davis, F. M., Rao, P. N.: Antibodies specific for mitotic human chromosomes. Exp. Cell Res. 137:381-386 (1982).

Dick, D., Johns, E. E.: The effect of two acetic acid containing fixatives on the histone content of calf thymus deoxyribonucleoprotein and calf thymus tissue. Exp. Cell Res. 51:626-632 (1968).

Dutrillaux, B., Lejeune, J.: Sur une nouvelle technique d'analysis du caryotype humain. C. R. Acad. Sci., Paris Ser. 272:2638-2640 (1971).

Ebstein, B. S.: Tritiated actinomycin D as a cytochemical label for small amounts of DNA. J. Cell Biol. 35:709-713 (1967).

_____. The distribution of DNA within the nucleoli of the amphibian oocyte as demonstrated by tritiated actinomycin D radioautography. J. Cell Sci. 5:27-44 (1969).

Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauser, V., Waxdal, M. J.: The covalent structure of an entire gG immunoglobin molecule. Proc. Natl. Acad. Sci. (U.S.A.) 68:78-85 (1969).

Evans, H. J., Buckland, R. A., Pardue, M. L.: Location of the genes coding for 18S and 28S ribosomal RNA in the human genome. Chromosoma 48:405-426 (1974).

Farber, S., Roch, R., Sears, E. M., Pinkel, D.: Advances in chemotherapy of cancer in man. Adv. Cancer Res. 4:1-71 (1956).

Forsgren, A., Sjoquist, J.: Protein A from S. aureus. J. Immunol. 97: 822-832 (1966).

Galbraith, W. M, Mellett, B.: Tissue disposition of [^{3}H]Actinomycin D (NSC-3035) in the rat, monkey and dog. Cancer Chem. Rep. Part 1, 59 (No. 6):1061-1969 (1975).

Gaubatz, J., Prashad, N., Cutler, R. G.: Ribosomal RNA gene dosage as a function of tissue and age for mouse and human. Biochim. et Biophys. Acta 418:358-375 (1976).

Goding, J. W.: Use of staphylococcal protein A as an immunological reagent. J. Immunol. Methods 20:241-253 (1978).

Greenwood, F. C., Hunter, W. M., Glover, J. S.: The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. 89:114-123 (1963).

Harper, M. E., Ullrich, A., Saunders, G. F.: Localization of the human insulin gene to the distal end of the short arm of chromosome II. Proc. Natl. Acad. Sci. (U.S.A.) 78:4458-4460 (1981).

Higuchi, R., Stang, H. D., Browne, J. K., Martin, M. O., Huot, M., Lipeles, J., Salser, W.: Human ribosomal RNA gene spacer sequences are found interspersed elsewhere in the genome. Gene 15:177-186 (1981).

Hubner, H., Kononowicz, A. K.: Autoradiographic studies on differentiation of chromatin in interphase nuclei and mitotic chromosomes. Acta Med. Pol. 18:251-260 (1977).

Huttner, K. M., Barbosa, J. A., Scangos, G. A., Pratcheva, D. D., Ruddle, F. H.: DNA mediated gene transfer without carrier DNA. Journal of Cell Biology 91:153-156 (1981).

Jacob, S. T., Sajdel, E. M., Munro, H. N.: Specific action of α-amanitin on mammalian RNA polymerase protein. Nature 225:60-62 (1970).

Johnson, G. D., Dorling, T.: Immunofluorescence and immunoperoxidase techniques, pp. 106-137, In: Techniques in Clinical Immunology, R. A. Thompson (ed.). Blackwell Scientific Pub., London, 1981.

Jones, G. H.: A rapid assay for the binding of actinomycin D to DNA. Anal. Biochem. 71:376-383 (1976).

Kanayama, Y., Yamakami, K., Inoue, T., Maeda, Y., Siota, K.: Interchromosomal connections as revealed by an immunoperoxidase technique applying anti-DNA antibody. Exp. Cell Res. 103: 439-442 (1976).

Kato, H., Moriwaki, K.: Factors involved in the production of banded structures in the mammalian chromosomes. Chromosoma 38:105-120 (1972).

Kristiansen, T.: Matrix bound antigens and antibodies, pp. 191-205, In: Affinity Chromatography, O. Hoffmann-Ostenhof, M. Breitenbach, F. Koller, D. Kraft, O. Scheiner (eds.). Pergamon Press, New York, 1967.

Kornvall, G., Frommel, D.: Definition of staphylococcal protein A reactivity for human immunoglobulin G fragments. Immunocytochemistry 7:124-127 (1970).

Levison, S., Kiergzenbaum, F., Dandliker, W.: Salt effects on antigen-antibody kinetics. Biochemistry 9:322-331 (1970).

Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., Rutter, W. J.: Specific inhibition of nuclear RNA polymerase II by α-amanitin. Science 170:447-449 (1970).

Lindell, T. J., O'Malley, A. F., Puglisi, B.: Inhibition of nucleoplasmic transcription and the translation of rapidly labeled nuclear proteins by low concentrations of actinomycin D in vivo. Proposed role of messenger RNA in ribosomal RNA transcription. Biochemistry 17:1154-1160 (1978).

Lindell, T. J.: Evidence for an extranucleolar mechanism of actinomycin D action. Nature 263:347-350 (1976).

_____. Inhibitors of mammalian RNA polymerases, pp. 111-137, In: Inhibitors of DNA and RNA Polymerases, P. S. Sarin, R. C. Gallo (eds.). Pergamon Press, New York, 1980.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275 (1951).

Lubit, B. W., Schreck, R. R., Miller, O. J., Erlanger, B. F.: Human chromosome structure as revealed by an immunoperoxidase staining procedure. Exp. Cell Res. 89:426-429 (1974).

Martin-DeLeon, P. A.: Location of the 18S and 28S rRNA cistrons in the genome of the domestic rabbit (Orgctolagus cunicalus L.). Cytogenet. Cell Genet. 28:34-40 (1980).

Matsukuma, S., Utakoji, T.: Uneven extraction of protein in Chinese hamster chromosomes during G-staining procedures. Exp. Cell Res. 97:297-303 (1976).

McKay, R. D. G.: The mechanism of G- and C-banding of mammalian metaphase chromosomes. Chromosoma 44:1-14 (1973).

Meienhofer, J.: Synthesis of peptide analogs of Actinomycin D (NSC-3053). Cancer Chemother. Rep., Part 1, 58:21-34 (1974).

Meienhofer, J., Atherton, E.: Structure-activity relationships in the actinomycins, pp. 428-529, In: Structure-Activity Relationships Among the Semisynthetic Antibiotics, D. Perlman (ed.). Academic Press, New York, 1977.

Mendelsohn, M. L., Mayall, B. H., Bogard, E., Moore, D. H., Perry, B. H.: DNA content and DNA-based centromeric index of the 24 human chromosomes. Science 179:1126-1129 (1973).

Miles, D. P.: Labeling and other effects of actinomycin D on human chromosomes. Proc. Nat'l. Acad. Sci. (U.S.A.) 65:585-592 (1970).

Miller, O. J., Schreck, R. R., Beiser, S. M., Erlanger, B. F: Immunofluorescent studies of chromosome banding with anti-nucleoside antibodies, pp. 43-48, In: Chromosome Identification Technique and Applications in Biology and Medicine, T. Caspersson, L. Zech (eds.), Nobel Symp. 23, Academic Press, New York, 1973.

Miller, O. J., Schnedl, W., Allen, J., Erlanger, B. F.: 5-Methycytosine localized in mammalian constitutive heterochromatin. Nature 251:636-637 (1974).

Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., Hungerford, D. A.: Chromosome preparations of leukocytes cultured from human peripheral blood. Exp. Cell Res. 20:613-616 (1960).

Morin, J., Marcollet, M., Leng, .: Reactivity of antinucleoside antibodies with metaphase chromosomes. Biochimie 61:865-868 (1979).

Muller, W., Crothers, D. M.: Studies of the binding of Actinomycin D and related compounds to DNA. J. Mol. Biol. 35:251-290 (1968).

Muller, W., Rosenkranz, W.: Rapid banding technique for human and mammalian chromosomes. The Lancet 1:898 (1972).

Murphy, R. F., Ashraf, I., Hughes, A., McGucken, M., Buchanan, K., Lonlon, J., Elmore, D.: Avoidance of strongly chaotropic eluents for immunoaffinity chromatography by chemical modification of immobilized ligand. Biochim. Biophys. Acta 420:87-96 (1976).

Nakane, P. K., Pierce, G. B., Jr.: Enzyme labeled antibodies for the light and electron microscopic localization of tissue antigens. J. Cell Biol. 33:307-318 (1967).

Nordheim, A., Pardue, M. L., Lafer, E. M., Moller, A., Stollar, B. D., Rich, A.: Antibodies to left-handed Z-DNA bind to interband regions of Drosophila polytene chromosomes. Nature 294:417-422 (1981).

Pardue, R. L., Brenner, S. L., Wible, L., Reading, C. L., Brinkley, B. R.: Tubulin antibody: A practical guide for immunization, purification and indirect immunofluorescence, Chapter 12, pp. 1-23, In: Lab Methods Manual for Hormone Action and Molecular Endocrinology, W. T. Schrader, B. W. O'Malley (eds.). Fifth Edition, Houston Biological Association, 1981.

Parker, C. W.: p. 239, In: Radioimmunoassay for Biologically Active Compounds. Prentice-Hall, Englewood Cliffs, NJ, 1976.

Pathak, S., Arrighi, F. E.: Loss of DNA following C-banding procedures. Cytogenet. Cell Genet. 12:414-422 (1973).

Perry, R. P.: The cellular sites of synthesis of ribosomal and 4S RNA. Proc. Natl. Acad. Sci. (U.S.A.) 48:2179-2186 (1962).

Perry, R. P.: Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. Nat. Cancer Inst. Monogr. 14:73-89 (1964).

_____. Regulation of ribosome content in eukaryotes. Biochem. Soc. Symp. 37:105-116 (1973).

Perry, R. P., Kelley, D. E.: Inhibition of RNA synthesis by Actinomycin D: Characteristics dose-response of different RNA species. J. Cell. Physiol. 76:127-140 (1970).

Pratt, W. B., Ruddon, R. W.: p. 323, In: The Anticancer Drugs. University Press, New York, Oxford, 1979.

Raso, V.: Radioimmunoassays for methotrexate, citrovorum factor, neocarzinostatin and actinomycin D. Cancer Treat. Rep. 61:585-590 (1977).

Ringertz, N. R., Darzynkiewicz, Z., Bolund, L.: Actinomycin binding properties of stimulated human lymphocytes. Exp. Cell Res. 56: 411-417 (1969).

Rocchi, A., Gigliani, F., Capoa, A., Archidiacono, N.: Labeling of human chromosomes with ^{3}H-AMD. Humangenetik 24:297-301 (1974).

Roeder, R. G., Rutter, W. J.: Specific nucleolar and nucleoplasmic RNA polymerases. Proc. Natl. Acad. Sci. (U.S.A.) 65:675-682 (1970).

Rogers, A. W.: pp. 183-284, In: Techniques of Autoradiography, Elsevier/ North Holland Biomedical Press, New York, 1979.

Ron, A., Presscott, D. M.: The efficiency of tritium counting with seven radioautographic emulsions, pp. 231-240, In: Methods in Cell Phys., D. M. Prescott (ed.), Vol. 4. Academic Press, New York, 1970.

Rutledge, M. H.: A simple procedure for obtaining autoradiographs of G-banded chromosomes. Chromosoma 70:259-262 (1979).

Schreck, R. R., Warburton, D., Miller, O. J., Beiser, S. M., Erlanger, B. F.: Chromosome structure as revealed by a combined chemical and immunochemical procedure. Proc. Natl. Acad. Sci. (U.S.A.) 70:804-807 (1973).

Schreck, R. R., Erlanger, B. F., Miller, O. J.: The use of antinucleoside antibodies to probe the organization of chromosomes denatured by ultraviolet irradiation. Exp. Cell Res. 88:31-39 (1974).

Schweizer, D.: Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58:307-324 (1976).

_____. R-banding produced by DNAase I digestion of chromomycin-stained chromosomes. Chromosoma 64:117-124 (1977).

Seabright, M.: A rapid banding technique for human chromosomes. The Lancet 1:971 (1971).

Sieger, M., Garweg, G., Schwarzacher, H. G.: Constitutive heterochromatin in Microtus agrestis: binding of actinomycin D and transcriptional inactivity. Chromosoma 35:84-98 (1971).

Siev, M., Weinberg, R., Penman, S.: The selective interruption of nucleolar RNA synthesis in HeLa cells by cordycepin. J. Cell. Biol. 41:510-520 (1969).

Simard, R.: The binding of actinomycin D-^{3}H to heterochromatin as studied by quantitative high resolution radioautography. J. Cell. Biol. 35:716-722 (1967).

Sjoquist, J., Melour, B., Hjelm, H.: Protein A isolated from Staphylococcus aureus after digestion with Lysostaphin. Eur. J. Biochem. 29:572-578 (1972).

Sternberger, L. A.: p. 354, In: Immunocytochemistry. John Wiley and Sons, New York, 1979.

Szabo, P., Lee, M. R., Elder, F. B., Prensky, W.: Localization of 5S RNA and rRNA genes in the Norway rat. Chromosoma 65:161-172 (1978).

Takusagawa, F., Dabrow, M., Neidle, S., Berman, H. M.: The structure of a pseudo intercalated complex between actinomycin and the DNA binding sequence d(GpC). Nature 296:466-469 (1982).

Tantravahi, R., Miller, D. A., Dev. V. G., Miller, O. J.: Detection of nucleolus organizer regions in chromosomes of human, chimpanzee, gorilla, orangutan and gibbon. Chromosoma 56:15-27 (1976).

Tata, J. R., Hamilton, M. J., Shields, D.: Effects of α-amanitin in vivo on RNA polymerase and nuclear RNA synthesis. Nature New Biology 238:161-164 (1972).

Tattersall, M. H. N., Sodergren, J. E., Sengrupta, S. K., Trites, D. H., Modest, E. J., Frei, E.: Pharmacokinetics of actinomycin D in patients with malignant melanoma. Clinical Pharmacology and Therapeutics 17:701-708 (1975).

Tsurugi, K., Morita, T., Gata, K.: Effects of the inhibition of ribosomal RNA synthesis on the synthesis of ribosomal structural proteins in regenerating rat liver. Eur. J. Biochem. 29:585-592 (1972).

Weir, D. M.: pp. 30-87, In: Immunology - An Outline for Students of Medicine and Biology. Churchill Livingstone, New York, 1977.

Yunis, J. J., Chandler, M. E.: Cytogenetics, pp. 801-856, In: Clinical Diagnosis and Management by Laboratory Methods, J. B. Henry (ed)., Sixteenth Edition. W. B. Saunders Co., Philadelphia, 1979.