PART I

SYNCHRONIZATION OF THE CELL DIVISION CYCLE OF HeLa CELLS IN SUSPENSION CULTURES

PART II

STUDIES ON CHROMOSOMAL PROTEINS OF HeLa CELLS DURING THE CELL DIVISION CYCLE

Thesis by

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to

Mataji and Pitaji

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At this time my thoughts go back almost six years when on a hot summer morning, I walked into the New Delhi hotel room of Prof. James Bonner and expressed my intention to be a graduate student at Caltech. A year later I was in the Kerckhoff basement.

I am grateful to Prof. James Bonner for the many ways in which he has inspired me during the last five years. Among these, his enthusiastic involvement with research work and always fresh, exciting ideas stand out in my mind. The pace of his research and of other academic activities will always remain a challenge for me hard to overcome.

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ABSTRACT

PART I

These studies investigate the potential of single and double treatments with either 5-fluorodeoxyuridine or excess thymidine to induce cell division synchrony in suspension cultures of HeLa cells. The patterns of nucleic acid synthesis and cell proliferation have been analyzed in cultures thus synchronized. Several changes in cell population during long incubation with 5-fluorodeoxyuridine or excess thymidine are also described. These results are subjected to detailed evaluation in terms of the degree and quality of synchrony finally achieved.

PART II

Histones and non-histone proteins associated with interphase and metaphase chromosomes of HeLa cells have been qualitatively and quantitatively analyzed. Histones were fractionated by chromatography on Amberlite CG-50 and further characterized by analytical disc electrophoresis and amino acid analysis of each chromatographic fraction. It is concluded that histones of HeLa cells are comprised of only a small number of major components and that these components are homologous to those of other higher organisms. Of all the histones, arginine-rich histone III alone contains cysteine and can polymerize through formation of intermolecular disulfide bridges between histone III monomers.

A detailed comparison by chromatography and disc electro-

phoresis established that interphase and metaphase histones are made up of similar components. However, certain quantitative differences in proportions of different histones of interphase and metaphase cells are reported. Indirect evidence indicates that a certain proportion of metaphase histone III is polymerized through intermolecular disulfide links, whereas interphase histone III occurs mainly in the monomeric form.

Metaphase chromosomes are associated with an additional acid-soluble protein fraction which is absent from interphase chromosomes. All of these additional acid-soluble proteins of metaphase chromosomes are shown to be non-histones and it is concluded that the histone/DNA ratio is identical in interphase and metaphase chromosomes. The bulk of acid-soluble non-histone proteins of metaphase chromosomes were found to be polymerized through disulfide bridges; corresponding interphase non-histone proteins displayed no evidence of similar polymerization.

The factors responsible for the condensed configuration and metabolic inactivity of metaphase chromosomes are discussed in light of these findings.

The relationship between histone and DNA synthesis in non-dividing differentiated chicken erythrocyte cells and in rapidly dividing undifferentiated HeLa cells is also investigated. Of all the histones, only arginine-rich histones are synthesized in mature erythrocytes. Histone synthesis in HeLa cells was studied in both unsynchronized and synchronized cultures. In HeLa cells, only part of the synthesis

of all histone fractions is dependent on concurrent DNA synthesis, whereas all histones are synthesized in varying degrees even in the absence of DNA synthesis.

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PART I

SYNCHRONIZATION OF THE CELL DIVISION CYCLE OF HeLa CELLS IN SUSPENSION CULTURES

INTRODUCTION

The cell division cycle of a wide variety of plants and animals has been demonstrated to consist of four distinct phases[1]: (a) mitosis (M); (b) a pre-DNA synthetic period (G_1) from the end of telophase to the beginning of DNA synthesis; (c) a period of DNA synthesis (S); and (d) a post-DNA synthetic period (G_2) from the end of DNA synthesis to the initiation of prophase. It is necessary to recognize at the outset that the above classification of the division cycle is biochemically precise only in so far as it defines the S phase in terms of the biosynthesis of a specific cellular constituent. In contrast, G_1 and G_2 refer simply to a collection of undefined biochemical events, presumably essential for initiation of DNA synthesis and mitosis. Indeed it will be seen later in the Discussion that certain unidentified events normally occurring in G_2 can take place in G_1 under special conditions.

Several workers have developed autoradiographic methods for analysis of the time-course of different phases in the cellular life cycle of cells growing in vivo or in vitro (e.g. see Howard and Pelc, 1953; Lajtha et al., 1954; Painter and Drew, 1959; Edwards et al., 1960; Puck, 1964a). In addition, autoradiographic studies have greatly contributed in establishing correlations between different phases of the division cycle and certain macromolecular events, such as DNA replication in different chromosomes at

[1] The terminology used to refer to the various phases was originally proposed by Howard and Pelc (1953).

different stages of the S phase, RNA synthesis and distribution at different stages, and sensitivity of the various phases to irradiation. However, in order to perform more detailed physical and chemical analysis of isolated cellular constituents whose syntheses, functions or transformations are confined to particular stages in the division cycle, it becomes necessary to use populations of cells in which a large majority of cells go through the different phases synchronously.

Populations of cells (or freely suspended nuclei) naturally dividing in a synchronous manner have been known in the literature for a long time. A classic example is that of cycad, Dioon, whose megaspore divides for eight generations giving rise to 256 nuclei with all mitoses synchronous (Chamberlain, 1935). Marked synchrony has been described in the early divisions of endosperm nuclei and of sporogenous tissue of anthers in many angiosperms (see Erickson, 1964, for more examples in plants). Similarly, synchronous mitoses have been observed in the developing eggs of a number of animals belonging to such diverse groups as coelenterates, amphibia, echinoderms, arthropods and others (see Agrell, 1964, for more examples in animals). These examples present excellent opportunities for studying the natural mechanisms involved in the control of synchronous divisions.

The naturally synchronous divisions, however, gradually become asynchronous after a few generations. Also, it is difficult to obtain large quantities of tissues of the same age and to control the physical and chemical factors of environment. To fulfill the

above needs, a concerted effort has been made in the last decade or so to develop methods which will produce mass populations of synchronously dividing cells cultured in vitro. Detailed descriptions of the large variety of techniques employed so far to synchronize bacterial, algal, protozoan and mammalian cells are available in the articles published in volumes edited by Zeuthen (1964) and Cameron and Padilla (1966). Some of the more commonly used methods involving environmental manipulations are: (a) temperature cycle and shock treatments; (b) exposure to alternating light and dark periods; (c) addition of enriched medium to starved cells; (d) growth in a medium lacking a specific nutrient required by a mutant strain, followed by addition of the nutrient; (e) change from a medium lacking a general nutritional source (e.g. carbon, nitrogen) to an enriched medium; and (f) accumulation of cells at a particular stage in the division cycle by the use of a specific metabolic block, followed by removal of the block. Alternatively, synchronous populations can be obtained without the severe treatments involved in the methods just referred to simply by selection of cells belonging to a specific stage in the cell cycle. This selection can be achieved in various ways, among them separation of smaller cells by sedimentation in density gradients or by filtration.

For synchronization of mammalian cell populations, probably the most sophisticated method developed so far is the one dependent on the tendency of the cells undergoing mitosis to detach themselves

from the glass surfaces used to grow monolayer cultures. Terasima and Tolmach (1963), exploiting this property, successfully collected synchronized HeLa cells from glass-attached cultures. Use of a calcium-deficient medium further improved this procedure by making the attachment of mitotic cells so tenuous that removal was possible by very mild agitation of the overlying medium (Robbins and Marcus, 1964; Robbins and Scharff, 1966). However, one major disadvantage of this particular selection method is an extremely small yield of synchronized cells since only a very small fraction (about 4% in HeLa cells) of a logarithmically growing culture undergoes mitosis at any given moment. It has been possible to increase the yield of mitotic cells by combining the selection procedure with other methods of synchronization (described below), such as cold shock (Sinclair and Morton, 1963) or excess thymidine treatment (Salb and Marcus, 1965). Both of the above modifications, although potentially useful for special purposes, diminish the unique advantage of the selection technique over other methods, namely the minimum environmental disturbance of normal cellular metabolism. A conceptually similar, but less useful, method utilizes high doses of tritiated thymidine to kill a majority of cells by permitting them to enter the S phase over a long enough period in such a way that only a small fraction of cells in late G_1 survives (Whitmore and Gulyas, 1966).

Newton and Wildy (1959) were able to synchronize a moderately high percentage of a HeLa population grown in mono-

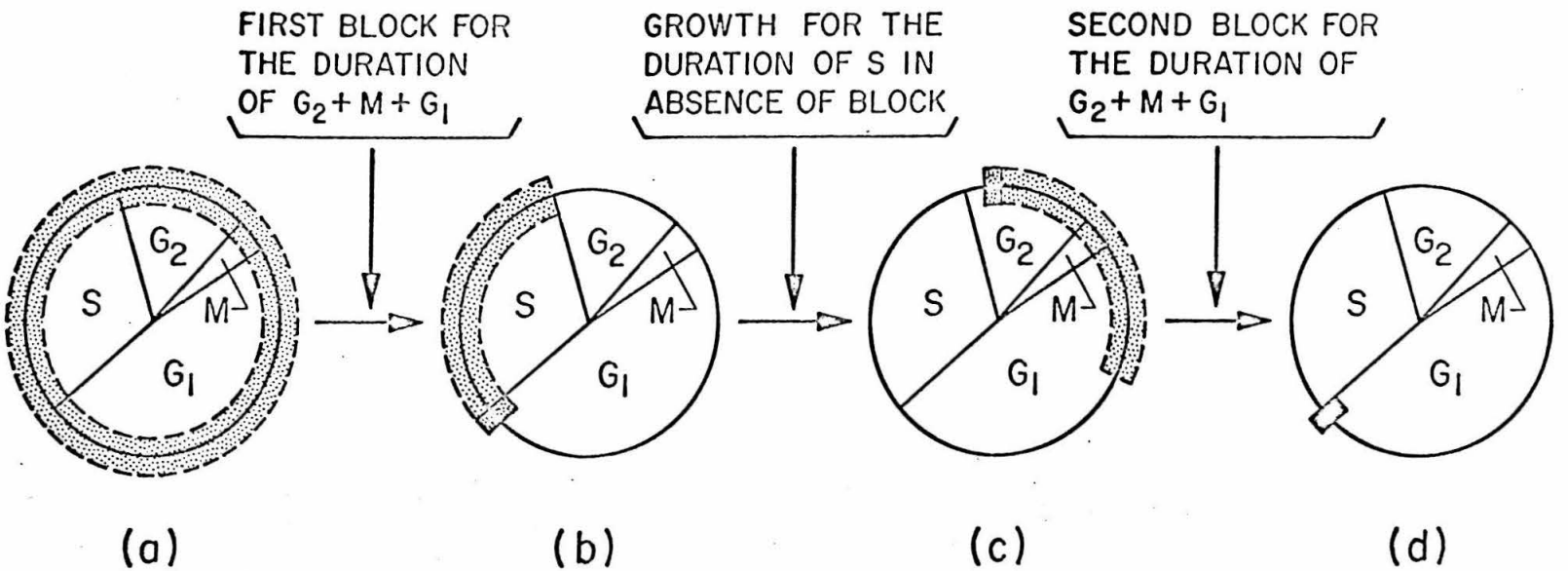
layers by subjecting the culture to 4°C for 1 hour followed by return to 37°C. However, later attempts to synchronize suspension cultures of L cells of mouse (Littlefield, 1962) and HeLa cells (Rao and Engelberg, 1966) as well as glass-attached cultures of human amnion cells (Miura and Utakoji, 1961) by similar temperature shocks have been unsuccessful, probably because the success of this method appears to be highly dependent upon the nutritional state, age and genetic strain of cells. More successful methods designed to synchronize large proportions of mammalian cell populations involve reversible inhibition of DNA synthesis. Thus mammalism cells have been synchronized by inhibition of DNA synthesis by substances like 5-fluorodeoxyuridine (Eidinoff and Rich, 1959; Rueckert and Mueller, 1960; Littlefield, 1962; Erikson and Szybalski, 1963; Till et al., 1963; Gold and Helleiner, 1964) or amethopterin (Rueckert and Mueller, 1960; Mueller et al., 1962; Schindler, 1963) for a certain length of time, followed by addition of thymidine. On the other hand, high concentrations (2mM or more) of thymidine when added alone also inhibit DNA synthesis and on removal result in well-synchronized populations (Xeros, 1962; Bootsma et al., 1964; Puck, 1964a,b; Petersen and Anderson, 1964; Galavazi et al., 1966; Rao and Engelberg, 1966; Vos et al., 1967). Similarly, deoxyadenosine (Xeros, 1962; Galavazi et al., 1966) and deoxyguanosine (Xeros, 1962; Mueller, 1963; Galavazi et al., 1966) have been successfully used to induce synchrony in mammalian cell cultures.

The synchrony obtained by single treatment with excess

thymidine in the earlier attempts was substantially improved by a carefully designed procedure involving double treatments with this compound. Since the present work has extensively utilized double treatments with both excess thymidine and 5-FUdr, the rationale for the double treatment method, independently developed by Bootsma et al. (1964) and Puck (1964a,b), is explained below in detail.

When a specific and reversible block of DNA synthesis (such as 5-fluorodeoxyuridine or excess thymidine) is added to a logarithmically growing population of cells, the cells which are at various points of the S phase at this moment will not proceed further as long as the blocking treatment is maintained. However, the cells which have finished DNA synthesis will continue through their life cycle unhindered unless the blocking agent has secondary effects on other phases. If the block is present for a period equal to the combined duration of G_2, M and G_1, all of the cells that were not in the S phase at the time of addition of the block will accumulate just before the S phase, whereas the cells which were synthesizing DNA at that time will be randomly distributed at various points along the S phase (Fig. 1b). On removal of the block, all of the cells which accumulated just before the S phase will enter this phase together but the cells blocked within the S phase itself will finish DNA synthesis earlier and will not be synchronized. Synchrony can be improved by allowing the cells to grow in the absence of the DNA block for a period equal to the normal duration of the S phase by

Figure 1. Schematic summary of the rationale for the synchronization method employing double treatments with a block of DNA synthesis. The figure shows distribution of cells in various phases of the cell division cycle during different periods of the double synchronization method.


FIRST BLOCK FOR THE DURATION OF $G_2 + M + G_1$
GROWTH FOR THE DURATION OF S IN ABSENCE OF BLOCK
SECOND BLOCK FOR THE DURATION OF $G_2 + M + G_1$
S
G_2
M
G_1
(a)
(b)
(c)
(d)

which time all cells will have completed DNA synthesis and no cell will be present in the S phase (Fig. 1c). As pointed out by Galavazi et al. (1966), "no cells will have reached the next S phase yet, if the duration of S is shorter than half of the total cycle and the variation in duration of the cycle of the individual cells is not large." A block of the DNA synthesis is again added at this point and maintained long enough so that all cells accumulate at the beginning of the S phase (i.e. at the end of G_1; Fig. 1d). When the second DNA block is removed, all cells will initiate the S phase synchronously.

The present experiments were initiated to investigate the full potential of single and double treatments with both 5-fluorodeoxyuridine (5-FUdr) and excess thymidine in induction of synchrony in suspension cultures of HeLa cells. In the case of 5-FUdr, the double treatment procedure was attempted for the first time. In order to attain the above objective, a detailed study of the pattern of nucleic acid synthesis and cell proliferation has been carried out in cultures synchronized by both single and double treatments with the two blocking agents. In addition, this work describes some of the interesting changes which the cell population undergoes during long incubation with 5-FUdr and excess thymidine. All of these results are evaluated in detail in terms of the degree and quality of synchrony which is finally achieved. Some implications of these findings on the various phases of the cell division cycle of unsynchronized as well as synchronized cells are also discussed. Most

of the earlier experience, with some exceptions (e.g. Rao and Engelberg, 1966), in synchronization of HeLa cells has been with glass-attached monolayer cultures. The practical difficulties involved in obtaining large uniformly grown quantities of cells from monolayer cultures prompted the use of suspension cultures in the present work. Therefore, the various details of handling the cells and the optimum conditions for synchronization of suspension cultures, developed in the course of this study, will also be described.

The knowledge of the time-course of various events in synchronized systems gained through these investigations has enabled us to meaningfully plan and interpret experiments utilizing synchronized cultures for investigation of the relationship between histone synthesis and the cell division cycle (see Chapter 2 in Part II).

MATERIALS AND METHODS

A. DESCRIPTION OF THE CELL LINE

HeLa cells, cultured in several laboratories since February, 1951, were derived from an epidermoid carcinoma of the human cervix by Gey and coworkers (Gey et al., 1952; Scherer et al., 1953). The clonal line S3, isolated by Puck and Fisher (1956) and shown to contain an average number of chromosomes expected for a triploid human female cell (Huberman and Attardi, 1967), was used in the present studies.

B. CULTIVATION OF CELLS

1. General Culture Methods and Maintenance of Stocks

HeLa S3 cells were grown in suspension culture in a modified Eagle's medium (Eagle, 1955, 1959) at 37 ± 1°C under sterile conditions. Four to five hundred ml of the stock cell culture were routinely maintained in logarithmic phase between cell concentrations of 0.5×10^5 and 2.5×10^5 cells/ml by diluting with fresh prewarmed (37°C) culture medium every 48 hours (about two generation periods) during weekdays. On weekends, however, the stock culture was diluted to a concentration of 0.3×10^5 cells/ml and allowed to proliferate for 72 hours (about three generation periods) before dilution.

Every two to three months, a batch of cells was frozen alive. These cells were to be used in case of accidental loss of the stock culture. For this purpose, the cell suspension was centrifuged at

500xg for 5 minutes at 5°C in an International Centrifuge Model PR-2 (International Equipment Company, Needham Hts., Mass., U.S.A.) and the pellet of live cells suspended in the modified Eagle's medium (MEP) supplemented with dialyzed calf serum (10%) and glycerine (10%) at a concentration of approximately 2×10^6 cells/ml. Suspension of the cell pellet was achieved by repeated squirting with a specially designed five ml pipette (15 cm long; Bellco Biological Glassware, Vineland, N.J., U.S.A.) equipped with a rubber bulb. Five ml aliquots of the above cell suspension were dispensed in small test tubes (Pyrex), stoppered, and cooled at a rate of 1°C/min from 5°C to -60°C in a Canalco Programmed Temperature Controller (Canal Industrial Corp., Bethesda, Md., U.S.A.), using a mixture of n-propanol and dry ice as a cooling agent. All the above operations were performed under sterile conditions. The cells frozen alive in this way were stored at -80°C for several months with no apparent damage. These cells could be regenerated by thawing quickly and washing them free of glycerin. On resuspension in MEP supplemented with 5% dialyzed calf serum, the cells started multiplying again after a lag period of a few hours.

The suspension culture system (Bellco Biological Glassware, Vineland, N.J., U.S.A.), based on the assembly devised by McLimans et al. (1957), consisted of cylindrical glass 'spinner' flasks of various capacities (250 ml, 500 ml, 1000 ml), each fitted with a magnetic stirring arrangement to allow continuous agitation of the cultures with a minimum generation of heat. The use of a

culture medium enriched in sodium bicarbonate and sodium phosphate monobasic (see below) improved the buffering capacity of the medium and obviated the need for a continuous flow of CO_2 over the cultures which was considered necessary for the maintenance of pH in the earlier work with mammalian cells in suspension cultures (e.g., McLimans et al., 1957).

2. Composition of the Culture Medium

As stated earlier, the cells were cultured in a modified Eagle's medium, enriched in phosphate ions (Eagle, 1955, 1959). Our medium (MEP) contained all of the 13 amino acids, except glutamine, in concentrations fourfold of those employed by Eagle (1955) and glutamine in twofold higher concentration; the mixture of 13 amino acids was further supplemented with glycine and serine (0.4 mM each). Similarly, all but one (inositol) of the vitamins were present in MEP in fourfold higher concentrations than those used by Eagle (1959); the medium was enriched in inositol 3.5-fold. While the concentrations of most of the salts (except $NaHCO_3$) in the present medium were identical to those used by Eagle (1959), MEP lacked calcium, contained a twofold higher concentration of $NaHCO_3$ and was further supplemented with $Fe(NO_3)_3 \cdot 9H_2O$ (0.25×10^{-3} mM). In addition to amino acids, vitamins and salts, MEP contained glucose (25 mM), penicillin (500 units/ml), streptomycin (0.1 mg/ml), n-butyl p-hydroxy-benzoate (antimycotic; 0.2×10^{-3} mg/ml), phenol red (1.5×10^{-2} mg/ml) and dialyzed calf syrum (5%, v/v). All of the components of MEP were dissolved in commercially distilled

water (Arrowhead Puritas Waters, Los Angeles, Calif., U.S.A.; Na < 0.03 mg/100 g of water).

3. Preparation, Filtration and Storage of the Culture Medium

MEP was normally prepared in 10 liter batches, flushed with CO_2 to pH 6.9-7.1, and filtered under air pressure through a specially devised filtering assembly employing a Millipore Filter Holder (Millipore Filter Corporation, Bedford, Mass., U.S.A.). The filter holder was equipped with Millipore Filter GSWP (pore size = 0.22 μ; Millipore Filter Corporation). All of the components used in this filter assembly were thoroughly sterilized beforehand. The filtered MEP was dispensed in 200 ml portions into presterilized Erlenmeyer flasks, stoppered, and stored at 4°C. Each fresh batch of filtered MEP was subjected to tests for any contamination by aerobic and anaerobic microorganisms by layering small aliquots on specially prepared test media for this purpose, followed by incubation at 37°C for 48 hours. A batch of media showing contamination, which occurred rarely, was either rejected or refiltered.

4. Dialysis, Filtration and Storage of Calf Serum

Whole calf serum (Hyland Laboratories, Los Angeles, Calif., U.S.A.), stored at -20°C, was thawed and poured into dialysis bags of large diameter. The dialysis bags had been washed earlier according to a special procedure involving soaking in EDTA (1%) $NaHCO_3$ (5%), and ethanol (95%) successively, followed by boiling and thorough rinsing in distilled water (E. O. Akinrimisi, personal

communication). Up to 2 liters of serum were dialyzed together against 5-8 changes of 10 liters each of salt solution (NaCl, 0.14 M; KCl, 0.005 M; Tris-HCl, 0.025 M; pH 7.4-7.5) supplemented with all the antibiotics at the same concentrations as in MEP. The total time spent during dialysis was between 60 and 80 hours. Dialyzed serum, supplemented further with penicillin (1000 units/ml) and streptomycin (0.2 mg/ml), was filtered under air pressure through a Millipore filter assembly in which a series of Millipore filters (glass filter pad; RAWP, 1.2 μ; HAWP, 0.45 μ; GSWP, 0.22 μ) of decreasing pore size was employed. Dialyzed and filtered serum was dispensed in 10 ml portions in presterilized test tubes, stoppered, and stored at -20°C. Filtered serum was routinely tested for contamination by aerobic and anaerobic microorganisms in the same way as described above for MEP.

C. SOME CHARACTERISTICS OF CELL POPULATIONS

Under ideal conditions of low cell concentration (less than 2×10^5 cells/ml) and small culture volume (less than 800 ml), 90 to 95% of the cells grown existed as single cells or a few as pairs of cells representing daughter cells not yet separated after mitosis. When encountered under the above conditions, clumps of cells usually consisted of a group of 3-5 cells only. However, when a larger volume of culture or a higher concentration of cells was grown, the frequency as well as the size of clumps increased considerably.

The generation time of HeLa S3 cells in the logarithmic phase ranged between 20 and 24 hours (usually close to 22 hours). The DNA and RNA content of the culture also increased at a rate identical to that of the increase in cell number and doubled along with cell number in 20 to 24 hours (see Fig. 3a).

D. DETERMINATION OF CELL CONCENTRATION

The concentration of cells was determined by removing samples of cells with presterilized Pasteur pipettes and counting the number of cells in a standard hemocytometer. Each observation is an average of cells in eight counting chambers of a hemocytometer, each with a volume capacity of 10^{-4} ml. All values reported here for cell concentration represent a mean of such observations made at least in triplicate and often in quadruplicate. The standard deviation (expressed as percentage of the mean) for the determination of cell counts was, however, quite large, ranging between 2 to 13% on different occasions. Although several modifications were made in the techniques of removing samples, the accuracy of the determinations could not be further improved. Some of the factors responsible for this variation are (1) different depths of the culture from which the samples are removed, (2) different time intervals between removal of samples and their injection into the counting chambers, and (3) the different angles at which the Pasteur pipettes are held on the hemocytometer during injection of samples into the counting chambers. In spite of the large standard deviation, it was found

possible to explore with considerable certainty the changes in cell concentrations by taking the mean of three or four observations.

E. DETERMINATION OF THE MITOTIC INDEX

The method of Huberman and Attardi (1966) was used for measurement of the frequency of metaphase cells in a given population. Normally, a 5 ml aliquot of the culture was centrifuged in a Clinical Centrifuge, the pellet of cells suspended in 1% sodium citrate for 10 minutes, fixed in acetic acid-ethanol (3:2) for 10 minutes and then stained in 1% orcein dissolved in lactic acid-acetic acid (1:1). The stained cells were examined under a microscope and the percentage of metaphase cells determined by counting 1000 cells. Each value for metaphase percentage reported here is a mean of two separate determinations.

F. RADIOACTIVE LABELING AND ASSAY OF NUCLEIC ACIDS

An 18 ml sample (cell concentration = 0.7 to 2.0×10^5 cells/ml) was removed from the culture under sterile conditions and incubated at 37°C for 20 minutes with continuous stirring in a presterilized 25 ml Erlenmeyer flask (equipped with a small magnetic bar) containing a radioactive nucleic acid precursor dissolved in 0.15 ml of sterile water at neutral pH. DNA was labeled with 0.083 μc/ml of [^{3}H]-methyl-thymidine (New England Nuclear Corp., Boston, Mass., U.S.A.; sp. act. = 6.7 c/mM), and RNA with 0.12 μc/ml of [^{3}H]-5-uridine (Schwarz Bio Research, Inc.,

Orangeburg, N.Y., U.S.A.; sp. act. = 23.0 c/mM).

At the end of the incubation period, a 15 ml aliquot was removed, quickly chilled in ice, and centrifuged in a Clinical Centrifuge at 4°C. All further operations were carried out at 0 - 4°C unless specified otherwise. The cell pellet was washed twice with isotonic salt solution (NaCl, 0.14 M; KCl, 0.005 M; Na_2HPO_4, 0.7×10^{-3} M; Tris-HCl, 0.025 M; pH 7.4-7.5), suspended in cold 10% trichloroacetic acid (TCA) and allowed to stand in ice for at least 4 hours. The TCA suspension of cells was homogenized in a motor-driven Teflon Potter-Elvehjem homogenizer with 10 strokes and centrifuged. The pellet was washed once with 10% TCA, twice with 5% perchloric acid (PCA) and finally once with ether-ethanol (1:1) and dried under air at room temperature. The dried pellet was treated according to the method described in the next section for separation of hydrolyzed fractions of DNA and RNA which were assayed for radioactivity in a Beckman Liquid Scintillation Spectrophotometer using a dioxane-based scintillation fluid.

In some experiments, the cells labeled with either radioactive thymidine or uridine were washed twice with isotonic salt solution, suspended in cold 10% TCA, and directly filtered through Bac-T-Flex (B-6) membrane filters (Schleicher and Schuell Co., Keene, N.H., U.S.A.). The membrane filters were repeatedly washed with several batches of cold 10% TCA and their radioactivity measured in a Beckman Liquid Scintillation Spectrophotometer.

G. QUANTITATIVE ANALYSIS OF NUCLEIC ACIDS

For determination of nucleic acid content of cells, the general method of Schmidt and Thannhauser (1945) was followed. Cells collected from a 15 ml aliquot of the culture were washed with isotonic salt solution, homogenized in 10% TCA, and subsequently washed with 10% TCA, 5% PCA, and ether-ethanol (1:1) and then air-dried as described in the previous section. The dried cell pellet was dissolved in 0.3 N KOH and the RNA hydrolyzed by incubation at 37°C for 18 hours. PCA (70%) was then added to a final concentration of 5% and the precipitate of DNA, protein, $KClO_4$ and other materials allowed to stand at 0°C for at least 1/2 hour. The samples were centrifuged in a Clinical Centrifuge and the supernatant saved. The precipitate (pellet) was washed once with a small volume of 5% PCA and the wash combined with the above supernatant. RNA in the pooled RNA hydrolysate solution was assayed by the orcinol method (Schneider, 1957) using commercially available yeast RNA as a standard.

DNA in the precipitate was hydrolyzed by boiling in 5% PCA at 100°C for 15 minutes and the supernatant containing hydrolyzed DNA removed after centrifugation. The remaining pellet was washed once with a small volume of 5% PCA and then pooled with the supernatant above. DNA in the hydrolystate was determined by the diphenylamine method either according to Dische (1930) or Burton (1956) using commercially available calf thymus DNA as a standard.

H. TECHNIQUES OF SYNCHRONIZATION

1. Synchronization by 5-Fluorodeoxyuridine (Fig. 2a)

5-Fluorodeoxyuridine (5-FUdr) dissolved in MEP at 10^{-3} M was added to a logarithmic phase culture (0.8 to 1.0 liter; 0.7 to 1.0×10^5 cells/ml) to a final concentration of 10^{-6} M. Incubation of the culture was continued for 15.5 hours and then thymidine (dissolved in MEP at 10^{-2} M) was added to a final concentration of 10^{-5} M.

A second treatment with 5-FUdr, whenever desired, was given after the growth of cells in the presence of thymidine for 15.5 to 16.5 hours (end of mitosis). For this second treatment, the thymidine and 5-FUdr added in the first step were removed by centrifugation of the culture at 500xg for 5 minutes at room temperature, followed by washing of the cell pellet twice with prewarmed (37°C) MEP. The final cell pellet was suspended at a cell concentration of 1.0×10^5 cells/ml in prewarmed MEP supplemented with 5-FUdr (10^{-6} M). The culture was incubated with 5-FUdr for another 15.5 hour period and then thymidine was added (10^{-5} M).

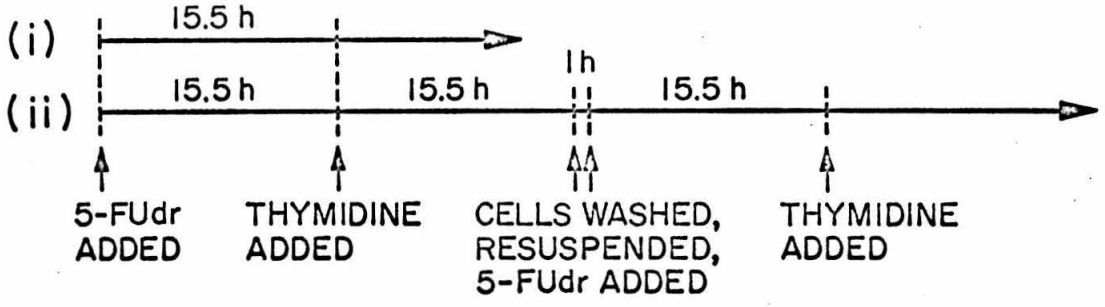
Determinations of cell concentration and DNA and RNA content were continuously made at hourly intervals during the periods of active DNA synthesis and cell proliferation, but at longer intervals during the presence of 5-FUdr.

2. Synchronization by Excess Thymidine (Fig. 2b)

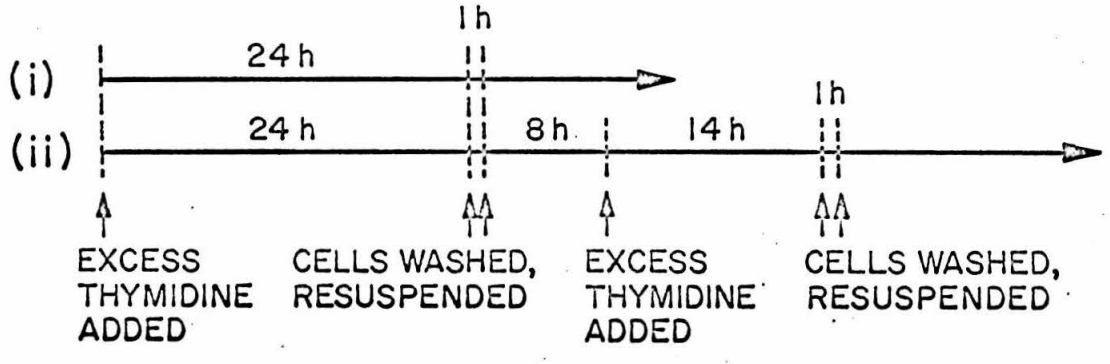
Thymidine (Calbiochem, grade A) was added to a logarithmic phase culture (0.8 to 1.0 liter; 0.7 to 1.5×10^5 cells/ml) to a final

Figure 2. Schemes for synchronizing cells. (a) By 5-FUdr: i. Single treatment; ii. Double treatment. (b) By excess thymidine: i. Single treatment; ii. Double treatment.

(a)


(i)
15.5 h
(ii)
15.5 h
15.5 h
1 h
15.5 h
5-FUdr ADDED
THYMIDINE ADDED
CELLS WASHED, RESUSPENDED, 5-FUdr ADDED
THYMIDINE ADDED

(b)


(i)
24 h
1 h
(ii)
24 h
8 h
14 h
1 h
EXCESS THYMIDINE ADDED
CELLS WASHED, RESUSPENDED
EXCESS THYMIDINE ADDED
CELLS WASHED, RESUSPENDED

concentration of 3.0 to 7.5 mM. Stock solutions of thymidine in MEP were routinely prepared at 10-fold higher concentration than that required for incubation. The stock solution thus prepared (normally one liter) was filtered in the same way as described earlier for MEP, dispensed in 100 ml batches in presterilized Erlenmeyer flasks, and stored at $4^{\circ}C$ for a period of 2 months with no noticeable loss of effectiveness. Preparation of fresh thymidine solutions just before use was found unnecessary (cf. Galavazi et al., 1966). Incubation with excess thymidine was continued for 24 hours, after which the cells were centrifuged at 500xg for 5 minutes at room temperature, washed thrice with 100 ml each of prewarmed MEP and finally resuspended (at 1.0×10^5 cells/ml) in prewarmed MEP.

If a double treatment with excess thymidine was desired, the culture was again supplemented with thymidine (concentration same as in the first step) 8 hours after release of the first thymidine block. At this point all cells have finished the S phase and are either in G_2, M, or G_1. The second thymidine treatment was continued for 14 hours and then the cells were centrifuged, washed and resuspended in MEP as before.

Determinations of cell concentration, DNA and RNA content, rate of incorporation of radioactive nucleic acid precursors and frequency of metaphase cells were regularly made at hourly intervals during the periods of active DNA synthesis and cell proliferation, but at longer intervals otherwise.

RESULTS

A. SYNCHRONIZATION BY 5-FLUORODEOXYURIDINE

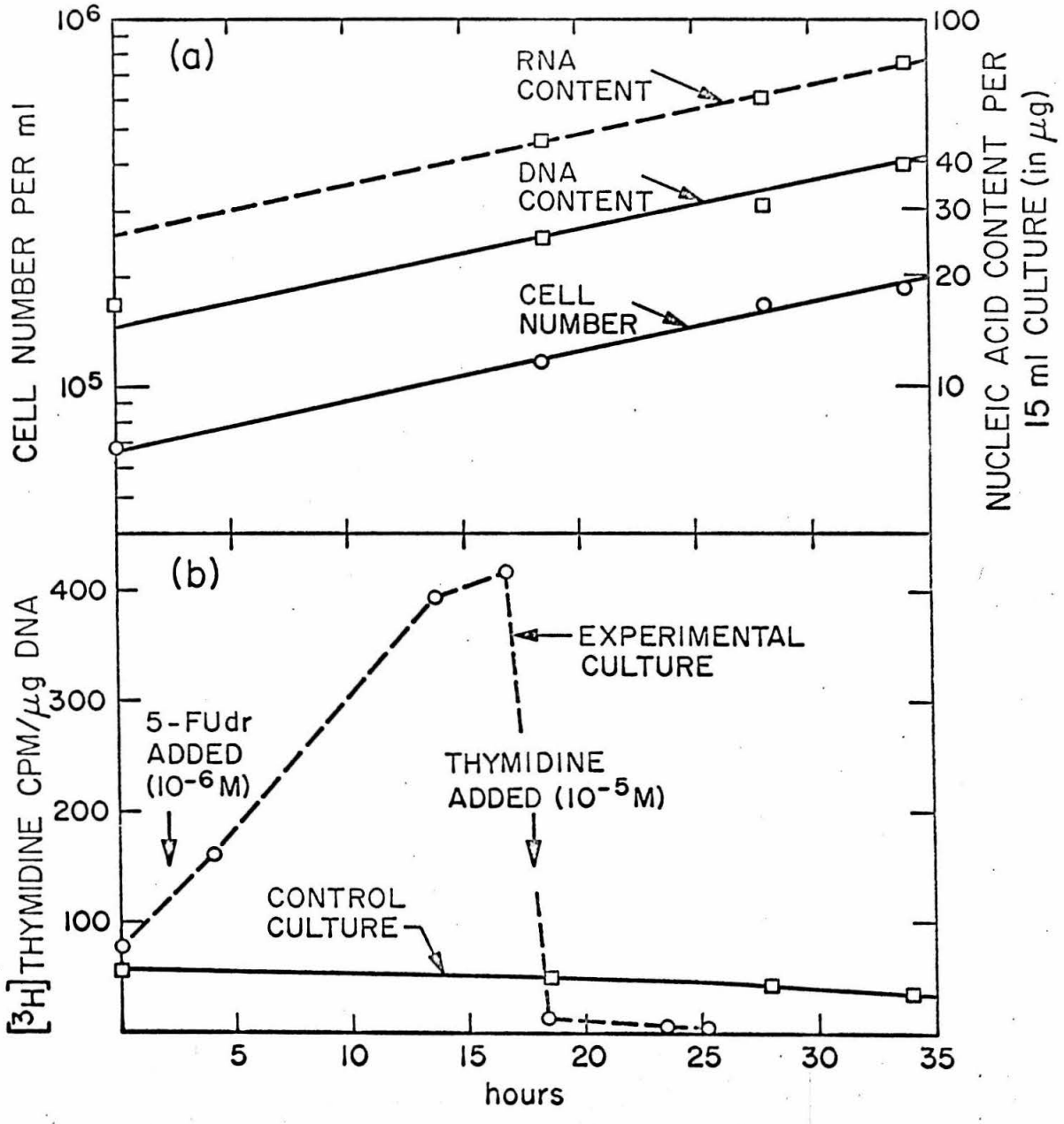
1. Induction of Thymidine (Tdr) Deficiency by 5-Fluorodeoxyuridine

Fig. 3b shows the results of pulse-labeling the DNA of a logarithmically growing population of HeLa cells with [^{3}H] Tdr in the absence or presence of 5-FUdr. In the absence of 5-FUdr, the rate of incorporation of [^{3}H] Tdr into DNA remains essentially constant over a period of 35 hours (approximately 1.5 generation periods). The slight but definite decrease in the rate of incorporation of [^{3}H] Tdr over this period probably reflects a diminishing concentration of the radioactive precursor in the cell pool. Such an effect is conceivable since cell number per ml is continuously increasing during this period (about 2.9-fold increase in 35 hours) while the same amount of [^{3}H] Tdr is added per ml of culture for pulse-labeling.

In contrast to the above situation, the addition of 5-FUdr (10^{-6} M) immediately results in a significant increase in the rate of [^{3}H] Tdr incorporation. Eidinoff and Rich (1959) using another human cell line have reported similar results. It may be noted that the cells incorporate [^{3}H] Tdr at a very high rate (5- to 6-fold higher than controls) even after prolonged incubation (15 hours) in the presence of 5-FUdr. This result clearly shows that the biochemical machinery of the cells for synthesizing DNA is not detectably damaged by long incubations with 5-FUdr and that the

Figure 3. (a) Some growth characteristics of a log phase HeLa S3 population. (⊢○—○⊣) cell number/ml; (⊢□—□⊣) DNA content; (⊢□--□⊣) RNA content.

(b) The rate of incorporation of [^{3}H]-methyl-thymidine into DNA by log phase HeLa S3 cells in the absence and presence of 5-FUdr. Aliquots of the culture were removed at the indicated intervals and incubated with tritiated thymidine (0.083 μc/ml; sp. act. = 6.7 c/mM) for 20 minutes each. See Materials and Methods for details. (⊢□—□⊣) control culture; (⊢○--○⊣) experimental culture.


(a)
RNA CONTENT
DNA CONTENT
CELL NUMBER
CELL NUMBER PER ml
NUCLEIC ACID CONTENT PER 15 ml CULTURE (in μg)
(b)
EXPERIMENTAL CULTURE
5-FUdr ADDED (10-6 M)
THYMIDINE ADDED (10-5 M)
CONTROL CULTURE
[3H] THYMIDINE CPM/μg DNA
hours

cells remain ready to enter or continue the S phase the instant Tdr is made available. This observation has obvious bearing on the applicability of long incubation times with 5-FUdr for blocking DNA synthesis and allowing accumulation of cells at the beginning of the S phase followed by instantaneous reversal of the block by Tdr resulting in synchronous entry of cells into the S phase. It can be concluded further that 5-FUdr remains an effective inhibitor of DNA synthesis for a period of at least 15 to 16 hours. Addition of non-radioactive Tdr (10^{-5} M) effectively dilutes the concentration of the radioactive precursor in the incubating medium and, as expected, leads to a significantly reduced rate of incorporation of [^{3}H] Tdr into DNA, despite the fact that most of the cells are actively synthesizing DNA under this condition (see Fig. 4a).

2. Cell Proliferation and Nucleic Acid Synthesis During Thymidine-deficient Growth

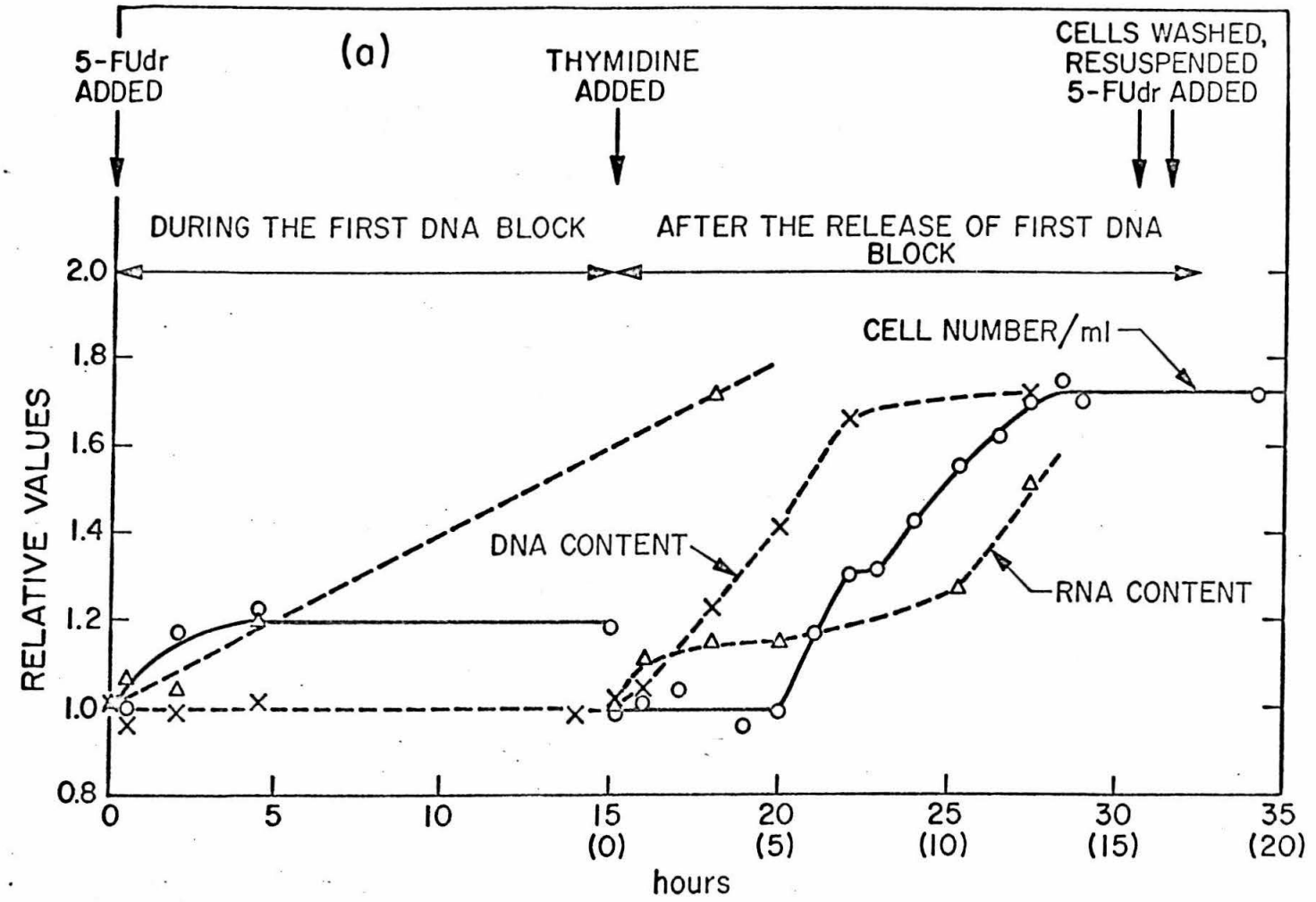
Synchronization is begun by adding 5-FUdr (10^{-6}M) to a culture of HeLa cells in logarithmic phase (initial cell concentration between 0.7 and 1.0×10^5 cells/ml) at 0 hours and the incubation is continued for 15.5 hours (Fig. 4a). During this period the DNA content of the culture remains unchanged. However, a slight increase in DNA content (5 to 10%) over a period of 15 hours was observed if cell concentrations higher than 1.0×10^5 cells/ml were incubated with 10^{-6}M 5-FUdr. A similar observation has also been made by Rueckert and Mueller (1960) while using glass-attached monolayer cultures of HeLa cells. In contrast to DNA content, RNA

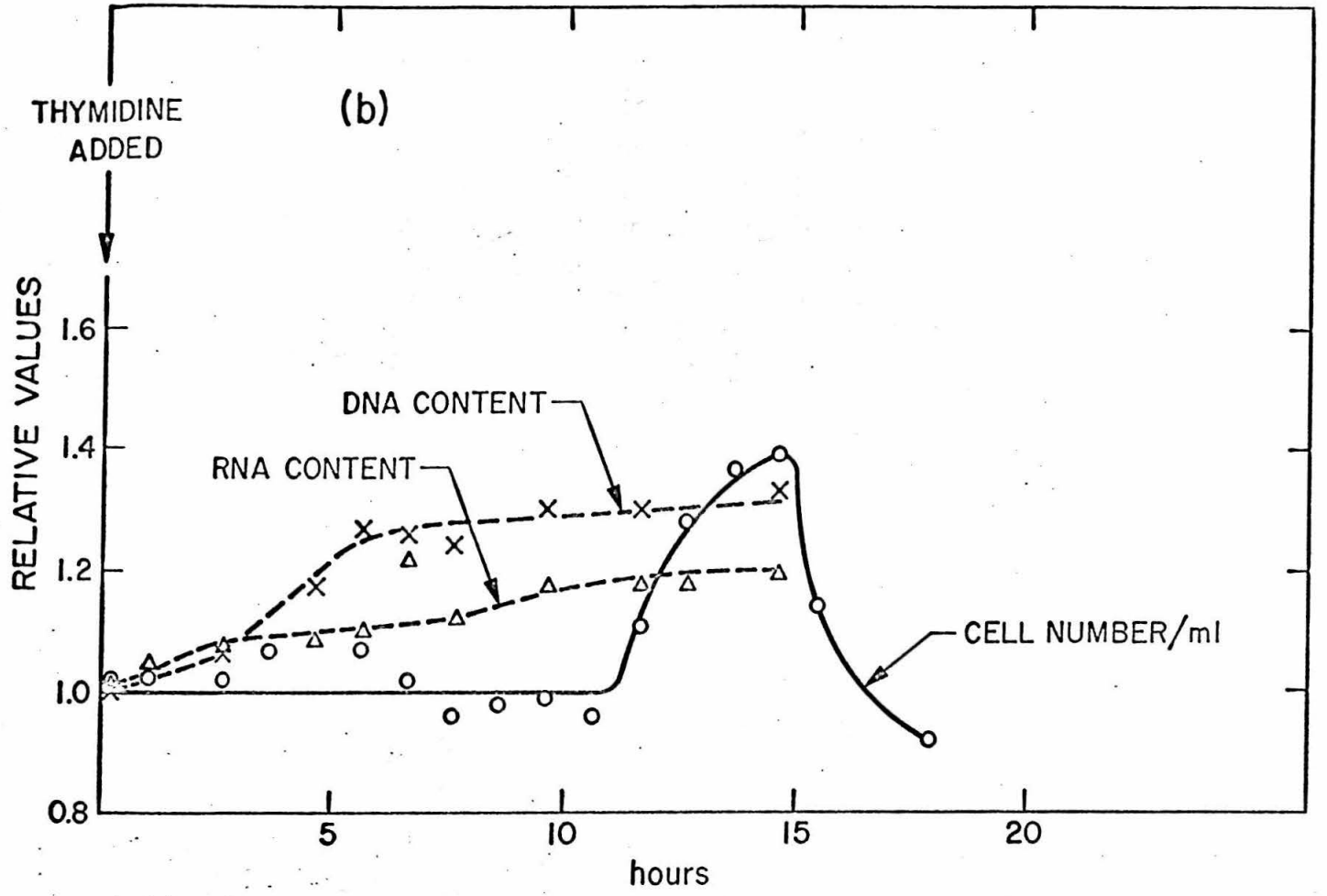
content continues to increase in the presence of 5-FUdr at a normal rate. Cell number per ml increases by about 20% during the first 4 to 5 hours after addition of 5-FUdr; no further increase in cell concentration takes place during the remaining 10 to 11 hours of incubation with the blocking agent.

3. Characteristics of a Synchronized Population (Figs.4a and b)

Inhibition of DNA synthesis is reversed by addition of Tdr (10^{-5}M) at the end of the 15.5 hour incubation with 5-FUdr (Fig. 4a). The DNA content of the culture begins to increase instantaneously and reaches a plateau in 7 to 8 hours. In different experiments DNA content increased 1.7- to 1.8-fold during this period. RNA content also steadily increases but the rate of increase slows down 4 hours after addition of Tdr. This period of slow increase in RNA content parallels the period of active cell proliferation and is followed by a sharp rise in RNA content. Cell number per ml, in contrast to nucleic acid content, remains unaltered for a period of 5 hours after reversal of the block. The burst of cell proliferation activity beginning at this point exhibits a biphasic mode and lasts about 8 hours. Approximately 30% of the cells divide in the first phase while in the second phase another 40 to 50% of the original cell population completes cell division. As a result of this, cell number per ml increases 1.7- to 1.8-fold and no further increase is observed during the next 8 to 9 hours. Notice the period in which DNA synthesis takes place with no accompanying increase in cell number as well as the period in which DNA synthesis and cell proliferation activities

Figure 4. Characteristics of a HeLa S3 population synchronized by 5-FUdr treatment. (—O—O—) cell number/ml; (--X---X--) DNA content/ml; (--Δ---Δ--) RNA content/ml. (a) Single treatment (during and after release of the first block). (b) Double treatment (after release of the second block).


(a)
5-FUdr ADDED
THYMIDINE ADDED
CELLS WASHED, RESUSPENDED 5-FUdr ADDED
DURING THE FIRST DNA BLOCK
AFTER THE RELEASE OF FIRST DNA BLOCK
CELL NUMBER/ml
DNA CONTENT
RNA CONTENT
RELATIVE VALUES
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
5
10
15 (0)
20 (5)
25 (10)
30 (15)
35 (20)
hours


THYMIDINE
ADDED
(b)
RELATIVE VALUES
1.6
1.4
1.2
1.0
0.8
DNA CONTENT
RNA CONTENT
CELL NUMBER/ml
5
10
15
20
hours

overlap each other.

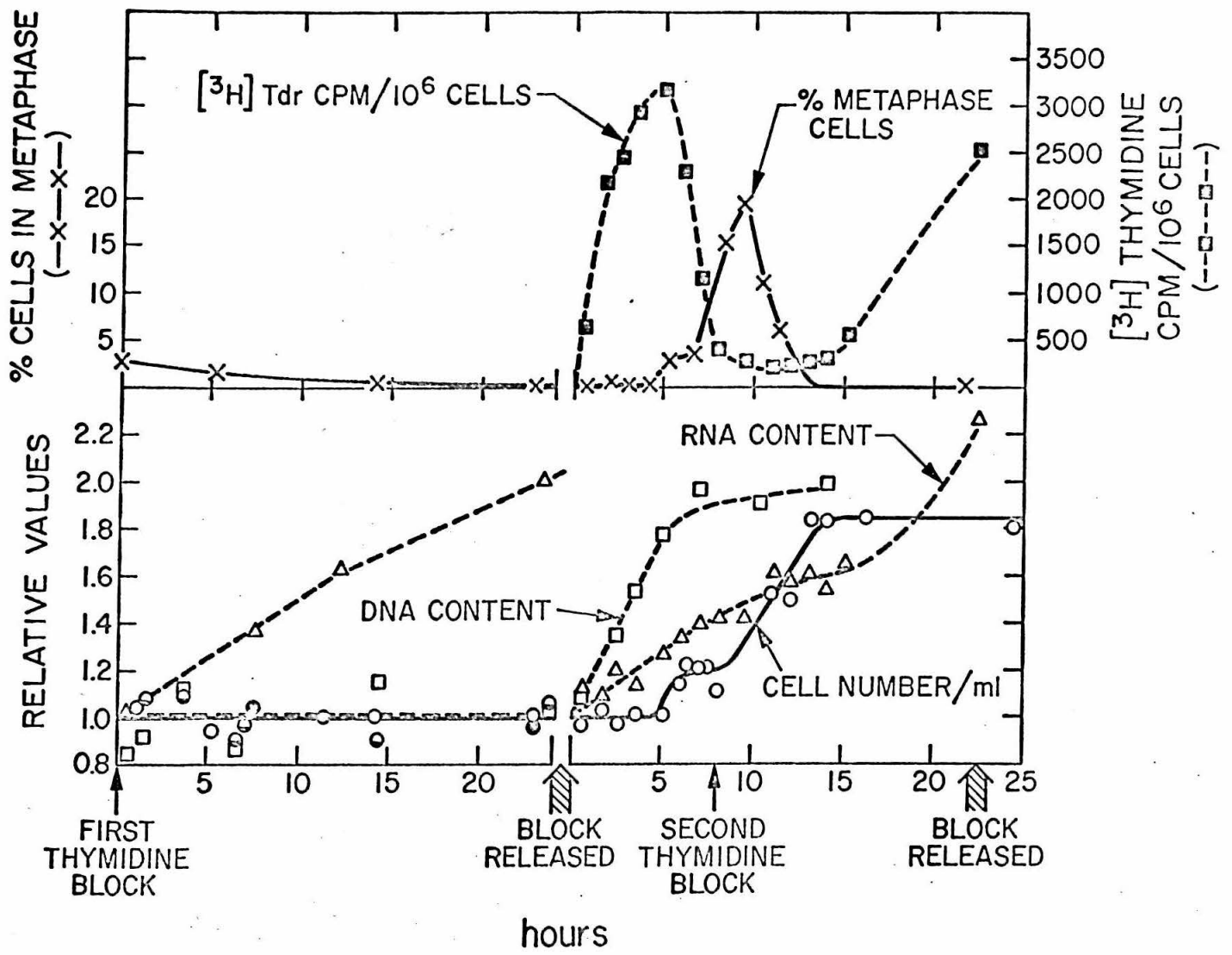
About 15 hours after reversal of the first DNA block, the blocking treatment is again initiated and continued for another 15.5 hours. In this period no increase in DNA content or cell concentration takes place, whereas RNA content continues to increase (results not shown here). At the end of this treatment, Tdr is added and a typical pattern of cell proliferation and nucleic acid synthesis after reversal of the second DNA block is shown in Fig. 4b. DNA content begins to rise first at a relatively lower rate and later at a faster rate, finally leveling off by 7 to 8 hours. However, the increase in DNA content is only 1.3- to 1.4-fold. The extent of rise in RNA content is also much lower than that noticed after reversal of the first DNA block. Cell number per ml shows no increase for about 11 hours and then increases in a single burst lasting about 4 hours; the biphasic mode of increase observed in Fig. 4a is lacking. In contrast to the increase which occurred after removal of the first block, cell number increases only 1.4-fold, followed by a sharp decline in cell concentration which is also accompanied by a definite increase in microscopically visible cell debris. The degree as well as the duration of decline in cell number, however, varies in different experiments.

B. SYNCHRONIZATION BY EXCESS THYMIDINE

1. The First Step (Fig. 5)

Synchronization is started by incubating a logarithmically

Figure 5. Characteristics of a HeLa S3 population synchronized by single treatment with excess thymidine. (—○—○—) cell number/ml; (○) 5.0 mM Tdr, 1.0×10^5 cells/ml; (◒) 7.5 mM Tdr, 1.0×10^5 cells/ml; (●) 7.5 mM Tdr, 2.0×10^5 cells/ml; (—×—×—) % cells in metaphase; (--□--□-) DNA content/ml; (--▣---▣--) [^{3}H] Tdr CPM/10^6 cells; (--△--△-) RNA content/ml.


% CELLS IN METAPHASE (—x—x—)
[3H] Tdr CPM/10^6 CELLS
% METAPHASE CELLS
[3H] THYMIDINE CPM/10^6 CELLS (--□--□--)
RELATIVE VALUES
RNA CONTENT
DNA CONTENT
CELL NUMBER/ml
FIRST THYMIDINE BLOCK
BLOCK RELEASED
SECOND THYMIDINE BLOCK
BLOCK RELEASED
hours

growing culture of HeLa cells (initial cell concentration between 0.7 and 1.5×10^5 cells/ml) with excess Tdr (3.0 to 7.5 mM) for 24 hours. For reasons to be discussed later, 5.0 mM was chosen as the optimum Tdr concentration for obtaining the best synchronization results. Therefore, the results described below were obtained by employing 5.0 mM Tdr unless specified otherwise.

As shown in Fig. 5, the DNA content of the culture does not change during incubation with excess Tdr whereas RNA content gradually rises at a normal rate. It may be recalled that cell number per ml increased by about 20% after addition of 5-FUdr in the absence of any increase in DNA content (see Fig. 4a). In contrast, such an increase in cell number, although theoretically expected, was never observed after addition of excess Tdr. Since this is a point of considerable interest because of its bearing on the length of G_2 as well as on the possibility of secondary effects of Tdr on the cell division cycle (see Discussion), the data showing a lack of any change in cell number during incubation at another Tdr concentration (7.5 mM) at two different cell concentrations are also presented in Fig. 5. After addition of excess Tdr, the percentage of metaphase cells steadily but slowly falls from its original value in log phase cultures and reaches a low value of 0.5% or less in about 12 hours, declining very slowly thereafter. Notice that at the end of 24 hour incubation with excess Tdr, 0.35% of cells are still in metaphase.

Following 24 hour incubation with excess Tdr, the cells are transferred to a Tdr-free medium. The DNA content of the culture

begins to rise immediately, almost leveling off in about 7 to 8 hours. In different experiments DNA content increased 1.8- to 1.9-fold during this period. The rate of incorporation of [^{3}H] Tdr into DNA per ml of culture rises very rapidly from zero on removal of excess Tdr, reaching a peak in about 4 to 5 hours, followed by a tenfold decline within the next 3 hours. The rate of [^{3}H] Tdr incorporation remains at this low level for another 4 to 5 hours and then rises again. The RNA content of the culture continues to increase after removal of the block; the rate of increase, however, slows down at about 7 hours. The period of slow rise in RNA content (6 to 7 hours) parallels the duration of mitotic activity and is then followed by a period of sharp increase.

Cell number per ml remains unchanged for the first 4 to 5 hours after release of the block and then increases in a biphasic mode similar to the one observed after reversal of the first 5-FUdr block (cf. Fig. 4a). Approximately 20% of the cells divide in the first phase and another 55 to 65% of the original cell population completes mitosis in the second phase. The wave of cell proliferation activity lasts about 9 hours, resulting in a 1.75- to 1.85-fold increase in cell number, followed by a period of 10 or more hours in which cell number remains essentially constant. The percentage of metaphase cells remains at an extremely low level ($\approx$ 0.2%) for about 4 hours after the cells are transferred to a Tdr-free medium, thereupon increasing in a biphasic fashion to a peak value of about 18 to 20%, followed by a sharp decline to a negligible value. An

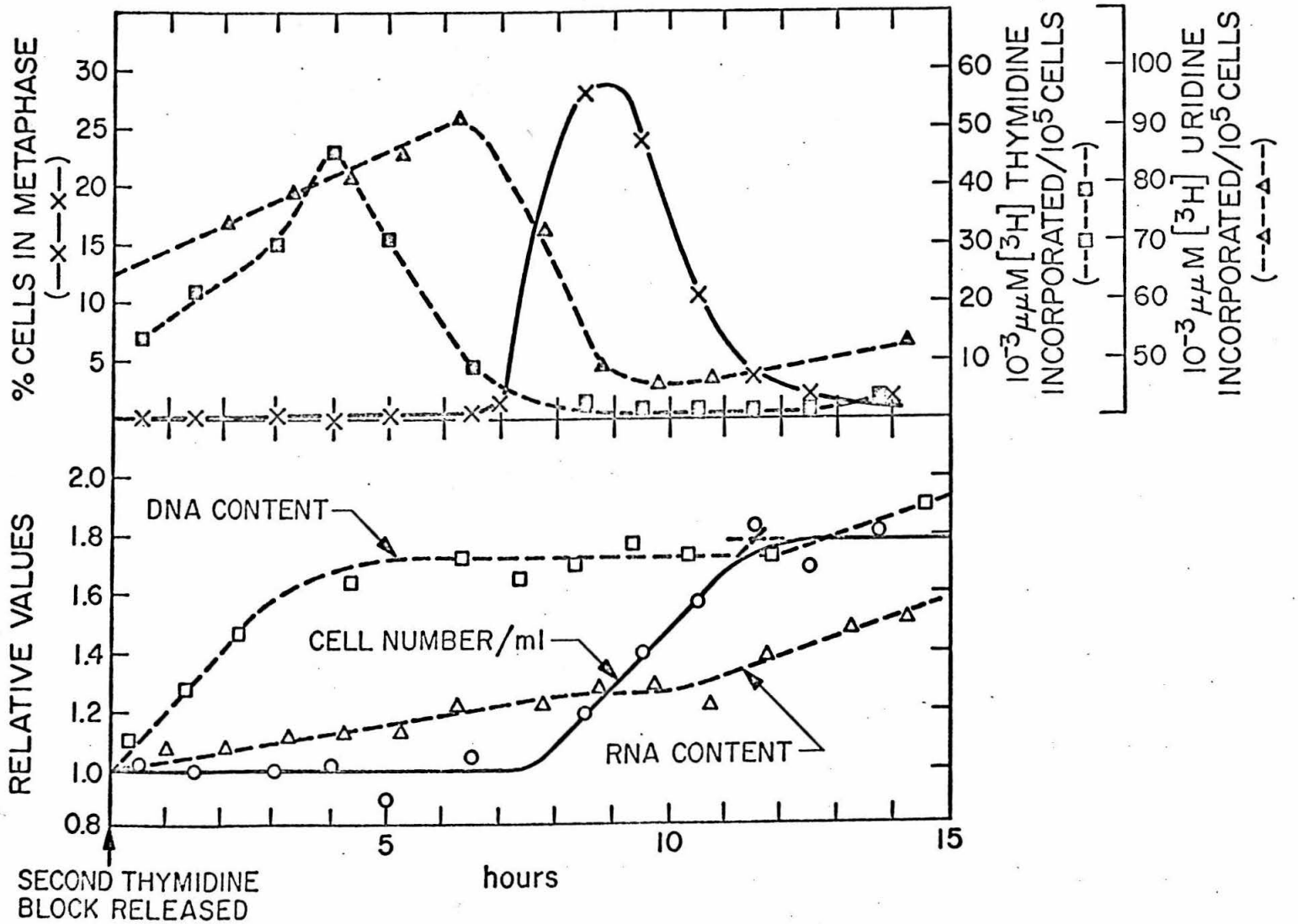
increase in the metaphase index usually precedes rise in cell number, and the burst of mitotic activity, like the period of cell proliferation, lasts about 9 hours.

2. The Second Step (Fig. 6)

Synchrony is improved by adding a second excess Tdr block 8 hours after removal of the first excess Tdr block, as pointed out in Materials and Methods and Fig. 1. At this stage DNA synthetic activity is at its minimum (cf. Fig. 5). Incubation is continued for another 14 hours. The presence of excess Tdr in this period has no detectable effect on the process of RNA synthesis described in the previous section but causes complete inhibition of the slow DNA synthetic activity beginning at 12 to 13 hours (cf. Fig. 5). As expected, cell number, after leveling off, and DNA content remain unchanged during this period whereas RNA content continues to increase. However, due to insufficient and somewhat irreproducible data, it is not presently clear how the mitotic and cell proliferation activities are affected by addition of excess Tdr while these activities are still going on; in some experiments at least the rise in cell number was restricted by the presence of excess Tdr during this period (i.e. between 8 and 15 hours following the termination of the first treatment).

On removal of the second excess Tdr block, DNA content rises rapidly, reaching a plateau in 5 to 6 hours (Fig. 6). Although the data are not sufficiently conclusive, the DNA content, having

Figure 6. Characteristics of a HeLa S3 population synchronized by double treatment with excess thymidine. (—○——○—) cell number/ml; (—X——X—) % cells in metaphase; (-□--□-) DNA content/ml; (-□--□-) 10^{-3} μμM [^{3}H] Tdr incorporated/10^{5} cells; (-△--△-) RNA content/ml; (-▲--▲-) 10^{-3} μμM [^{3}H] uridine incorporated/10^{5} cells.


% CELLS IN METAPHASE
(—x—x—)
30
25
20
15
10
5
10^{-3} μμM [^{3}H] THYMIDINE INCORPORATED/10^5 CELLS
(--□--□--)
60
50
40
30
20
10
10^{-3} μμM [^{3}H] URIDINE INCORPORATED/10^5 CELLS
(--Δ--Δ--)
100
90
80
70
60
50
RELATIVE VALUES
2.0
1.8
1.6
1.4
1.2
1.0
0.8
DNA CONTENT
CELL NUMBER/ml
RNA CONTENT
5
10
15
hours
SECOND THYMIDINE BLOCK RELEASED

remained at the plateau for about 6 hours, has been observed in a few experiments to slowly but steadily rise. The rate of DNA synthesis in the culture, measured by the incorporation of [^{3}H] Tdr, first increases at a relatively low rate, then at a higher rate, reaching a peak in 4 to 5 hours, followed by a tenfold decline within the next 3 hours. DNA synthetic activity remains at a low level for about 5 hours and then begins to rise again.

RNA content continues to rise but the rise is slowed down when mitotic activity begins (i.e. at 7 to 8 hours), accelerating again 3 to 4 hours later. The rate of RNA synthesis in the culture, measured by [^{3}H] uridine incorporation, steadily rises for about 6 to 7 hours after removal of the block and then starts to fall down sharply. When mitotic activity in the culture is at maximum (at 9 to 10 hours), the level of RNA synthetic activity is 30% lower than the initial level at the time of release of the second block (obtained by extrapolating the curve of [^{3}H] uridine incorporation to 0 hours in Fig. 6). The rate of RNA synthesis begins to rise by the time mitotic activity comes to an end.

Cell number per ml shows no change until about 2 hours after the end of the DNA synthesis period, thereafter increasing in a single wave which levels off within 5 hours (cf. the partial overlap of DNA synthesis and cell proliferation as well as the biphasic mode of the latter after release of the first block in Fig. 5). During this period about 80% of the cells complete mitosis. The percentage of metaphase cells also remains low and constant for the whole duration of

DNA synthesis and for an additional 2 hours, and then increases very rapidly, reaching a peak of 28%, followed by a decline. The peak of the metaphase index curve obtained after release of the second block is always higher than the one obtained after release of the first block (cf. Fig. 5), indicating better synchrony as a result of double treatment. Similarly, the double blocking treatment produces a mitotic and cell proliferation activity period of shorter duration than the one produced by the single blocking treatment.

3. Effect of Different Concentrations of Tdr on Synchronization

When synchronization was achieved by employing 3.0 mM or 7.5 mM Tdr, only 55 to 70% of the cells completed mitosis in a single wave after release of the second block. This is in contrast to the 80% which divided when 5.0 mM Tdr was used for synchronization. Similarly, lower peaks of metaphase indices were obtained when concentrations of Tdr higher (7.5 mM) or lower (3.0 mM) than 5.0 mM were used to synchronize cells. Therefore, 5.0 mM was chosen as the optimum concentration of Tdr for synchronization of cells grown in suspension cultures. Although the use of 3.0 mM or 7.5 mM Tdr resulted in lower degrees of synchrony than when 5.0 mM Tdr was used, the duration of the DNA synthesis period and of mitotic and cell proliferation activities after release of the second block were similar and highly reproducible in all cases.

C. THE DEGREE OF SYNCHRONIZATION

The method of Engelberg (1961) has been used for quantitative estimation and comparison of the degrees of synchronization in differently synchronized cultures. This method utilizes a growth parameter R, the normalized rate of cell division, such that $R = (dn/dt)/n$, where n is the number of cells at time t. In a randomly dividing (logarithmic) culture, R will be a constant, R_u, which is independent of time.

An R vs. t plot is constructed by calculating various values of R at different time intervals from the data of a growth curve. A representative calculation for the cell proliferation curve obtained after release of the second excess Tdr block (Fig. 6) is presented in Table 1. R vs. t plots calculated in this way for the cell proliferation curves in Figs. 4a, 5 and 6 are presented in Fig. 7. The height of the horizontal line R_u is equal to $(\ln 2)/T_g$, where T_g is the generation (doubling) time of this culture (see Engelberg, 1961, for details). The area under R which lies above the horizontal line R_u is called the overlying area. The degree (percentage) of synchronization, S, of a culture is defined as follows:

$$S = \frac{\text{Overlying area}}{\text{Total area under R}} \times 100$$

The values of S thus calculated from the R vs. t plots in Fig. 7 for cultures synchronized by different methods in the present study are tabulated in Table 2.

Table 1. Computation of Various Values of R at Different Time Intervals from the Cell Proliferation Data in Fig. 6 (after Engelberg, 1961)

Time Interval (hours)	No. of Cells Which Divided (Δn)	Mean No. of Cells (n)	$R = \frac{1}{n}\frac{\Delta n}{\Delta t}$	Time (hours)
7 to 8	8,500	104,250	0.0815	7.5
8 to 9	19,500	118,250	0.1649	8.5
9 to 10	20,000	138,000	0.1449	9.5
10 to 11	19,000	157,500	0.1206	10.5
11 to 12	10,000	172,000	0.0581	11.5
12 to 13	1,000	177,500	0.0056	12.5

Figure 7. Calculation of the degree of synchronization (S) by the method of Engelberg (1961). R, normalized rate of cell division [$R = (dn/dt)/n$]; R_u, normalized rate of cell division for a logarithmic culture with a doubling time of 22.4 hours [$R_u = (\ln 2)/22.4$]. S (expressed as percentage) is the ratio of the area under R lying above R_u to the total area under R. Area measurements were made with a planimeter. (-□--□--) after release of the first 5-FUdr block, data from Fig. 4a; (-□——□-) after release of the first excess Tdr block, data from Fig. 5; (-○——○-) after release of the second excess Tdr block, data from Fig. 6; (————) R_u.

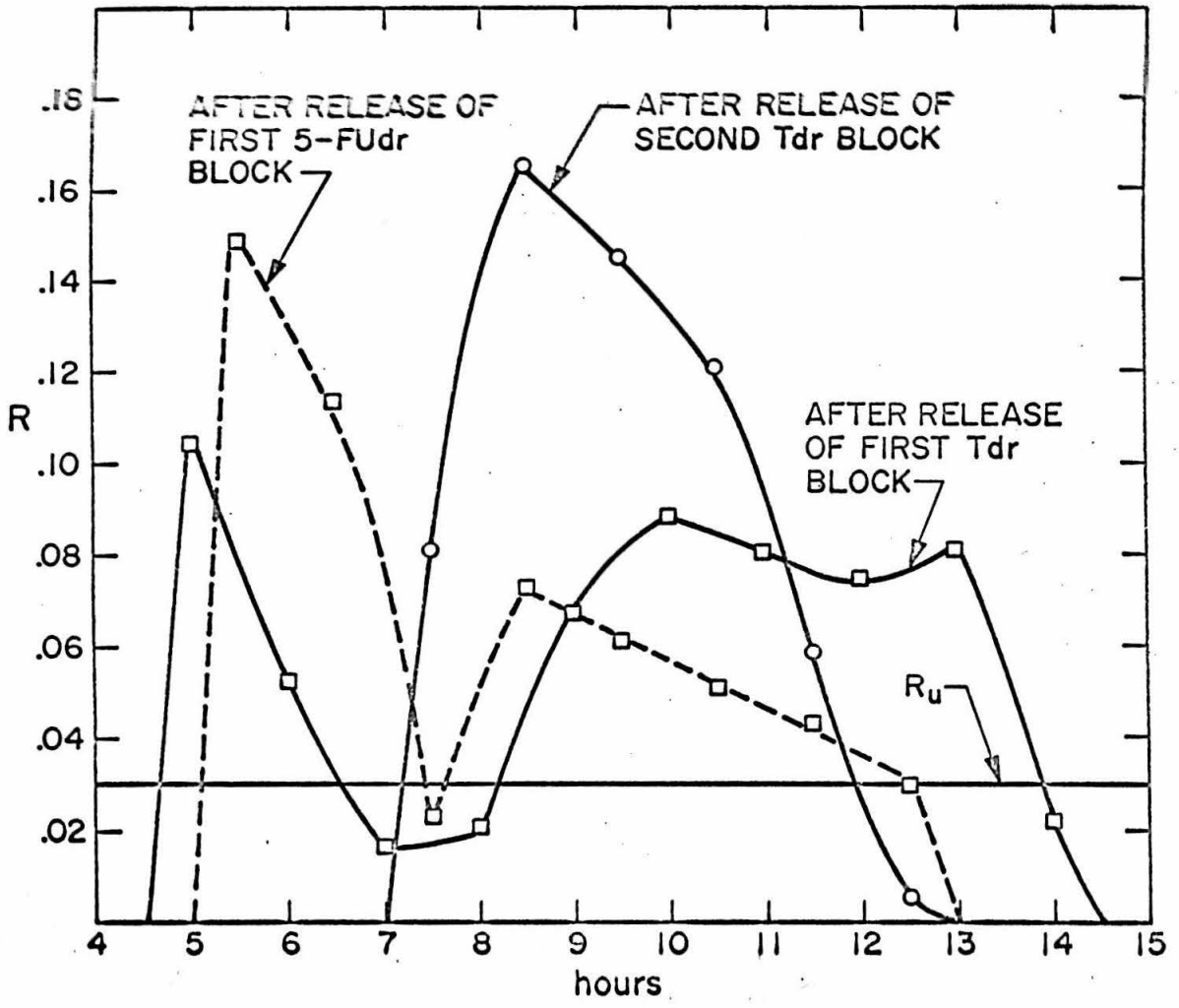

.18
.16
.14
.12
.10
.08
.06
.04
.02
R
AFTER RELEASE OF FIRST 5-FUdr BLOCK
AFTER RELEASE OF SECOND Tdr BLOCK
AFTER RELEASE OF FIRST Tdr BLOCK
Ru
4
5
6
7
8
9
10
11
12
13
14
15
hours

Table 2. Degrees (Percentages) of Synchronization (S) Calculated From the R vs. t Plots in Fig. 7

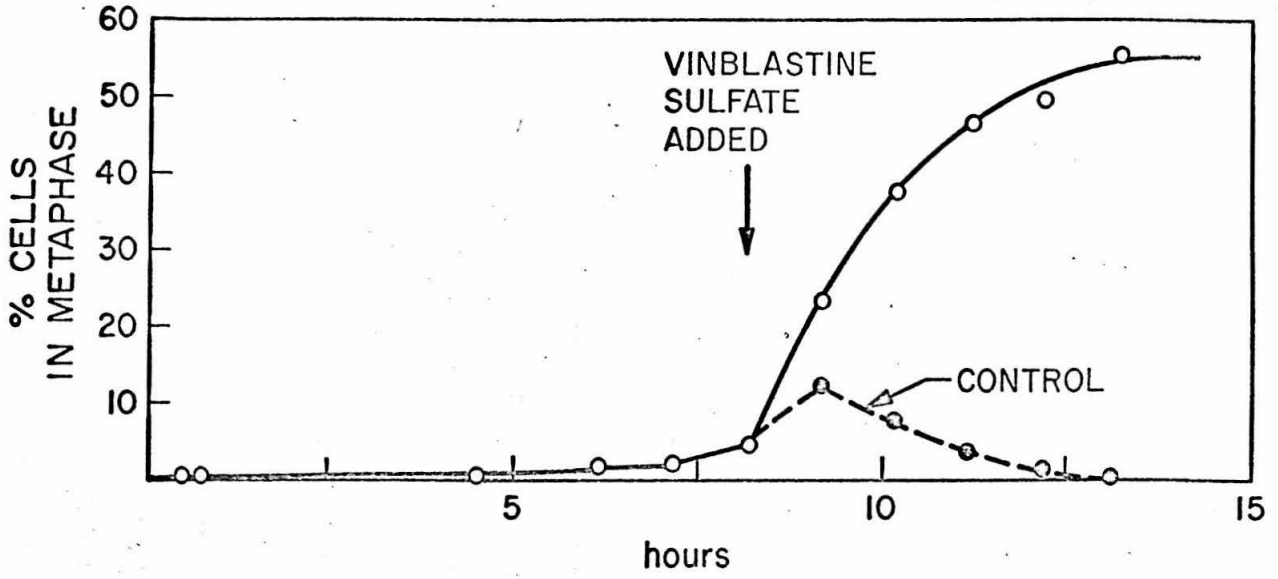
Method of Synchronization	S (%)
Single treatment with 5-FUdr	55.3
Single treatment with 5.0 mM Tdr	53.2
Double treatment with 5.0 mM Tdr	72.2

D. A METHOD FOR ACCUMULATION OF METAPHASE CELLS

Vinblastine sulfate (Eli Lilly and Co.) has been used to arrest cells in metaphase. When a logarithmic phase culture of HeLa cells is incubated with vinblastine sulfate (0.01 μg/ml) for 15 hours, 50 to 80% of the cells are blocked in metaphase (Maio and Schildkraut, 1967; Huberman and Attardi, 1967). However, in this procedure many of the cells are held in metaphase for very long periods. Since the effect of such long periods of metaphase arrests on the cell metabolism is not fully understood, it can be advantageous to devise a method which will allow accumulation of comparable percentages of cells blocked in metaphase in shorter periods. One such method has been worked out and its results are presented below.

A HeLa culture was synchronized by double blocking treatment with excess thymidine (3.0 mM) as outlined in Fig. 2b. The metaphase index of the culture reached a high value about 8 hours after release of the second block. At this stage, vinblastine sulfate was added to a final concentration of 0.01 μg/ml. As shown in Fig. 8, 55% of the cells were found in metaphase within 5 hours. In contrast, the control culture (synchronized but without vinblastine sulfate) had a negligible number of cells in metaphase at this time.

Figure 8. Accumulation of HeLa cells arrested in metaphase by addition of vinblastine sulfate to a synchronized culture. Cells were synchronized by double treatment with excess thymidine (3.0 mM). Vinblastine sulfate was added to half of the culture 8.16 hours after release of the second block and the other half used as a control. (-●--●-) control; (—○——○—) vinblastine sulfate treated.


60
50
40
30
20
10
% CELLS IN METAPHASE
VINBLASTINE SULFATE ADDED
CONTROL
5
10
15
hours

DISCUSSION

It is well established that 5-FUdr blocks the synthesis of thymidine 5'-monophosphate from deoxyuridylate by inhibiting thymidylate synthetase (Cohen et al., 1958; Heidelberger, 1963; Huennekens et al., 1963). If methylation of deoxyuridylate catalyzed by thymidylate synthetase is the major pathway for the biosynthesis of thymidine in HeLa cells, it is expected that addition of 5-FUdr to cultures will produce thymidine deficiency in the cell pool. A deficiency of thymidine in cells is clearly shown by the observation that addition of 5-FUdr instantaneously results in a significantly increased rate of incorporation of exogenously added radioactive thymidine per unit DNA (Fig. 3b). Assuming that inhibition of thymidylate synthetase is the primary effect of 5-FUdr, the above observation indicates that methylation of deoxyuridylate is indeed the major source of thymidine in HeLa cells. Since incorporation of exogenous thymidine into DNA is very high even near the end of 15 hour incubation with 5-FUdr, it can be argued that no other biosynthetic pathway capable of replenishing normal (control) levels of thymidine in cells is induced in spite of extended periods of thymidine deficiency. As already stated, these results show further that the cellular machinery for the biosynthesis of DNA is not adversely affected during 15 hours of incubation with 5-FUdr and that DNA synthesis can begin instantaneously as thymidine is supplied to cells.

The rate of incorporation of [^{3}H] thymidine per unit of DNA steadily increases during the presence of 5-FUdr, being about 3-fold higher at the end of 15 hours of incubation than it is 30 minutes after addition of 5-FUdr (Fig. 3b). This finding suggests two possible interpretations:

a) Depletion of thymidine from the cell pool in the presence of 5-FUdr is slow and gradual, causing a continuous increase in the specific activity of exogenously added labeled thymidine in the cell pool. Consequently, the cells which were in the S phase at the time 5-FUdr was added are capable of incorporating progressively increasing quantities of radioactivity into DNA over a period of 15 hours.

b) Depletion of endogenous thymidine from the cell pool is immediate and rapid so that the cells attain maximal capacity to utilize exogenously supplied labeled thymidine for DNA synthesis within a short time after addition of 5-FUdr. This signifies a constant specific activity of [^{3}H] thymidine in the cell pool throughout the duration of incubation with 5-FUdr. Therefore, the steady increase in the incorporation of labeled thymidine per unit DNA indicates a progressive increase in the number of cells achieving biochemical competence for DNA synthesis as incubation with 5-FUdr continues.

The former of the two interpretations can be easily ruled out since no detectable increase in the DNA content of the culture takes place after addition of 5-FUdr (Fig. 4a), indicating that intracellular thymidine is completely depleted shortly after the inhibitor is added.

Complete lack of any increase in DNA content during the presence of 5-FUdr has also been reported in bacteria (Cohen et al., 1958) and in monolayers of HeLa cells (Rueckert and Mueller, 1960), and another human cervical carcinoma cell line (Eidinoff and Rich, 1959). Therefore, the increasing utilization of labeled thymidine per unit DNA during the progress of incubation with 5-FUdr is indeed a result of a larger number of cells being triggered into DNA synthesis on being supplied with exogenous thymidine. Since about 32% of the cells in a logarithmically growing HeLa culture are in the S phase (Rao and Engelberg, 1966), a 3-fold increase in the utilization of labeled thymidine after 15 hours of incubation with 5-FUdr (Fig. 3b) suggests that about 95% of the cells are either at the beginning of or at some point along the S phase. Using autoradiography involving pulse-labeling with [^{3}H] Tdr, Mueller et al. (1962) in HeLa cells treated with amethopterin and Till et al. (1963) in L cells treated with 5-FUdr have directly demonstrated progressive accumulation of cells just before the S phase as incubation with the inhibitors continues. It can be concluded, therefore, that the cells which had finished DNA synthesis at the time of addition of 5-FUdr are able to complete their remaining cellular life cycle in the presence of 5-FUdr, resulting at the end of incubation in accumulation of a majority of cells having a capacity for DNA synthesis if thymidine is exogenously supplied.

The above results specifically demonstrate that the presence of 5-FUdr for 15 hours does not prevent cells in G_1 from carrying

out various metabolic activities essential for preparation for initiation of DNA synthesis. Similarly, direct evidence showing that cells in G_2 and M are able to complete mitosis normally in the presence of 5-FUdr has been obtained in the present studies. Rao and Engelberg (1966) have estimated that in logarithmically growing suspension cultures of HeLa cells, G_2 and M respectively occupy 16% and 4% of the mean generation time. On the basis of these figures the mean durations of G_2 and M in our HeLa cell line (mean generation time = 22.4 hours) are about 3.6 and 0.9 hours respectively. If the presence of 5-FUdr does not prevent cells in G_2 and M from normal progression through cell division, we expect the addition of 5-FUdr to be followed by an increase in cell number of about 20% in 4.5 hours (the total duration of G_2 + M), no more cell division occurring thereafter. The data presented in Fig.4a are in excellent agreement with the above prediction. Indeed, this result suggests that addition of 5-FUdr to a logarithmic population of cells followed by hourly scoring of cell number can be used as a rapid and reliable method for estimating the length of G_2 (the length of mitosis, easily estimated by determining the mitotic index of a logarithmic population, can be subtracted from the total length of G_2 + M to give the length of G_2). A similar increase in cell number after addition of 5-FUdr to cultures of mammalian cells has not been recorded in many of the previous studies because observations on cell number were not made in the hours immediately following addition of 5-FUdr (cf. Rich et al., 1958; Eidinoff and Rich, 1959; Rueckert

and Mueller, 1960; Littlefield, 1962), whereas in other studies in which such observations were recorded, results analogous to those in Fig. 4a were obtained (cf. Till et al., 1963; Gold and Helleiner, 1964). However, Eidinoff and Rich (1959) using monolayer cultures of a human carcinoma cell line reported complete disappearance of mitotic cells within 3 to 4 hours of addition of 5-FUdr. This result is expected from the reasoning just presented, although these workers themselves did not recognize the above significance of their finding. Once again, scoring of the mitotic index after addition of 5-FUdr is another method for estimating the length of G_2. The precision of this method will be low since the initial percentage of cells in mitosis in a logarithmic culture is rather small to begin with. The accuracy of this method, however, can be improved by addition of a metaphase-arrest agent (e.g. colchicine) along with 5-FUdr followed by scoring of the increasing percentage of metaphase cells with progressively higher precision.

The observation that RNA content continues to increase at a normal rate in the presence of 5-FUdr (Fig. 4a) is in accord with the findings of Cohen et al. (1958) in bacteria, Rueckert and Mueller (1960) in HeLa cells grown in monolayer cultures, and Till et al. (1963) in L cells grown in suspension cultures. Thus the complete cessation of DNA synthesis but continued synthesis of RNA and proteins (cf. Cohen et al., 1958; Rueckert and Mueller, 1960; Till et al., 1963; Erikson and Szybalski, 1963; Gold and Helleiner, 1964) in the presence of 5-FUdr produces in HeLa cells the classic syndrome of

thymidineless unbalanced growth first described in a thymidine-requiring bacterial strain by Cohen and Barner (1954).

Addition of thymidine to cells incubated with 5-FUdr for 15 hours was immediately followed by a single wave of DNA synthesis as expected from our earlier conclusion that this incubation permits accumulation of about 95% of the cells in a state of readiness to enter the S phase (Fig. 4a). We have never observed the two waves of DNA accumulation, one premitotic and the other commencing while cell proliferation is still going on, reported by Rueckert and Mueller (1960) after addition of thymidine to amethopterin-treated HeLa cells. Also in contrast to the 1.7- to 1.8-fold increase in DNA content observed in our work, DNA content increased in the first wave about 1.5-fold only in HeLa cells synchronized by amethopterin by Rueckert and Mueller (1960). In accord with the observations reported in the above-mentioned study, we have also observed that the initial rate of DNA accumulation is lower than the rate which follows (Fig. 4a). Mueller et al. (1962) and Stubblefield and Mueller (1962) have presented evidence suggesting that the acceleration of DNA synthesis from its initial slower rate requires protein synthesis and is correlated with the activation of new sites of DNA synthesis along the chromosomes. This phenomenon, observed in both 5-FUdr- and amethopterin-synchronized HeLa cells, can be utilized for investigating the control mechanisms and localized changes in chromosomal proteins involved in the initiation of DNA synthesis at different loci in chromosomes.

We have successfully demonstrated a period of about 5 hours following reversal of the block in which DNA synthesis takes place unaccompanied by any cell proliferation (Fig. 4a). In contrast, another period of 5 hours exists (beginning 8 hours after addition of thymidine) in which almost no DNA synthesis occurs in the culture, all the cells being either in G_2, M, or G_1. About 15 hours after release of the block, essentially all cells are found in G_1.

Consistent with the theoretical rationale used for synchronization, as discussed in the Introduction (see Fig. 1 for schematic explanation), it is proposed that the biphasic curve of cell proliferation observed in Fig. 4a is due to the presence of two kinds of cell populations at the time of reversal of the DNA block. It is expected that at the end of 15 hour incubation with 5-FUdr, one set of population (about 32% of the cells) will be distributed along the S phase while the remaining cells will accumulate at the beginning of the S phase. The observation that 30% of the cells divide in the first phase of growth confirms the above prediction. This suggests that the height of the increase in cell number in the first phase can be used to estimate the number of cells which were actually blocked in the S phase without losing their viability during the synchronization treatment. Also the length of the interval between the end of the first synchronization treatment and the beginning of cell proliferation is an estimate of the total length of G_2 + M of cells blocked in the S phase for long periods by 5-FUdr (or excess thymidine as shown in Fig. 5). The length of G_2 + M (5 hours) of cells blocked in the S phase is

thus estimated and found to be almost equal to the mean length of G_2 + M (4.5 hours) estimated for unsynchronized HeLa cells on the basis of data provided by Rao and Engelberg (1966). An interesting and significant implication of this finding will be discussed later.

Our attempt to improve the degree of synchronization by the use of a double treatment with 5-FUdr on the basis of the rationale proposed in the Introduction (see Fig. 1) has not given encouraging results. As shown in Fig. 4b, DNA content and cell number increased by only 1.4- to 1.5-fold after reversal of the second block, followed by actual death and disintegration of cells. Rapid loss of viability of mammalian cells after 24 hour incubation with 5-FUdr has already been described by Eidinoff and Rich (1959), Rueckert and Mueller (1960), and Till et al.(1963). The probable causes of decreased viability are a) metabolic conversion of 5-FUdr to fluorouracil and the latter's incorporation into RNA leading to errors in protein synthesis, and b) increased frequency of chromosome breaks in the presence of 5-FUdr as reported by Taylor (1963). These undesirable effects of 5-FUdr can gradually accumulate during incubation with the inhibitor and become particularly significant in cells synchronized by double treatment due to the extended periods of contact with 5-FUdr involved in the process. It may be noticed, however, that in the experiment described in Fig. 4b, cell proliferation did not begin for almost 11 hours after release of the second block, thus allowing a distinct separation between the periods of DNA synthesis and cell proliferation by a well-defined interval (G_2). As

shown in Fig. 4a, such a high quality of synchronization can not be achieved by single treatment with 5-FUdr. Indeed, the original objective of double treatment, namely accumulation of all cells at the beginning of the S phase (with none of the cells within the S phase) was achieved in spite of the complications caused by 5-FUdr. Therefore, it seems worthwhile to try to reduce the extent of the adverse effects of 5-FUdr. It may be possible to alleviate the damage caused by incorporation of fluorouracil into RNA by conducting both the first and second incubations with 5-FUdr in the presence of excess quantities of uridine.

Several workers have presented evidence demonstrating that a phosphorylated derivative of thymidine, if present in high concentrations, blocks the conversion of cytidylic acid to 2'-deoxycytidylic acid by feedback inhibition (Morris and Fischer, 1960; Reichard et al., 1960; Xeros, 1962; Morris and Fischer, 1963; Morris et al., 1963; Galavazi et al., 1966). Consistent with the above action of high concentrations of thymidine, DNA content of HeLa cell cultures has been observed to remain constant during the first (24 hour) as well as the second (14 hour) blocking treatment with excess thymidine (Fig. 5 and unreported results). This suggests that in HeLa cells essentially all of the 2'-deoxycytidylic acid is synthesized by reduction of cytidylic acid. That thymidine does not inhibit the synthesis of any of the ribonucleotides is shown by its lack of effect on RNA accumulation (Fig. 5).

Using human kidney cells cultured in monolayers, Galavazi

and Bootsma (1966) have observed that the mitotic index does not decrease to zero during treatment with excess thymidine but instead declines to a very low constant value. We have confirmed the above finding in our studies (Fig. 5). However, the sudden dip in the mitotic activity following the addition of thymidine reported by the above authors was not observed by us. Most probably, this is because addition of thymidine in our suspension culture system does not involve a medium renewal as was the case with the monolayer system of these authors. Galavazi and Bootsma (1966) interpreted the failure of the mitotic index to reach zero as indicating that DNA synthesis was not stopped completely by excess thymidine but slowed down considerably (they estimated a 10-fold decline in the rate of DNA synthesis). However, this interpretation is difficult to accept in view of our inability to detect any definite increase in DNA content of the cultures after addition of excess thymidine. Nevertheless, more precise data are needed to detect DNA accumulation at rates less than 10% of the controls.

An alternative interpretation of the above behavior of the mitotic index in the presence of excess thymidine may be presented. We wish to propose that excess thymidine, when added to a logarithmically growing population, interferes with the normal progression of cells through mitosis in some unknown manner. In support of this proposal, we cite the following experimental results:

1. As shown in Fig. 5, no increase in cell number has been observed after the addition of excess thymidine (5.0 and 7.5 mM).

This strongly suggests that cells which were in G_2 (16%) and M (4%) at the onset of incubation with thymidine fail to complete (or possibly even to enter) mitosis. An expected 20% increase in cell number, similar to that recorded after addition of 5-FUdr (Fig. 4a), will certainly have been detected in these studies. Data on cell number during incubation with excess thymidine have not been reported in any of the earlier studies so that no comparison with our results can be made.

2. If the progression of mitosis is normal it is expected that 4.5 hours (the length of G_2 + M) after addition of excess thymidine, the metaphase index will drop sharply. No such drop has been observed either in our studies or in those of Galavazi and Bootsma (1966).

3. Using phase-contrast cinematography, Hughes (1952) demonstrated in chick tissue cultures that adenosine and adenylic acid (0.6 to 1.2 mM) inhibit cells from entry into prophase and also cause reversible fragmentation of nucleoli of interphase cells; in the same studies adenine (3.7 mM) was shown to prevent cleavage and inhibit anaphase movement. Agents that inhibit DNA synthesis, such as deoxyadenosine and cytosine arabinoside (Nichols et al., 1964) and 5-FUdr (Taylor, 1963), are generally known to cause chromosome breaks; excess thymidine could conceivably cause similar breaks because of its capacity to inhibit DNA synthesis. Bootsma et al. (1964) have reported scattering of chromosomes in human kidney cells treated with excess thymidine. Evidence suggesting

that excess thymidine prolongs metaphase in HeLa cells has also been presented (Barr, 1963). Prolonged metaphases can slowly reconstruct into nuclei without proceeding to telophase as demonstrated by Hughes (1952) in chick tissue cultures.

The alternative proposals forwarded by Galavazi and Bootsma (1966) and by us can be used only as working hypotheses until more investigations have been carried out on the effects of excess thymidine on metabolic processes other than DNA synthesis. The relevance of this question to the duration of various phases of the cell division cycle in synchronized cells will be discussed later.

The pattern of nucleic acid synthesis and cell proliferation in cultures synchronized by single treatment with excess thymidine is almost superimposable on that of cultures synchronized by single treatment with 5-FUdr (cf. Figs. 4a and 5). The biphasic curve of cell proliferation in Fig. 5 is interpreted in the same way as the similar curve obtained after reversal of the first 5-FUdr block. However, only 20% of the cells divided in the first phase in contrast to the 32% which were theoretically expected to divide. The cause of this discrepancy is yet unknown. The biphasic increase in metaphase index also suggests that excess thymidine indeed blocked one set of population in the S phase and allowed accumulation of the other at the beginning of the S phase.

Double blocking treatment with excess thymidine gave results expected from the theoretically worked out rationale outlined in Fig. 1. The absence of rise in cell number and metaphase index at 5 hours

and the increase in both parameters in a single wave (Fig. 6) clearly shows that at the release of the second block all cells were accumulated just before the S phase; the presence of cells also within the S phase, as is the case at the end of the first block, will certainly have produced a rise in mitotic activity at about 5 hours (the normal length of $G_2 + M$ in unsynchronized cells) or earlier (if the length of $G_2 + M$ was shorter after the synchronization treatments). Puck (1964b) also concluded that 98% of the cells were accumulated at the end of the second blocking treatment within a region no greater than 5% of the total life cycle. Thus double treatment with excess thymidine produces a synchronized culture in which there is no overlap between the periods of DNA synthesis and cell proliferation following release of the second block (Fig. 6). It should be noticed that in Fig. 6 there is almost a 2 hour interval separating completion of DNA synthesis and the beginning of mitotic activity. Cells in this period belong to G_2 and can be utilized for biochemical studies with essentially no contamination with cells from other phases. It may be pointed out that such a well-defined G_2 period has not been explicitly demonstrated in any of the synchronized systems described so far for a variety of mammalian cells utilizing different methods including excess thymidine treatments (cf. Newton and Wildy, 1959; Rueckert and Mueller, 1960; Littlefield, 1962; Terasima and Tolmach, 1963; Petersen and Anderson, 1964; Galavazi et al., 1966; Rao and Engelberg, 1966; Robbins and Scharff, 1966).

It is not yet possible to specify the cause (or causes) for the

failure of DNA content as well as of cell number to increase exactly 2-fold (the increase ranges between 1.7- and 1.9-fold) in cultures synchronized by either single treatment with 5-FUdr or by single and double treatments with excess thymidine. The possibility of some cells escaping synchronization treatment due to some obscure reasons can not be denied. It is more likely, however, that this failure is due to adverse effects of these blocking agents on cell viability or to their interference with normal cellular metabolism. The death of cells after prolonged incubation with 5-FUdr and its possible biochemical causes have already been described and discussed. Similarly, toxic effects of higher concentrations of excess thymidine have been observed in the present studies as well as in those of Galavazi et al. (1966). The question of interference with the progression of mitotic phases by excess thymidine discussed earlier is also relevant in this connection. An additional reason for failure of growth parameters to show a 2-fold increase is the presence of up to 9% dead cells even in logarithmically growing HeLa cell cultures (Puck and Steffen, 1963).

Several workers have demonstrated that RNA synthesis completely stops in cells undergoing mitosis (Taylor, 1960; Prescott and Bender, 1962; Konrad, 1963; Feinendegen and Bond, 1963). In accord with the above observations, a depression in the rate of RNA accumulation has been repeatedly observed at the time mitotic activity begins in cultures synchronized by either 5-FUdr or excess thymidine (Figs. 4a, 5 and 6). This depression is followed by an increase in

the rate of RNA accumulation as the mitotic activity decreases. RNA synthesis in the culture never completely stops since at no time more than a certain percentage of cells go through mitosis; the remaining cells, being in phases either just before or after mitosis, continue to make RNA. In cultures synchronized by double treatment with excess thymidine (Fig. 6), the rate of incorporation of [^{3}H] uridine into RNA has also been measured and it was shown that a decline in the rate parallels the rise in metaphase index. The observation that uridine incorporation by the culture is about 30% lower than the normal level when approximately 30% of the cells are in metaphase is consistent with the view that no RNA is synthesized by metaphase cells.

The degree of synchronization (S), estimated by the method of Engelberg (1961), is quite comparable in cultures synchronized by single treatments with either 5-FUdr or excess thymidine (see Fig. 7 and Table 2), indicating practically equal effectiveness for both chemicals in synchronizing cells. As expected, double treatment with excess thymidine results in a significant increase in the degree of synchronization (Table 2), giving a value (72%) for S which is comparable to the value (73%) reported by Galavazi and Bootsma (1966) for monolayers of human kidney cells synchronized by the same method. In contrast, however, much lower degrees of synchronization (S between 50 and 60%) have been reported by Petersen and Anderson (1964) for Chinese hamster ovary cells synchronized by double treatment with excess thymidine and by

Terasima and Tolmach (1963) for HeLa cells synchronized by selection of mitotic cells from glass-attached cultures.

The degree of synchronization (S), although a very useful quantitative measure, fails to reveal certain other features of synchronized systems. It should be noted that almost perfect synchrony with respect to DNA synthesis has been achieved in all cases of synchronized cultures described in this work. The mean estimated length of the S phase in logarithmically growing HeLa cells is about 7.2 hours, and, as can be seen in Figs. 4a, 5 and 6, DNA synthesis activity (measured by either DNA accumulation or rate of incorporation of labeled thymidine) is essentially completed in 7 to 8 hours. This high degree of synchronization for the S phase, however, rapidly decays by the time cells begin to enter the first mitotic division. A qualitative appreciation of the extent of desynchronization shortly after completion of the S phase can be gained as follows. The duration of mitosis in randomly dividing HeLa cells is of the order of one hour (Puck, 1964a; Rao and Engelberg, 1966), whereas the first cell proliferation activity lasts about 8 to 9 hours in cultures synchronized by single treatment with either 5-FUdr or excess thymidine and for 5 hours in cultures synchronized by double treatment with excess thymidine. The apparently higher degree of desynchronization after release of the first block than after release of the second block is actually due to the greater spread of cells which already exists at the time of termination of the first blocking treatment. If the cells which divide in the second phase only after the first synchronization treat-

ment (i.e. the cells which accumulate at the beginning of the S phase) are taken into account, then it becomes obvious that cell proliferation activities obtained following release of both the first and second blocks last for comparable periods (5 to 6 hours). Therefore, it can be concluded that double block treatment, although providing a higher degree of synchronization than single treatment, does not reduce the tendency of a culture to desynchronize. Similar rapid desynchronization by the time cell proliferation begins has been observed in almost all attempts to synchronize mammalian cells with reversible inhibitors of DNA synthesis. In contrast, when a population is synchronized by selection of cells undergoing mitosis, the mitotic cells complete the first cell division within 1 to 2 hours (Terasima and Tolmach, 1963; Robbins and Scharff, 1966). However, DNA synthesis activity following the first division in the above synchronized system lasts for a very long period (12 to 14 hours); the period of DNA synthesis in cultures synchronized by 5-FUdr or excess thymidine lasts 7 to 8 hours or less. Therefore, different methods of synchronization can be useful for obtaining populations optimally synchronized with respect to different phases of the cellular life cycle. For instance, the selection technique mentioned above provides populations excellently synchronized with respect to M but poorly synchronized for the S phase; the reverse is true for populations synchronized by the use of reversible inhibitors of DNA synthesis.

Two possible causes can be suggested for the rapid desynchro-

nization which occurs by the time cell proliferation begins in systems synchronized by 5-FUdr or excess thymidine.

a) The population of cells accumulated just before the S phase is physiologically very heterogeneous, consisting of cells having been arrested at the juncture of G_1 and S for different lengths of time.

b) Random variability in the lengths of G_1 and G_2 has been demonstrated by several authors (cf. Engelberg, 1964; Siskin and Morasca, 1965).

The above-mentioned variability often can be very large. For instance, DNA synthesis in HeLa cultures, synchronized by selecting mitotic cells, began within 2 hours after completion of the first division (Terasima and Tolmach, 1963; Robbins and Scharff, 1966). This suggests that in some cells of the above population, G_1 is as short as 2 hours in comparison to the mean length of G_1 in HeLa cells of 9 to 10 hours. In this connection, it may be noted in Figs. 5 and 6 that no DNA synthesis occurred for several hours after completion of the first wave and then it resumed at 14 hours. Since the mean combined length of S + G_2 + M in HeLa cells is about 12 hours, the resumption of DNA synthesis 14 hours after release of the block suggests that even in synchronized populations some cells have very short G_1 and/or G_2.

In unsynchronized HeLa cells the mean length of S + G_2 is 10.8 hours. However, as shown in Fig. 6, cells arrive at mitosis only 7 hours after release of the second excess thymidine block.

Similarly, the combined mean length of S + G_2 + 1/2M in unsynchronized cells is about 11.25 hours, whereas the time between release of the first thymidine block and the peak of the metaphase index is just 9.5 hours (Fig. 5). Furthermore, Fig. 6 shows that the peak of the metaphase index after the end of the second block comes even earlier (i.e. at 8.8 hours). Analogous observations have been reported by Galavazi et al. (1966) in human kidney cells and by Rao and Engelberg (1966) in HeLa cells. Galavazi and Bootsma (1966) have estimated the lengths of different phases in cells synchronized by double treatment with excess thymidine. They report that G_1 and G_2 are shorter in synchronized cells than in unsynchronized cells; in contrast, the lengths of S and M remain unaltered. It has been proposed that G_1 and G_2 are shortened in synchronized cells because part of the metabolic activities normally occurring in G_1 and G_2 of randomly dividing cells occur while the cells accumulate and wait just before the S phase (Galavazi and Bootsma, 1966; Rao and Engelberg, 1966). Consequently, the cells synchronized by inhibitors of DNA synthesis are able to complete G_2 and begin mitosis in less time than unsynchronized cells. The above interpretation suggests that G_1 and G_2 of synchronized cells, with part of their metabolic functions already taken care of, are physiologically different from the corresponding phases of unsynchronized cells. This conclusion should be kept in mind while using synchronized populations for biochemical studies.

It has been observed repeatedly that mitotic activity begins

4 to 5 hours after release of the first DNA block (5-FUdr or excess thymidine) and never earlier (Figs. 4a and 5). Since the total length of $G_2 + M$ in HeLa cells is 4.5 hours, this observation suggests that the mean length of G_2 in the cells originally blocked in the S phase is not shortened in spite of long incubations with DNA blocks. Analogous data presented in all of the previous studies with cultures synchronized by either 5-FUdr, amethopterin or excess thymidine support the above conclusion. In light of the conclusion drawn in the preceeding paragraph, it can be argued that while cells accumulated at the end of G_1 (i.e. just before the S phase) are able to carry out part of the metabolic activities normally taking place in G_2, the cells blocked within the S phase are not able to do so. This interpretation is in marked contrast to that of Galavazi and Bootsma (1966) who contend that excess thymidine allows DNA synthesis at a very low level, resulting in accumulation of cells within the S phase (as opposed to just before the S phase) so that some of the activities normally occurring in G_1 and G_2 take place during the extremely elongated S phase. Although further studies are obviously needed, it is tentatively proposed that the metabolic activities of G_2, essential for the preparation for mitosis, can proceed in G_1 but not in the S phase.

REFERENCES

Agrell, I. 1964. In "Synchrony in Cell Division and Growth" (Zeuthen, E., ed.), Interscience Publishers, New York, N.Y., p. 39.

Barr, H. J. 1963. J. Cell and Comp. Phys. 61, 119.

Bootsma, D., Budke, L., and Vos, O. 1964. Exptl. Cell Res. 33, 301.

Burton, K. 1956. Biochem. J. 62, 315.

Cameron, I. L., and Padilla, G. M. (eds.) 1966. "Cell Synchrony" Academic Press, New York.

Chamberlain, C. J. 1935. "Gymnosperms, Structure and Evolution," Univ. of Chicago Press, Chicago, Ill.

Cohen, S. S., and Barner, H. D. 1954. Proc. Natl. Acad. Sci. U.S. 40, 885.

Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R., and Lichtenstein, J. 1958. Proc. Natl. Acad. Sci. 44, 1004.

Dische, Z. 1930. Mikrochemie 8, 4.

Eagle, H. 1955. Science 122, 501.

Eagle, H. 1959. Science 130, 432.

Edwards, J. L., Koch, A. L., Youcis, P., Freese, H. L., Laite, M. B., and Donalson, J. T. 1960. J. Biophys. Biochem. Cytol. 7, 273.

Eidinoff, M. L., and Rich, M. A. 1959. Cancer Res. 19, 521.

Engelberg, J. 1961. Exptl. Cell. Res. 23, 218.

Engelberg, J. 1964. Exptl. Cell Res. 36, 647.

Erickson, R. O. 1964. In "Synchrony in Cell Division and Growth" (Zeuthen, E., ed.), Interscience Publishers, New York, N.Y., p. 11.

Erikson, R. L., and Szybalski, W. 1963. Rad. Res. 18, 200.

Feinendegen, L. E., and Bond, V. P. 1963. Exptl. Cell Res. 30, 393.

Galavazi, G., and Bootsma, D. 1966. Exptl. Cell Res. 41, 438.

Galavazi, G., Schenk, H., and Bootsma, D. 1966. Exptl. Cell Res. 41, 428.

Gey, G. O., Coffman, W. D., and Kubicek, M. T. 1952. Cancer Res. 12, 264.

Gold, M., and Helleiner, C. W. 1964. Biochim. Biophys. Acta 80, 193.

Heidelberger, C. 1963. Exptl. Cell Res. Suppl. 9, 462.

Howard, A., and Pelc, S. R. 1953. In "Symposium on Chromosome Breakage" (suppl. to Heredity 6), Charles C. Thomas, Springfield, Ill.

Huberman, J. A., and Attardi, G. 1966. J. Cell Biol. 31, 95.

Huberman, J. A., and Attardi, G. 1967. J. Mol. Biol. In press.

Huennekens, F. M., Bertino, J. R., Silber, R., and Gabrio, B. W. 1963. Exptl. Cell Res., Suppl. 9, 441.

Hughes, A. 1952. Expt. Cell Res. 3, 108.

Konrad, C. G. 1963. J. Cell Biol. 19, 267.

Lajtha, L. G., Oliver, R., and Ellis, F. 1954. Brit. J. Cancer 8, 367.

Littlefield, J. W. 1962. Exptl. Cell Res. 26, 318.

Maio, J. J., and Schildkraut, C. L. 1967. J. Mol. Biol. 24, 29.

McLimans, W. F., Davis, E. V., Glover, F. L., and Rake, G. W. 1957. J. Immunol. 79, 428.

Miura, T., and Utakoji, T. 1961. Exptl. Cell Res. 23, 452.

Morris, N. R., and Fischer, G. A. 1960. Biochim. Biophys. Acta 42, 183.

Morris, N. R., and Fischer, G. A. 1963. Biochim. Biophys. Acta 68, 84.

Morris, N. R., Reichard, P., and Fischer, G. A. 1963. Biochim. Biophys. Acta 68, 93.

Mueller, G. C. 1963. Exptl. Cell Res. Suppl. 9, 144.

Mueller, G. C., Kajiwara, K., Stubblefield, E., and Rueckert, R. R. 1962. Cancer Res. 22, 1084.

Newton, A. A., and Wildy, P. 1959. Exptl. Cell Res. 16, 624.

Nichols, W. W., Levan, A., and Kihlman, B. A. 1964. In "Cytogenetics of Cells in Culture" (Harris, R. J. C., ed.), Academic Press, New York, N.Y., p. 255.

Painter, R. B., and Drew, R. M. 1959. Lab Invest. 8, 278.

Petersen, D. F., and Anderson, E. C. 1964. Nature 203, 642.

Prescott, D. M., and Bender, M. A. 1962. Exptl. Cell Res. 26, 260.

Puck, T. T. 1964a. Cold Spring Harbor Symp. Quant. Biol. 29, 167.

Puck, T. T. 1964b. Science 144, 565.

Puck, T. T., and Fisher, H. W. 1956. J. Exptl. Med. 104, 427.

Puck, T. T., and Steffen, J. 1963. Biophysic. J. 3, 379.

Rao, P. N., and Engelberg, J. 1966. In "Cell Synchrony" (Cameron, I. L., and Padilla, G. M., eds.), Academic Press, New York, N.Y., p. 332 .

Reichard, P., Canellakis, Z. N., and Canellakis, E. S. 1960. Biochim. Biophys. Acta 41, 558.

Rich, M. A., Bolaffi, J. L., Knoll, J. E., Cheong, L., and Eidinoff, M. L. 1958. Cancer Res. 18, 730.

Robbins, E., and Marcus, P. I. 1964. Science 144, 1152.

Robbins, E., and Scharff, M. 1966. In "Cell Synchrony" (Cameron, I. L., and Padilla, G. M., eds.), Academic Press, New York, N.Y., p. 353.

Rueckert, R. R., and Mueller, G. C. 1960. Cancer Res. 20, 1584.

Salb, J. M., and Marcus, P. I. 1965. Proc. Natl. Acad. Sci. U.S. 54, 1353.

Scherer, W. F., Syverton, J. T., and Gey, G. O. 1953. J. Exptl. Med. 97, 695.

Schindler, R. 1963. Biochem. Pharmacol. 12, 533.

Schmidt, G., and Thannhauser, S. J. 1945. J. Biol. Chem. 161, 83.

Schneider, W. C. 1957. In "Methods in Enzymology" (Colowick, S. P., and Kaplan, N. O., eds.), Academic Press, New York, N.Y., vol. 3, p. 680.

Sinclair, W. K., and Morton, A. A. 1963. Nature 199, 1158.

Siskin, J. E., and Morasca, L. 1965. J. Cell Biol. 25 (part 2), 179.

Stubblefield, E., and Mueller, G. C. 1962. Cancer Res. 22, 1091.

Taylor, J. H. 1960. Ann. N. Y. Acad. Sci. 90, 409.

Taylor, J. H. 1963. Exptl. Cell Res. Suppl. 9, 99.

Terasima, T., and Tolmach, L. J. 1963. Exptl. Cell Res. 30, 344.

Till, J. E., Whitmore, G. F., and Gulyas, S. 1963. Biochim. Biophys. Acta 72, 277.

Vos, O., Schenk, H. A. E. M., and Bootsma, D. 1967. Int. J. Rad. Biol. 12, 89.

Whitmore, G. F., and Gulyas, S. 1966. Science 151, 691.

Xeros, N. 1962. Nature 194, 682.

Zeuthen, E. (ed.) 1964. "Synchrony in Cell Division and Growth," Interscience Publishers, New York, N.Y.

PART II

STUDIES ON CHROMOSOMAL PROTEINS OF HeLa CELLS DURING THE CELL DIVISION CYCLE

CHAPTER 1

THE CHROMOSOMAL PROTEINS OF INTERPHASE AND METAPHASE CELLS

CHAPTER 2

THE RELATIONSHIP BETWEEN HISTONE AND DNA SYNTHESIS

CHAPTER 1. THE CHROMOSOMAL PROTEINS OF INTERPHASE AND METAPHASE CELLS

INTRODUCTION

The entry of a cell into mitosis is characterized by dramatic changes in chromosomal configurations. In this process, chromosomes undergo extensive coiling and become condensed bodies of distinct shapes. These structural changes are accompanied by a complete inhibition of RNA synthesis during metaphase (Taylor, 1960; Prescott and Bender, 1962; Konrad, 1963; Feinendegen and Bond, 1963; also data presented in Part I of this thesis, Fig. 6). The understanding of the factors which cause the transformation of extended and metabolically active interphase chromosomes to condensed and metabolically inactive metaphase chromosomes is one of the most challenging problems of modern cell biology.

Intuitively, it is expected that the characteristics of metaphase chromosomes are directly determined by the properties of the proteins associated with DNA. On the basis of the knowledge gained from the study of interphase chromosomes, three broad types of chromosomal proteins may be recognized in higher organisms. These are a) histones, b) acid-soluble non-histone proteins, and c) acid-insoluble non-histone proteins. Of these, histones have attracted the greatest attention in the recent past, mainly because of their highly basic character which in turn enables them to bind to DNA. Several workers have presented a wide variety of evidence

demonstrating direct involvement of histones in determining the structure of nucleoproteins (Zubay and Doty, 1959; Wilkins et al., 1959; Peacocke and Preston, 1961; Zubay, 1962; Giannoni and Peacocke, 1963; Izawa et al., 1963; Littau et al., 1965; Ohba, 1966; Tuan, 1967). At the same time, histones have been shown to repress DNA-dependent RNA synthesis (Huang and Bonner, 1962; Bonner and Huang, 1963; Allfrey et al., 1963; Barr and Butler, 1963; Hindley, 1963; Huang et al., 1964; Marushige and Bonner, 1966; Holoubek, 1966). These findings suggest the possibility that some kind of alteration in histones may be responsible for the condensed configuration of metaphase chromosomes as well as for the latter's inability to synthesize RNA. However, little effort has been made to date to study the characteristics of other chromosomal proteins. The possible involvement of acid-soluble and acid-insoluble non-histone proteins in the chromosomal structure and function remains by and large an unexplored area.

The present studies were initiated to gain some insight into the nature of histones and acid-soluble non-histone proteins associated with interphase and metaphase chromosomes. Through such investigations it was hoped that some understanding of the factors responsible for the change from interphase to metaphase chromosomes might be reached. HeLa S3 cells grown in suspension culture were chosen for these studies because they provide an excellent source of undifferentiated interphase cells and, at the same time, mass quantities of these can be accumulated in meta-

phase by the use of suitable metaphase blocks.

Before attempting to study the acid-soluble proteins of metaphase chromosomes, it was considered important that the histones of interphase chromosomes be characterized. Therefore, this Chapter will first present the data obtained on the chemistry of HeLa interphase histones along with several observations of general significance to histone chemistry. In this connection, it is appropriate to stress one point often ignored in such studies. All the work to be reported in this Chapter on interphase histones or chromosomes refers to preparations isolated from logarithmically growing cell populations. Consequently, the so-called 'interphase' histones are in fact mixtures of histones associated with chromosomes of cells in four different stages of cell division, namely, G_1, S, G_2 and M which respectively comprise 48%, 32%, 16% and 4% of total cell population (cf. Part I). Almost nothing is known regarding the differences, if any, among the chromosomal proteins of cells in these different stages. Nevertheless, the consideration of histones obtained from such a population as interphase histones is valid for the purposes of the present studies. This is because 96% of a logarithmic HeLa cell population possesses chromosomes in the extended and metabolically active form and therefore its histones and other proteins can be meaningfully compared with those isolated from metaphase cells containing condensed and metabolically inactive chromosomes.

The present investigation of the properties of acid-soluble

proteins associated with interphase and metaphase chromosomes has not enabled us so far to precisely answer the original question regarding the factors responsible for the structure and function of metaphase chromosomes but it has allowed us to rule out what are most probably not among these factors. In addition, many interesting differences have been observed among the proteins of interphase and metaphase chromosomes which make further work along these lines a worthwhile enterprise.

MATERIALS AND METHODS

A. CULTIVATION AND HARVEST OF CELLS

HeLa S3 cells were grown in suspension culture at 37±1°C in a modified Eagle's medium (Eagle, 1955, 1959) supplemented with 5% dialyzed calf serum. The methods involved in the preparation of medium and serum and in the culture of cells have been described in detail in Part I of this thesis.

The cells were harvested from a logarithmically growing suspension culture (cell concentration between 0.7 to 2.5×10^5 cells/ml) by centrifugation at 500xg for 5 minutes in an International PR-2 centrifuge (International Equipment Company, Needham Hts., Mass., U.S.A.). The harvested cell pellet was washed twice with cold isotonic salt solution (0.137 M NaCl, 5.0×10^{-3} M KCl, 7.0×10^{-4} M Na_2HPO_4, 0.025 M Tris-HCl, pH 7.4-7.5) and either frozen at -80°C for later use or used immediately for fractionation of cell constituents. If frozen, the cell pellet was quickly thawed at 37°C and washed once with isontonic salt solution just before use.

B. PREPARATION OF NUCLEI

All operations were carried out at 0 to 4°C. The washed cell pellet, consisting of 0.4 to 1.0×10^9 cells, was suspended in 80 ml of hypotonic buffer containing divalent cations (5×10^{-3} M Tris-HCl, 3×10^{-3} M $MgCl_2$, pH 7.4). The suspension was allowed to stand for 5 minutes and gently stirred intermittantly. All cells slowly swelled in this medium with some occurrence of lysis. The

suspension was then homogenized in a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle. This operation ruptured cell membranes releasing clean and unbroken neclei. Homogenization was continued until about 95% of the cells were broken (usually 20 to 25 strokes). The progress of homogenization was checked with a microscope. The homogenate was centrifuged in an International PR-2 centrifuge (head No. 284) at 500xg for 10 minutes. The supernatant was carefully discarded without disturbing the loosely packed crude nuclear pellet which was suspended in 60 ml of the Tris-Mg hypotonic buffer and homogenized (10 strokes) as above. At this stage the nuclei in the suspension were almost free of microscopically visible cytoplasmic debris. The suspension was mixed with 60 ml of 1.8 M sucrose dissolved in Tris-Mg buffer and centrifuged at 8000xg for 15 minutes in a Servall centrifuge (rotor type SS-34). The resulting nuclear pellet was resuspended by homogenization in 60 ml of Tris-Mg buffer containing 0.9 M sucrose.

The clean nuclei collected by centrifugation of the above suspension at 8000xg for 15 minutes were again suspended in 20 ml of Tris-Mg buffer with brief homogenization to break any clumps. Ten ml portions of this nuclear suspension were separately layered in two Spinco SW-25 celluose nitrate tubes over 15 ml of 2.2 M sucrose in 0.01 M Tris-HCl, 3×10^{-3} M $MgCl_2$ (pH 7.4). Two-thirds of the contents in each tube were gently stirred to form a rough sucrose gradient and the tubes then centrifuged at 18,000 rpm

for 1 hour in a Spinco SW-25 rotor. The supernatant consisting of cytoplasmic materials was discarded and the purified nuclear pellet washed once with 0.01 M Tris-HCl (pH 7.4) containing 3×10^{-3} M $MgCl_2$. In some experiments these nuclei were further extracted twice with 20 ml of 0.15 M NaCl, 3×10^{-3} M $MgCl_2$, 0.01 M Tris-HCl (pH 7.4). Approximately 75% of the DNA present in the original cell pellet was recovered in a purified nuclear preparation. Nuclei thus purified were either directly utilized for histone extraction or subjected to further fractionation for isolation of chromatin material.

C. PREPARATION OF INTERPHASE CHROMOSOMES

Two separate methods, described below, were employed for isolation of interphase chromosomes (also referred to as chromatin) from logarithmically growing HeLa cells. The Magnesium Precipitation Method (Method A), adapted from Kabat (1967), involved as an intermediary step the preparation of purified nuclei which were then ruptured to release chromatin. The Sucrose Sedimentation Method (Method B), on the other hand, is analogous to the procedures used for preparation of chromatin from pea bud (Huang and Bonner, 1965) and rat liver (Marushige and Bonner, 1966). In this method the cells and nuclei were directly ruptured without purification of nuclei and the chromatin thus released in the homogenate was successively purified by centrifugation. All operations in both of the methods were carried out at 0 to 4°C.

1. Magnesium Precipitation Method (Method A)

The nuclei purified according to the procedure described in the previous section were suspended in 20 ml of SSC/40 (a forty-fold dilution of 0.15 M NaCl, 0.015 M sodium citrate) by homogenization (25 strokes) in Potter-Elvehjem homogenizer with a motor-driven Teflon pestle. The nuclear suspension was then sheared in a Virtis homogenizer at 30 V for 90 seconds, rupturing all nuclei and releasing the chromatin. The homogenate was centrifuged at 10,000xg for 15 minutes; the supernatant, containing 70 to 80% of the nuclear DNA, was carefully removed and adjusted to 0.0024 M magnesium acetate. The precipitated sheared chromatin was allowed to settle for 30 minutes and then sedimented by centrifugation at 10,000xg for 15 minutes. The chromatin pellet was finally purified by extraction with 20 ml of 0.15 M NaCl, 0.0024 M magnesium acetate, 0.01 M Tris-HCl, pH 8.0.

2. Sucrose Sedimentation Method (Method B)

The washed cell pellet, consisting of 0.4 to 1.0×10^9 cells, was suspended in 80 ml of deionized water and allowed to stand for 10 minutes. The suspension of swollen cells was centrifuged at 700xg for 10 minutes and the supernatant discarded. The resulting pellet was suspended in 40 ml of deionized water and exhaustively homogenized (30 to 35 strokes) in a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle. At the end of homogenization, approximately half of the nuclei were ruptured with their chromatin released in the homogenate whereas the other half of

the nuclei were still intact but without cell membranes. The homogenate (40 ml) was mixed with 20 ml of 3-fold concentrated saline-EDTA buffer (saline-EDTA buffer consisted of 0.03 M Tris-HCL, 0.025 M NaCl, 0.008 M disodium ethylene diamine tetraacetate, pH 7.6) and 60 ml of 2.2 M sucrose in saline-EDTA buffer. This was centrifuged at 10,000xg for 30 minutes and the pellet resuspended in 40 ml of saline-EDTA buffer by homogenization (25 strokes). About 80 to 85% of the nuclei were broken at this point. To the homogenate were added 40 ml of 2.2 M sucrose in saline-EDTA buffer and the mixture centrifuged at 10,000xg for 30 minutes. The pellet was suspended in 15 ml of 0.01 M Tris-HCl (pH 8.0) by homogenization (25 strokes) and then sheared in a Virtis homogenizer at 10 V for 10-15 seconds to rupture the remaining intact nuclei. Microscopic examination revealed that all chromatin was free of nuclear membranes at this stage. Five ml portions of this homogenate were layered on 25 ml of 1.7 M sucrose in 0.03 M Tris-HCl (pH 8.0) in three separate tubes. The upper two-thirds of the contents in each tube were gently mixed to form a rough sucrose gradient. The tubes were then centrifuged at 22,000 rpm for 4 hours in a Spinco SW-25 rotor. The pellet thus obtained consisted of purified unsheared chromatin.

For preparation of sheared chromatin, the above pellet was resuspended in 15 ml of 0.01 M Tris-HCl (pH 8.0) and dialyzed against 2-3 changes of 100 volumes of the same buffer. The dialyzed

suspension of chromatin was sheared in a Virtis homogenizer at 25 V for 90 seconds, stirred for 30 minutes, and centrifuged at 10,000xg for 30 minutes. About 60-65% of the DNA in the unsheared chromatin was recovered in the supernatant as sheared chromatin.

D. PREPARATION OF METAPHASE CHROMOSOMES

As stated in Part I of this thesis, only about 2.5% of the cells in a logarithmically growing HeLa population are in metaphase at any given moment. Therefore, it is necessary to increase the fraction of a population in metaphase if isolation of mass quantities of metaphase chromosomes is desired. This was accomplished in the present studies by arresting cells in metaphase by incubating logarithmic phase cultures with either colchicine (at a final concentration of 1×10^{-5} M) or vinblastine sulfate (at a final concentration of 0.01 μg/ml) for 15 hours. Colchicine treatment allowed accumulation of 30 to 40% of the cells in metaphase by the end of incubation. Higher percentages (50 to 70%) of cells were found in metaphase when vinblastine sulfate was employed. The cells were harvested from these cultures and washed in isotonic salt solution as described earlier. Purification of metaphase chromosomes was carried out either at acid or neutral pH according to the methods given below. All operations in both methods were carried out between 0 and 4°C.

1. Preparation at Acid pH

This method, devised by Huberman and Attardi (1966), was

used in initial experiments only. Chromosomes were prepared and supplied by Dr. Joel A. Huberman. An outline of the method is presented below:

The cell pellet was suspended in 15 volumes of 0.1 M sucrose, 7×10^{-4} M $CaCl_2$, 3×10^{-4} M $MgCl_2$ and cells were allowed to swell for 5 minutes. The pH of the cell suspension was adjusted to 3.0 by addition of three volumes of 0.1 M sucrose, 7×10^{-4} M $CaCl_2$, 3×10^{-4} M $MgCl_2$, 3.3×10^{-3} M HCl. The cells were then homogenized in a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle until all interphase cells were ruptured. The homogenate, consisting of released chromosomes, intact nuclei and cytoplasmic debris, was centrifuged at 900xg in an International PR-2 centrifuge (head No. 269) for 30 minutes and each ml of pellet resuspended by homogenization in about 40 ml of HCM (1×10^{-3} M HCl, 7×10^{-4} M $CaCl_2$, 3×10^{-4} M $MgCl_2$). Twenty ml of this suspension were layered over 200 ml of a 0.1 to 0.8 M linear sucrose gradient in HCM (pH 3.0) in a 250 ml glass centrifuge bottle. The gradient was centrifuged in an International PR-2 centrifuge (head No. 284) at 450xg for 20 minutes. Acceleration and deceleration were performed slowly at a rate of 500 rpm per minute. Twenty ml of the gradient from the top, containing cytoplasmic debris, were discarded. All the rest of the gradient, except the last 10 ml at the bottom containing nuclei, were removed, thoroughly mixed and recentrifuged at 850xg in an International PR-2 centrifuge for 90 minutes. The resulting pellet, consisting mainly

of metaphase chromosomes, was resuspended in HCM (15-20 ml) by homogenization and layered over 10 ml of 2.2 M sucrose in HCM in a Spinco SW-25 plastic tube. The upper three-fourths of the tube were stirred to form a rough gradient and the purified chromosomes sedimented in a pellet by centrifugation at 20,000 rpm for 1 hour.

2. Preparation at Neutral pH

Purification of metaphase chromosomes at neutral pH was conducted according to the method of Maio and Schildkraut (1967).

The cell pellet consisting of 1 to 2×10^9 cells, was suspended in 13 volumes of TM ($CaCl_2$, $MgCl_2$ and $ZnCl_2$, each at 1×10^{-3} M in 0.02 M Tris-HCl, pH 7.0). Twenty minutes later, saponin (5% solution previously filtered through 2 layers of Whatman No. 1 paper) was added to this suspension of swollen cells to a final concentration of 0.05% and allowed to stand for 5 minutes. Forty ml portions of the suspension were homogenized (about 35-40 strokes) in a Dounce homogenizer (equipped with a small-clearance pestle) rupturing almost all cells and releasing both chromosomes and intact nuclei. A phase contrast microscope was employed to follow the effectiveness of the various operations.

Two volumes of TMS (TM containing 0.05% saponin) were added to the homogenate and 10 to 12 ml portions distributed in glass centrifuge tubes (25 × 117 mm). The tubes were centrifuged for 5 minutes at 120xg (700 rpm in the International PR-2 centrifuge, head No. 269). The supernatant, containing chromosomes and fine cellular debris, was carefully decanted from each tube and pooled

together. The pellet, consisting of nuclei and unruptured cells, was resuspended in 10 to 12 ml of TMS in each tube and entrapped chromosomes were then separated by repeating centrifugation at 120xg for 5 minutes. All supernatant fractions containing chromosomes were combined and recentrifuged, as described above at 120xg to remove contaminating nuclei. The pooled chromosomal suspension was then centrifuged at 2500xg for 10 minutes (Servall centrifuge, SS-34 rotor) and the pellets resuspended in 0.02 M Tris-HCl (pH 7.0) containing 0.1% saponin. This was again followed by recentrifugation at 2500xg for 10 minutes. The pellets of chromosomes were then suspended by homogenization in 54 ml of 2.2 M sucrose (sp. gr. 1.28) in 0.02 M Tris-HCl (pH 7.0) containing 0.1% saponin and 18 ml portions layered over 10 ml of 2.2 M sucrose solution in three separate Spinco SW-25 plastic tubes. The interfaces were stirred to form a rough gradient without disturbing 1 cm high layer of sucrose solution at the bottom of each tube. The tubes were centrifuged at 50,000xg for 1 hour resulting in pellets of pure chromosomes which were washed once in TM.

E. CHEMICAL ANALYSIS OF NUCLEOPROTEINS

Quantitative analyses of nucleic acids (DNA and RNA) and proteins (histones and non-histones) were carried out on separate aliquots of nucleoprotein materials (unsheared and sheared chromatin, metaphase chromosomes) suspended in 0.01 M Tris-HCl, pH 8.0. All the operations described below were performed at

0-4°C unless specified otherwise.

For determination of nucleic acid content, the general method of Schmidt and Thannhauser (1945) was followed. An aliquot (2 to 4 ml) of nucleoprotein suspension containing about 200 to 300 μg of DNA was adjusted to 10% trichloroacetic acid (TCA) and allowed to stand in ice for about 4 hours. The precipitated nucleoprotein material was collected by centrifugation in a Clinical centrifuge, and successively washed once with 10% TCA, twice with 5% perchloric acid (PCA) and finally once with ether:ethanol (1:1), each washing step involving resuspension followed by centrifugation. The washed pellet was air-dried at room temperature, dissolved in 1 ml of 0.3 N KOH and RNA hydrolyzed by incubation at 37°C for 18 hours. Precooled PCA (70%) was then added to a final concentration of 5% and the precipitate of DNA, proteins and $KClO_4$ allowed to stand in ice for about 1/2 hour. The samples were centrifuged in a Clinical centrifuge and the supernatant saved. The precipitate (pellet) was washed once with a small volume of 5% PCA and the wash combined with the supernatant above. RNA in the pooled RNA hydrolysate fraction was assayed by the orcinol method (Schneider, 1957) using commercially available yeast RNA as a standard.

DNA in the precipitate was hydrolyzed by boiling it in 5% PCA at 100°C for 15 minutes and the supernatant containing hydrolyzed DNA removed after centrifugation. The remaining pellet was washed once with a small volume of 5% PCA and the wash pooled with the

above supernatant. DNA in the hydrolysate was determined by the diphenylamine method either according to Dische (1930) or Burton (1956) using commercially available calf thymus DNA as a standard.

For quantitative determination of proteins, an aliquot (2 to 4 ml) of nucleoprotein suspension containing 400 to 500 μg of DNA was mixed with ice-cold 2N H_2SO_4 to a final concentration of 0.4 N H_2SO_4 [1]. The acidified suspension was allowed to stand in ice for 30 minutes with occasional stirring and then centrifuged at 10,000xg for 30 minutes. The supernatant containing histones was quantitatively removed with a Pasteur pipette and saved. The pellet was extracted once more with 0.4 N H_2SO_4 (one-half of the volume of the first extract) and the two supernatant fractions combined. The final pellet consisted of non-histone proteins and nucleic acids. The pooled acid extract containing histones was adjusted to 20% TCA, allowed to stand in ice for 30 minutes, and the histones collected by centrifugation. The pellets of both histones and non-histone proteins thus obtained were successively washed once with 20% TCA and once with ether:ethanol (1:1), air-dried at room temperature, and then dissolved in 0.5 to 1.0 ml of 1 N NaOH. The quantity of protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard (the linear portions of the standard curves of bovine serum albumin and calf thymus histones yielded identical $O.D._{750}$/μg protein/ml values).

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[1] In some experiments, histones were extracted with 0.2 N H_2SO_4 or 0.2 N HCl as specified later in the Results.

F. PREPARATION OF HISTONES

Histones were extracted from either purified nuclei, purified unsheared chromatin or metaphase chromosomes. For extraction of histones, one of the above-mentioned preparations was suspended in cold 0.01 M Tris-HCl, pH 8.0, (and sometimes dialyzed also) and then diluted to a concentration of 300 to 500 μg DNA/ml with the Tris buffer. All subsequent operations were carried out between 0 and 4°C unless mentioned otherwise.

The suspension was transferred to a Potter-Elvehjem glass homogenizer and slowly mixed with one-fourth volume of 2N H_2SO_4 to give a final concentration of 0.4 N H_2SO_4 while homogenization with a motor-driven Teflon pestle was continued. The acidified homogenate was stirred on ice for 30 minutes and then centrifuged at 15,000xg for 30 minutes. The supernatant was carefully removed and saved. The pellet was once more extracted with 0.4 N H_2SO_4 (one-fourth to one-fifth volume of the first extract) by homogenization followed by centrifugation as described above. The supernatants from the first and second extractions were pooled, mixed with 5-6 volumes of ice-cold absolute ethanol and allowed to stand at -20°C for at least 24 hours. Histone sulfate precipitate slowly appeared at -20°C and was sedimented by centrifugation at 10,000xg for 15 minutes. The precipitate was washed three times with absolute ethanol, and once with ether, vacuum-dried in a desiccator and stored at 2-4°C.

In some experiments, both extractions of histones were

made with either 0.2 N H_2SO_4 or 0.2 N HCl instead of 0.4 N H_2SO_4; the significance of the use of different acids will become clear from the data to be presented later. Since histone chlorides are not completely precipitable from acid extracts by ethanol (author's own experience; see also Fambrough, 1968), hydrochloric acid extract of histones was dialyzed against 5-6 changes of 100 volumes of 0.1 M acetio acid. The dialyzed preparation was lyophilized to dryness and the histone chlorides stored at 2-4°C.

G. PREPARATION OF NON-HISTONE PROTEINS

Non-histone proteins associated with interphase chromatin were obtained by selective removal of histones with acid according to a procedure devised by Dr. Keiji Marushige (personal communication). Purified unsheared or sheared chromatin was thrice extracted with 0.4 N H_2SO_4 as described above. The final pellet, consisting of non-histone proteins and nucleic acids, was suspended in 3-5 ml of 0.5 M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate. This suspension was incubated at 37°C for 12 hours, then cooled in ice and centrifuged at 10,000xg for 30 minutes in cold. All subsequent operations were performed at 0-4°C. The supernatent was removed and saved. The pellet, containing the 'insoluble' fraction of non-histone proteins, was extracted twice with 0.05 M Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate, and all of the supernatant fractions were pooled together to form the 'soluble' fraction. The pooled extract was adjusted to 20% TCA, allowed to stand for

2 hours, and the proteins of the 'soluble' fraction were collected by centrifugation at 10,000xg for 30 minutes. The pellets of both the 'soluble' and 'insoluble' non-histone proteins were washed once with 20% TCA, twice with ether:ethanol (1:1) and then air-dried at room temperature.

H. COLUMN CHROMATOGRAPHY OF HISTONES

Histones were fractionated into their individual classes by chromatography on a weak cation exchange resin, Amberlite CG-50, using a non-linear gradient of guanidinium chloride at pH 6.8 (Luck et al., 1958; Satake et al., 1960; Rasmussen et al., 1962). Separation of histones is based on differences in size and charges of various histone species. This procedure has been recently described in detail by Bonner et al. (1967a); however, the various operations involved are again outlined below along with modifications made during the course of present investigations.

1. Preparation of Resin and Column

An aqueous slurry of Amberlite CG-50 (200-400 mesh, chromatographic grade; Mallinckrodt Chemical Works) was allowed to stand for a few minutes to permit the heavier particles to settle and the suspension of finer particles then decanted. The resin was successively washed, followed each time by filtration through 2 layers of filter paper (using a Buchner funnel under suction), in the following series of solutions: 2N HCl; distilled water; 2N NaOH; distilled water; 2N HCl; distilled water; 2M NaCl; 2M NaCl (at this

step the resin was titrated to pH 7.0 with NaOH); 8% guanidinium chloride (GuCl) dissolved in 0.1 M sodium phosphate buffer at pH 6.8 (GuCl-PO_4). The resin was once more suspended in fresh 8% GuCl-PO_4 and the slurry employed for packing columns.

Two kinds of columns were used in these studies: the preparative column (2.5 × 60 cm) and the analytical column (0.6 × 60 cm). Both columns were packed tightly with the resin to a height of 55 cm and were then washed once with one column volume of 40% GuCl-PO_4 and once with two column volumes of 8% GuCl-PO_4 before use. The columns were used repeatedly throughout this work by flushing them successively, after the fractionation of each histone sample, with 40% and 8% GuCl-PO_4 as just described.

2. Purification and Preparation of Guanidinium Chloride Solutions

Practical grade GuCl (Eastman Organic Chemicals) was normally purified by mixing 1 to 2 liters of 60-65% GuCl solution in 0.1 M sodium phosphate buffer (pH 6.8) with sufficient activated charcoal (#655 Matheson Chem. Co.). The mixture was stirred for 2-5 hours at room temperature and then filtered through 2 layers of filter paper (using Buchner funnel under suction). This treatment with activated charcoal was repeated until the GuCl-PO_4 solution was colorless. Certain batches of GuCl which could not be completely purified in this manner were filtered through a column containing a mixture (2:1 w/w) of Celite (#545 Johns-Manville) and activated charcoal, as suggested by Bonner et al. (1967a).

The refractive index ($n^{25°}$) of a GuCl-PO_4 solution was used to determine GuCl concentration in the solution; a previously prepared linear curve of refractive index vs. percentage of GuCl (w/v) in 0.1 M sodium phosphate buffer (pH 6.8) was utilized as a standard for correlation. Solutions of various GuCl concentrations were prepared by appropriately diluting the concentrated GuCl-PO_4 solution (60-65%) with 0.1 M sodium phosphate buffer (pH 6.8), followed by finer adjustment of each solution with the aid of refractive index.

In the course of this study it was repeatedly found that the dilution of a GuCl-PO_4 solution resulted in significant lowering of the pH from the original value of 6.8, even though the concentrated solution being diluted as well as the buffer used for dilution were both at pH 6.8. The greater the extent of dilution, the larger was the decrease in pH (pH values as low as 6.2 have been observed). Since the effect on histone fractionation of the pH of the GuCl gradient has not been explored yet, GuCl-PO_4 solutions of different concentrations, prepared by dilution of 60-65% GuCl-PO_4, were routinely readjusted to pH 6.8 with 10 N NaOH. The extra time consumed in readjustment of pH of each GuCl-PO_4 solution was more than compensated for by the excellent reproducibility it ensured for the chromatographic patterns of histones.

3. Preparation of Histone Samples for Chromatography

Histone sulfates and histone chlorides can be directly dissolved in 8% GuCl-PO_4 for application to the column (see Bonner et al., 1967a; Fambrough, 1968). However, it was found more

suitable to dissolve histones in the following manner which ensured complete solubility of aggregated samples as well as of otherwise insoluble samples prepared from different sources by different methods. A small volume of 40% GuCl-PO_4 (0.1 ml for analytical column and 0.6 ml for preparative column) was added to the histone sample 8 hours before fractionation was to be started. The samples were allowed to stand in the presence of 40% GuCl-PO_4 at 2-4°C and were completely dissolved in 8 hours. This solution was then diluted 5-fold with 0.1 M sodium phosphate buffer (pH 6.8) to give a final concentration of 8% GuCl-PO_4. The diluted solution was then centrifuged at 10,000xg for 10 minutes and the clean colorless solution removed for application to the column.

4. Operation of the Preparative Column (2.5 × 60 cm)

50 to 80 mg of histones dissolved in 3 ml of 8% GuCl-PO_4, as described above, were allowed to be absorbed on the column bed, and then washed in with three 1 ml portions of 8% GuCl-PO_4. Five ml of 8% GuCl-PO_4 were then layered on the column bed and elution with the gradient initiated.

Lysine-rich and slightly lysine-rich histones were eluted from the column by employing a non-linear continuous gradient of GuCl-PO_4 as follows: 8% GuCl-PO_4 (250 ml) → 10.5% GuCl-PO_4 (300 ml) → 13% GuCl-PO_4 (350 ml). Arginine-rich histones were eluted by flushing the column with 150 ml of 40% GuCl-PO_4. The column was regenerated for reuse by washing it with 200 ml of 8% GuCl-PO_4.

A flow-rate of 30 ml per hour was normally maintained. The first 75 to 80 ml of the effluent were collected in a graduated cylinder and discarded. During this initial run, flow-rate was adjusted and the drop-volume calculated. Approximately 200 fractions of 5 ml each were collected.

5. Operation of the Analytical Column (0.6 × 60 cm)

3 to 6 mg of histones were dissolved in 0.5 ml of 8% GuCl-PO_4 as described earlier. The sample was absorbed on the column bed and then washed in with three 0.1 ml portions of 8% GuCl-PO_4. 0.5 ml of 8% GuCl-PO_4 were then layered on the bed and elution with non-linear gradient of GuCl-PO_4 carried out as follows: 8% GuCl-PO_4 (15 ml) → 10.5% GuCl-PO_4 (19 ml) → 13% GuCl-PO_4 (24 ml). This gradient, successfully used for fractionation of pea bud and calf thymus histones, led to very poor resolution of lysine-rich and slightly lysine-rich species of HeLa cells. However, a fairly acceptable resolution of histones of HeLa cells was achieved by employing the following non-linear gradient of GuCl-PO_4: 8.5% GuCl-PO_4 (15 ml) → 11.5% GuCl-PO_4 (19 ml) → 13% GuCl-PO_4 (24 ml). Therefore this gradient was routinely used for fractionation of HeLa histones in this study. Arginine-rich histones were easily eluted by flushing the column with 15 ml of 40% GuCl-PO_4. The column was regenerated for reuse by passing through it 25 ml of 8.5% GuCl-PO_4.

A flow-rate of 4 to 5 ml per hour was found suitable. The initial 6 ml of the effluent were collected in a graduated centrifuge

tube and later discarded. During this time, adjustment of the flow-rate and calculation of the volume of drops were carried out. Approximately 200 fractions of 0.3 ml each were collected.

6. Analysis of the Proteins in the Fractions

The protein content of the 5 ml fractions collected from the preparative column was determined as follows: An 0.3 ml aliquot from each (or every second) fraction was removed with a pipette into a separate small test tube. One ml each of 1.43 M TCA was added to groups of 10 tubes (final TCA concentration = 1.1 M) and the tubes were then shaken vigorously. Turbidity in the tubes was allowed to develop for 13 minutes after which optical density at 400 mμ was measured quickly.

It may be noted that dilution of histone fractions with water before addition of TCA, as suggested by Bonner et al. (1967a) and Fambrough (1968), can be avoided if the analysis is done in the above manner. In this quicker procedure, no precipitation of GuCl crystals occurs until the last one or two fractions containing arginine-rich histones are eluted which, as may be seen in Figs. 2, 3 or 4 causes no real problem in the interpretation of results.

The 0.3 ml fractions, collected from the analytical column, were analyzed directly (without dillution with water) by addition of 1 ml of 1.43 M TCA to groups of 10 tubes followed by measurement of optical density at 400 mμ after 13 minutes as described above.

7. Recovery of Histones

A comparison of the amount of histones applied to the column with their recovery (calculated from the area under the curve of $O.D._{400}$ vs. fractions obtained from the column, such as the one shown in Fig. 3) showed that all of the histones were quantitatively eluted. The following two methods were employed to recover histones from the fractions for further uses, such as amino acid composition studies and disc electrophoresis:

a) The fractions belonging to the peak of each histone species were pooled and dialyzed against 4 to 5 changes of 100 volumes of 0.1 M acetic acid at 2-4°C. The dialysis bags had been previously washed according to a special procedure involving soaking in EDTA (1%), $NaHCO_3$ (5%) and ethanol (95%) separately, followed by boiling and thorough rinsing in distilled water (E. O. Akinrimisi, personal communication). The volume of each dialyzate was then reduced to 7-5 ml by flash evaporation (this was not necessary in the case of the analytical column). The concentrated solutions were again exhaustively dialyzed against 0.1 M acetic acid in cold, lyophilized to dryness and stored at 2 to 4°C.

b) When the total amount of histones in the pooled fractions was rather small (as it was in the case of analytical columns), it was often preferable to avoid losses inherent in the above method. This was accomplished by precipitating histones from the pooled fractions by adding TCA to a final concentration of 1.1 M. The precipitated histones were collected by sedimentation in a Clinical centrifuge and

repeatedly washed with 1.1 M TCA to remove GuCl. The GuCl-free precipitates were washed once with either ether:ethanol (1:1) or acidified acetone (200 ml acetone:0.1 ml conc. HCl), and finally vacuum-dried in a desiccator.

I. DISC ELECTROPHORESIS OF HISTONES

Histones were fractionated by disc electrophoresis according to a modification (Bonner et al., 1967a; Fambrough, 1968) of the method of Reisfeld et al. (1962).

Eight gels consisting of 15% acrylamide in 6 M urea at pH 4.3 were prepared as follows: 1 ml of Temed solution (48 ml 1 N KOH, 17.2 ml glacial acetic acid, 4 ml N,N,N',N'-tetramethylethylene-diamine, deionized water to 100 ml) was mixed with 5 ml of 0.2% (w/v) ammonium persulfate dissolved in freshly prepared 10 M urea solution in deionized water. To this mixture were added 2 ml of acrylamide solution (60 g acrylamide, 0.4 g N,N'-methylene bis acrylamide, deionized water to 100 ml). This 15% acrylamide solution was rapidly mixed with a Pasteur pipette with sufficient bubbling to delay polymerization and then 0.9 ml aliquots were quickly transferred to pieces of glass tubing (length 6.5 cm, ID 5 mm) taking care to avoid the formation of any air bubbles. The aliquots were gently overlaid with 0.1 ml of freshly prepared 3 M urea solution to allow polymerization under anaerobic conditions.

After 45 minutes of polymerization, this layer was removed and the histone sample dissolved in 5 to 30 µl of 10 M urea at a

concentration of about 1 mg/ml was applied to the gel. The optimum amount of HeLa cell histones for application to a gel was found to be 8 to 10 μg per band or 15 to 25 μg of whole histones. The sample was overlayered with the tray buffer (31.2 g β-alanine, 8 ml glacial acetic acid, distilled water to 1 liter) and the gels placed in the standard electrophoretic chambers containing the tray buffer. Electrophoresis was performed for 90 minutes by applying a constant current of 4 milliamperes per gel in such a way that the negative electrode was away from the origin of the gels (histones migrate towards the negative electrode).

Gels were removed from the tubes and stained for 8 to 10 hours in 1% amidoschwarz 10 b dissolved in 40% ethanol - 7% glacial acetic acid aqueous solution. After staining, the gels were placed in the destaining solution (40% ethanol, 7% glacial acetic acid) for 2 to 4 hours and then destained by reverse electrophoresis at a constant current of 2 milliamperes per gel. A trace of stain was usually added to the destaining solution in the upper chamber to prevent discoloration of the stained histone bands. The destained gels were stored in screw-cap vials containing destaining solution. Any background stain in the gels was slowly removed by dialysis against the destaining solution.

Densitometer tracings of the gels were prepared by scanning the gels at a constant speed in a Canalco Model E Microdensitometer (Canal Industrial Corp., Rockville, Md.).

J. REDUCTION OF DISULFIDE LINKAGES IN HISTONES

To reduce their disulfide bands, histones were dissolved in freshly prepared 10 M urea at a concentration of about 1 mg/ml. β-mercaptoethanol was then added to this solution to a final concentration of 0.1 M. Complete reduction was achieved by incubating the above mixture at 37°C for 1 hour. Reduced samples were directly examined by disc electrophoresis to study the role played by disulfide bonds in the aggregation of histones.

K. AMINO ACID ANALYSIS

1. Protein Hydrolysis

Histone samples for amino acid analysis were obtained from the pooled fractions eluted from the Amberlite CG-50 column either by dialysis against 0.1 M acetic acid followed by lyophilization or by precipitation with TCA as described under 'Recovery of Histones' (page 99). Non-histone proteins were obtained according to the procedure outlined earlier under 'Preparation of Non-histone Proteins' (page 92).

Constant boiling HCl (0.5 to 1 ml) was added to the protein samples (0.5 to 3 mg) in hydrolysis tubes. The tubes were evacuated, freed of oxygen by repeated flushing with nitrogen followed by evacuation, and then sealed. Hydrolysis of the proteins was accomplished by incubation at 105°C for 24 hours. After hydrolysis, the sealed tubes were broken and the hydrolysates dried under vacuum in a desiccator. One-half ml of distilled water was added to each

dried sample, and once again dried as above to remove traces of HCl.

2. Column Chromatography

Amino acid analyses of the hydrolyzed protein samples were performed in a Beckman/Spinco Automatic Amino Acid Analyzer (Model 120B) using a pH gradient in sodium citrate buffer for elution of amino acids from the column. In the earlier analyses (performed by Joyce Bullock), two separate columns were used for fractionation of amino acids: one for basic amino acids and the other for acidic and neutral amino acids. In the later analyses (performed by John Racs), however, all amino acids were fractionated on a single column.

3. Paper Electrophoresis

High voltage paper electrophoresis was carried out according to the method of Dreyer and Bynum (1967). The hydrolyzed protein samples were applied to a Whatman No. 3MM chromatography grade paper and the amino acids separated in a 6.7% formic acid (pH 1.62)-Varsol system by electrophoresis at 8000 volts (current between 450 and 490 milliamperes) for 105 minutes at 45°C. The electrophoregrams were dried in a ventilated oven at 80°C for 30 minutes and dipped in cadmium acetate-ninhydrin reagent (20 ml distilled water, 4 ml glacial acetic acid, 200 mg cadmium acetate, 200 ml acetone, 2 g ninhydrin). The stain of the amino acids was developed by drying in an ammonia-free bath for 16 hours. Quantitative analyses were

performed by elution of the spots of stained amino acids from the paper with absolute methanol followed by determination of optical density at 500 mμ. Staining constants for amino acids were obtained by electrophoresing and staining standards.

4. Performic Acid Oxidation of Proteins

Traces of oxygen whenever present during acid hydrolysis of proteins cause breakdown of some amino acids. In particular, cysteine (or cystine) is partially oxidized to cysteic acid and pyruvic acid. During electrophoretic and chromatographic separations of amino acids, part of the cysteine thus appears as cysteic acid yielding an inaccurate quantitative analysis. Small quantities of cysteine may even remain undetected. In addition to cysteine degradation, methionine can also be partially converted to methionine sulfone and sulfoxide in the presence of traces of oxygen and can thus be underestimated.

For accurate quantitative estimation of cysteine and methionine, therefore, it is necessary to minimize the oxidative reactions mentioned above. This is accomplished by oxidation of proteins with performic acid before acid hydrolysis. Such oxidation quantitatively converts all cysteine to cysteic acid and all methionine to methionine sulfone; both cysteic acid and methionine sulfone remain stable during acid hydrolysis and are estimated as such.

The following procedure was employed to oxidize unhydrolyzed protein samples with performic acid (Dr. W. R. Gray, personal communication): 0.1 ml of 30 vol. hydrogen peroxide were mixed with 1.9 ml of 98% formic acid and the mixture allowed to stand for

2 hours at room temperature. Performic acid (0.2 ml) thus formed was cooled in ice and added to a precooled solution (0.1 ml) of the protein sample (0.5 to 3 mg) in 98% formic acid. This mixture was incubated for 1 hour at 0°C, diluted 10- to 20-fold with distilled water and then freeze-dried under vacuum in a desiccator over sodium hydroxide. To the dried sample were added 1/2 to 1 ml of distilled water which was again dried as above to remove all traces of performic acid. The oxidized protein sample thus prepared was acid hydrolyzed and subjected either to column chromatography or to paper electrophoresis according to the procedures described earlier.

RESULTS

A. PROPERTIES OF INTERPHASE AND METAPHASE CHROMOSOMES

1. Spectral Properties

The ultraviolet absorption spectra of unsheared and sheared interphase chromosomes (chromatin) prepared by the Sucrose Sedimentation Method (Method B) are shown in Fig. 1a. The two spectra are almost indistinguishable. Interphase chromosomes prepared by the Magnesium Precipitation Method (Method A) give a similar spectrum. This spectrum is also very similar to those of chromatin preparations obtained by other workers in this laboratory from rat liver and pea bud (see Bonner et al., 1967a). Since chemical characterization of HeLa cell histones was in general carried out on histone preparations from the unsheared chromatin isolated by Method B, it is relevant to briefly discuss some of the notable features of such chromatin.

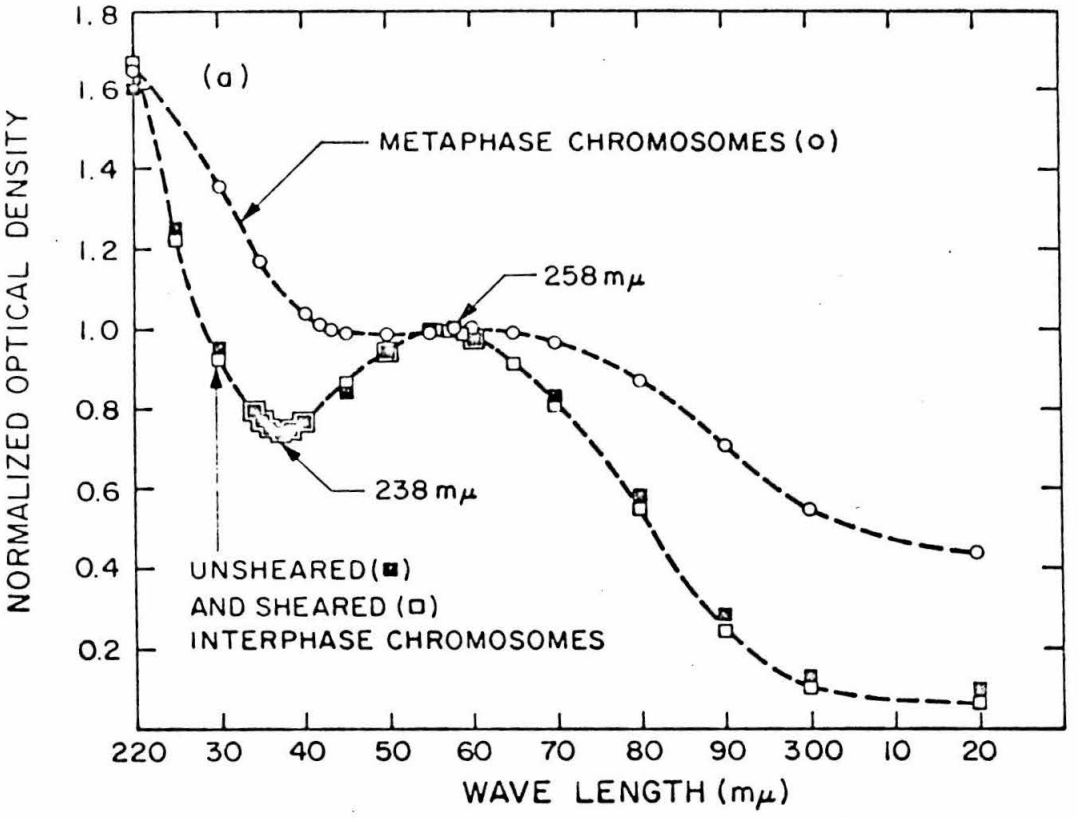
a) Interphase chromosomes have an absorption maximum at 258 mμ.

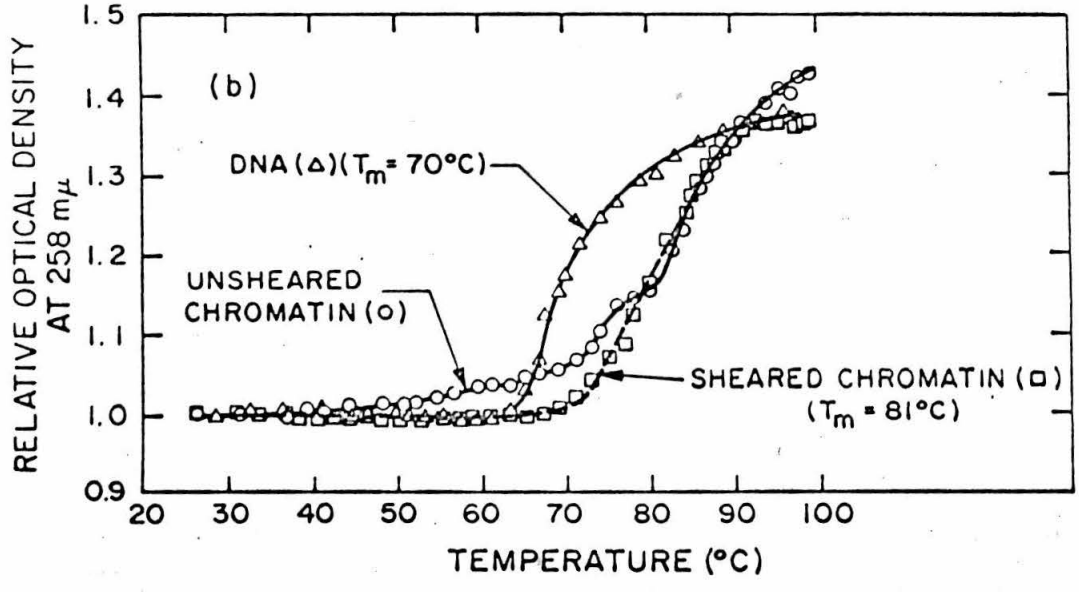
b) The spectrum shows in addition a trough in the lower wavelength regions with an absorption minimum at 238 mμ.

c) In the case of sheared chromatin prepared by Method B, the ratio $O.D._{238}:O.D._{258}:O.D._{280}$ was 0.75:1.00:0.55. In contrast, the chromatin prepared by Method A exhibited somewhat higher absorption at 238 mμ and 280 mμ ($O.D._{238}:O.D._{258}:O.D._{280}$ = 0.80:1.00:0.63) which suggests a higher protein content of the

Figure 1. (a) Ultraviolet absorption spectra of interphase and metaphase chromosomes. Interphase chromosomes were suspended in 0.01 M Tris-HCl, pH 8.0. Metaphase chromosomes were suspended in either distilled water or TM ($CaCl_2$, $MgCl_2$, and $ZnCl_2$, each at 1×10^{-3} M in 0.02 M Tris-HCl, pH 7.0). All optical density values are expressed as relative to $O.D._{258}$.

(b) Melting profiles of deproteinized DNA and interphase chromosomes in DSC (0.015 M NaCl, 0.0015 M sodium citrate). Both unsheared and sheared interphase chromosomes were prepared by the Sucrose Sedimentation Method (see Materials and Methods for details).


(a)
METAPHASE CHROMOSOMES (o)
258 mμ
238 mμ
UNSHEARED (■)
AND SHEARED (□)
INTERPHASE CHROMOSOMES
NORMALIZED OPTICAL DENSITY
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
220 30 40 50 60 70 80 90 300 10 20
WAVE LENGTH (mμ)


(b)
DNA (Δ) (T_m = 70°C)
UNSHEARED
CHROMATIN (o)
SHEARED CHROMATIN (□)
(T_m = 81°C)
RELATIVE OPTICAL DENSITY
AT 258 mμ
1.5
1.4
1.3
1.2
1.1
1.0
0.9
20 30 40 50 60 70 80 90 100
TEMPERATURE (°C)

chromatin prepared by this method. This conclusion was verified by direct chemical determination of protein content (see Table 1).

d) A reasonably purified chromatin preparation has very low turbidity as shown by the low optical density at 320 mμ (≤ 0.1 of $O.D._{258}$). Shearing of chromatin resulted in a slightly lower absorption at 320 mμ. As nucleic acids and proteins do not absorb at this wavelength, the low optical density is an indication of lack of aggregation of chromatin. In spite of higher protein content, the chromatin prepared by Method A showed no more turbidity than the sheared chromatin prepared by Method B ($O.D._{320}$ was 0.07 of $O.D._{258}$ in both cases).

The metaphase chromosomes, prepared at neutral pH by the method of Maio and Schildkraut (1967) and suspended in TM ($CaCl_2$, $MgCl_2$ and $ZnCl_2$, each at 1×10^{-3} M in 0.02 M Tris-HCl, pH 7.0), exhibited relatively much higher optical density at 320 mμ (Fig. 1a; $O.D._{320}$:$O.D._{258}$ was 0.42-0.44:1). This indicates an extensive aggregation of chromosomes which was not caused by the presence of divalent cations in chromosomal suspension since a similar spectrum was obtained with metaphase chromosomes suspended in distilled water. Due to this aggregation the optical densities at 238 mμ and 280 mμ cannot be used to estimate their protein content. The absence of an absorption minimum at 238 mμ in the spectrum of metaphase chromosomes can probably be attributed to increased scattering at lower wavelengths.

2. Melting Profile

Fig. 1b presents the melting profiles of unsheared and sheared interphase chromosomes together with that of deproteinized DNA in DSC (0.015 M NaCl, 0.0015 M sodium citrate). These results are similar to those reported for calf thymus and pea bud chromatin preparations (Bonner et al., 1967a). The unsheared chromatin exhibits a 2- or 3-step melting behavior in contrast to the 1-step melting profile of sheared chromatin. The higher T_m of sheared chromatin (81°C) as compared to that of DNA (70°C) indicates a more stable state of DNA in sheared chromatin. The melting profile of chromatin prepared by the Magnesium Precipitation Method is identical to that of sheared chromatin (prepared by the Sucrose Sedimentation Method) shown in Fig. 1b. The melting behavior of metaphase chromosomes can not be similarly studied because of the complications caused by scattering.

3. Chemical Composition

The mass ratios of DNA, RNA, histones and non-histone proteins in interphase and metaphase chromosomes are presented in Table 1. The data reveal that one important effect of shearing of interphase chromosomes (prepared by Method B) is loss of almost half of the non-histone proteins. More will be said about this non-histone protein component later in this Chapter (cf. p. 184). It may be also noted that chromatin prepared by the Magnesium Precipitation Method has a higher total protein:DNA ratio than sheared chromatin prepared by the Sucrose Sedimentation Method. In contrast, chromatin

Table 1. Chemical Composition[a] of Interphase and Metaphase Chromosomes

Type of Chromosomal Preparation	DNA	RNA	Protein	Acid Soluble Protein		Acid Insoluble Protein		Acid Soluble Protein/ Acid Insoluble Protein
				HCl	H_2SO_4	HCl	H_2SO_4	
1. Interphase chromatin prepared by the Sucrose Sedimentation Method								
Unsheared	1.0	0.12	2.85	-	1.29	-	1.56	0.83
Sheared	1.0	0.10	1.75	-	1.03	-	0.72	1.43
Sheared	1.0[b]	0.15	2.10	1.1	-	1.0	-	1.10
2. Interphase chromatin prepared by the Magnesium Precipitation Method	1.0	0.72	3.42	-	1.05	-	2.37	0.44
3. Metaphase chromosomes prepared at acid pH	1.0	0.56	4.85	-	0.89	-	3.96	0.22
	1.0[b]	0.66	4.70	2.0	-	2.7	-	0.74
4. Metaphase chromosomes prepared at neutral pH	1.0	0.67	4.50	-	1.33	-	3.17	0.42
	1.0	0.67	4.50	2.55	-	1.95	-	1.31
	1.0[c]	0.71	4.39	2.54	-	1.85	-	1.37

[a]Expressed as mass ratios of different chromosonal components.

[b]Huberman and Attardi (1966).

[c]Maio and Schildkraut (1967).

preparations isolated by both methods have the same ratio of H_2SO_4-soluble protein to DNA. In view of the varying ratios of H_2SO_4-insoluble protein (non-histone) to DNA found in HeLa chromatin prepared by different methods, the in vivo non-histone protein content of interphase chromosomes can not be unambiguously estimated. In addition, the non-histone protein content of chromatin prepared from a number of plant and animal species has been found to be highly variable whereas the acid (H_2SO_4 or HCl) soluble protein:DNA ratio of the chromatin from these species is relatively much less variable (see Bonner et al., 1967b).

An unexpected and interesting observation was made in the course of the studies on the chemical composition of metaphase chromosomes. It was found that 0.2 N HCl extracts twice as much protein from metaphase chromosomes as does 0.2 N H_2SO_4 (see Table 1). This was true whether metaphase chromosomes were prepared at acid pH by the method of Huberman and Attardi (1966) or at neutral pH by the method of Maio and Schildkraut (1967). Consequently, the HCl-soluble protein:DNA ratio (between 2.0 and 2.5) was twice that of H_2SO_4-soluble protein:DNA ratio (between 0.9 and 1.3). In contrast, the ratios of HCl-soluble protein to DNA and of H_2SO_4-soluble protein to DNA were not significantly different in the case of interphase chromosomes and ranged between 0.9 and 1.1. Histone:DNA ratios of a similar magnitude (0.9 to 1.1) have also been reported for chromatin prepared from logarithmically growing ascites tumor cells in which histone was extracted with 0.2 N H_2SO_4

(Dahmus and Bonner, 1965) as well as for chromatin prepared from the embryonic axis of pea (Huang and Bonner, 1962) and different developmental stages of sea urchin eggs (Marushige and Ozaki, 1967) in which the histone fraction was extracted with 0.2 N HCl. In all of the above-mentioned examples most of the cells used for the preparation of chromosomes were in interphase with a very small number in metaphase. These facts suggest that metaphase chromosomes contain an HCl-soluble protein fraction which is absent from interphase chromosomes.

This conclusion leads to important questions regarding the chemical nature and role of the HCl-soluble protein fraction specific to metaphase chromosomes. Experiments giving some insight into these questions will be described in a later section on the 'Histones and Other Acid-Soluble Proteins of Metaphase Chromosomes' (page 151).

B. HISTONES OF INTERPHASE CHROMOSOMES

1. Fractionation of Histones by Column Chromatography

In the initial phase of this work, attempts were made to fractionate histones of HeLa cells, calf thymus and pea bud on Amberlite CG-50 in an analytical column (0.6 × 60 cm) by using a gradient of 8% GuCl-PO_4 → 10.5% GuCl-PO_4 → 13% GuCl-PO_4 to elute lysine-rich (Iabc) and slightly lysine-rich (IIab) histones, followed by 40% GuCl-PO_4 to elute arginine-rich histones (III-IV). As in the previously reported work of Fambrough and Bonner (1966),

this gradient led to a very successful resolution of lysine-rich and slightly lysine-rich histones of pea bud (Fig. 2a) and calf thymus (Fig. 2b). However, only a very poor resolution of these histone species isolated from interphase chromosomes of HeLa cells could be obtained (Fig. 2c). As can be seen from these results, a higher concentration of guanidinium chloride is required to elute the lysine-rich histones of HeLa cells than is required for elution of corresponding species of pea bud and calf thymus histones. Since histones of HeLa cells are very similar to those of pea bud and calf thymus with respect to amino acid composition and electrophoretic behavior, as will be demonstrated later in this Chapter, the above results suggest that lysine-rich histones of HeLa cells may differ from those of pea bud and calf thymus in some very subtle manner, such as the degree of phosphorylation. It may be interesting to explore whether this increased retention of HeLa lysine-rich histones on Amberlite CG-50 reflects a characteristic of rapidly dividing undifferentiated cells.

A much improved resolution of HeLa histones was achieved when lysine-rich (Ia and Ib) and slightly lysine-rich (O and II) species were eluted from an analytical column by a different gradient of guanidinium chloride, namely, 8.5% GuCl-PO_4 → 11.5% GuCl-PO_4 → 13% GuCl-PO_4, followed by elution of arginine-rich histones (III-IV) with 40% GuCl-PO_4. A typical result of such a fractionation is presented in Fig. 3. An even better resolution of HeLa histones is obtained when these are fractionated on a preparative column (2.5 × 60 cm) as described in Materials and Methods (Fig. 4). In particular,

Figure 2. Fractionation of histones by chromatography on Amberlite CG-50 (analytical column). Lysine-rich (Iabc) and slightly lysine-rich (IIab) histones were eluted by a non-linear gradient of guanidinium chloride as follows: 8% → 10.5% → 13%. This was followed by elution of arginine-rich histones (III-IV) with 40% guanidinium chloride. Protein concentration (—○—○—) was measured by precipitation at 1.1 M TCA and determination of O.D. at 400 mμ as described in Materials and Methods. Guanidinium chloride concentration (-□--□-) of the effluent was measured by determination of refractive index. The names assigned to various peaks are indicated. The chromatographic peak A is also referred to as the run-off peak. (a) Pea bud histones, (b) Calf thymus histones, (c) Interphase histones of HeLa cells.

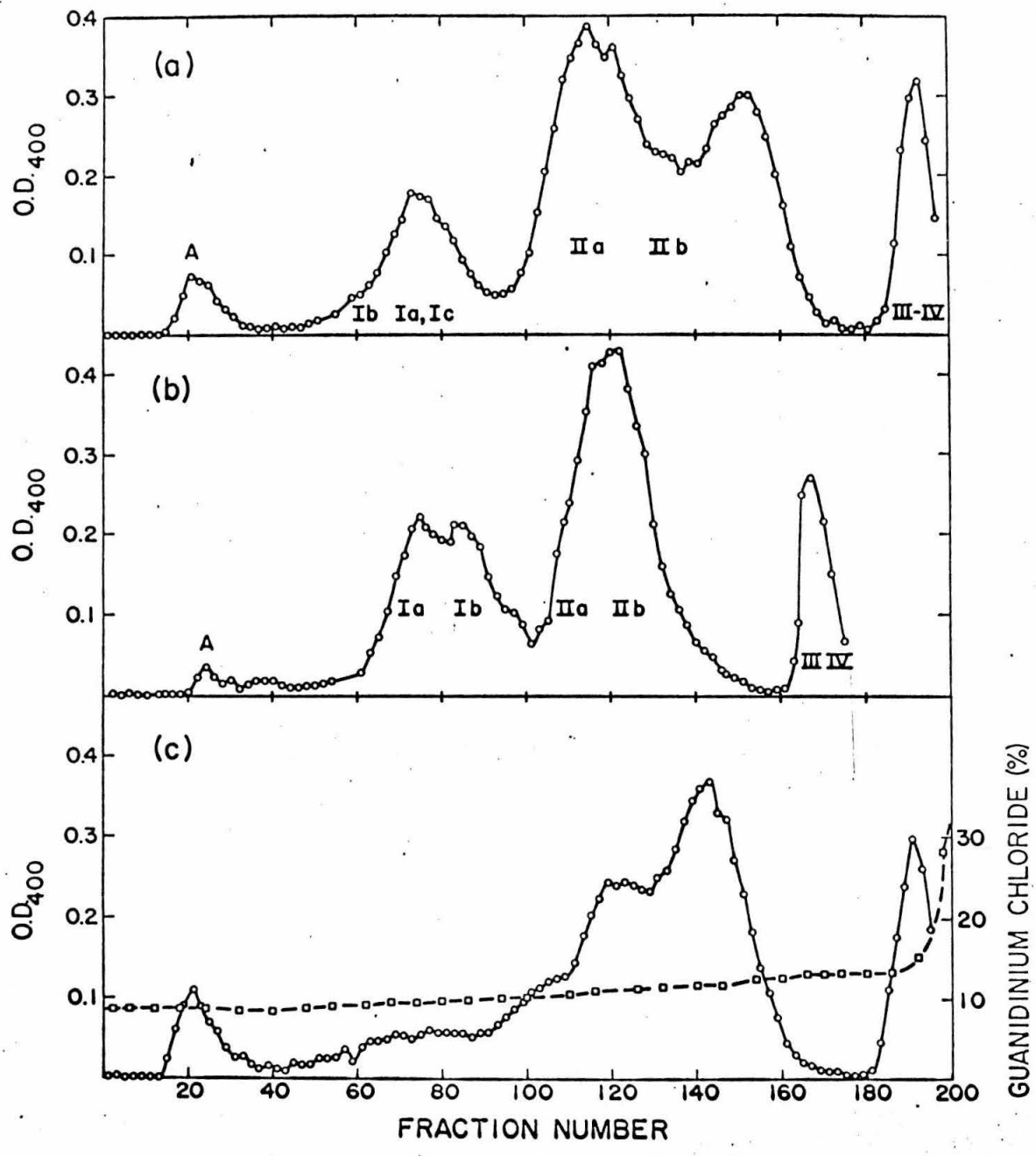

(a)
(b)
(c)
O.D.400
A
Ib
Ia, Ic
IIa
IIb
III-IV
Ia
Ib
IIa
IIb
III IV
0.4
0.3
0.2
0.1
20
40
60
80
100
120
140
160
180
200
FRACTION NUMBER
GUANIDINIUM CHLORIDE (%)
30
20
10

Figure 3. Fractionation of interphase histones of HeLa cells on Amberlite CG-50 (analytical column). Lysine-rich (Iab) and slightly lysine-rich (O, II) histones were eluted by a non-linear gradient of guanidinium chloride as follows: 8.5% → 11.5% → 13%. This was followed by elution of arginine-rich histones (III-IV) by 40% guanidinium chloride. Protein concentration (—O——O—) and guanidinium chloride concentration (-□--□-) were measured as described in Materials and Methods. Vertical lines indicate the scheme in which the chromatogram was partitioned for disc electrophoretic studies. The names assigned to various peaks are indicated. The chromatographic peak A is also referred to as the run-off peak.

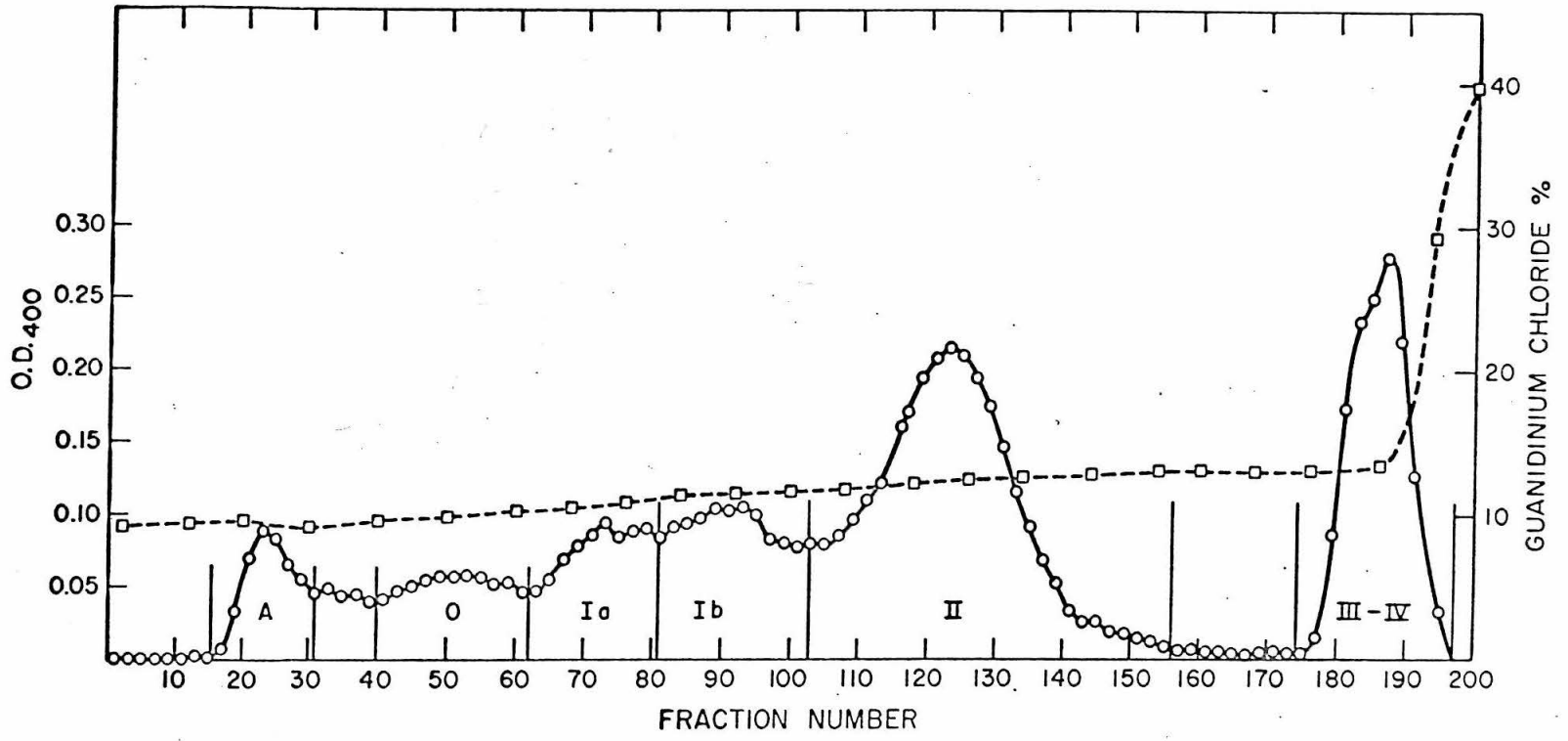
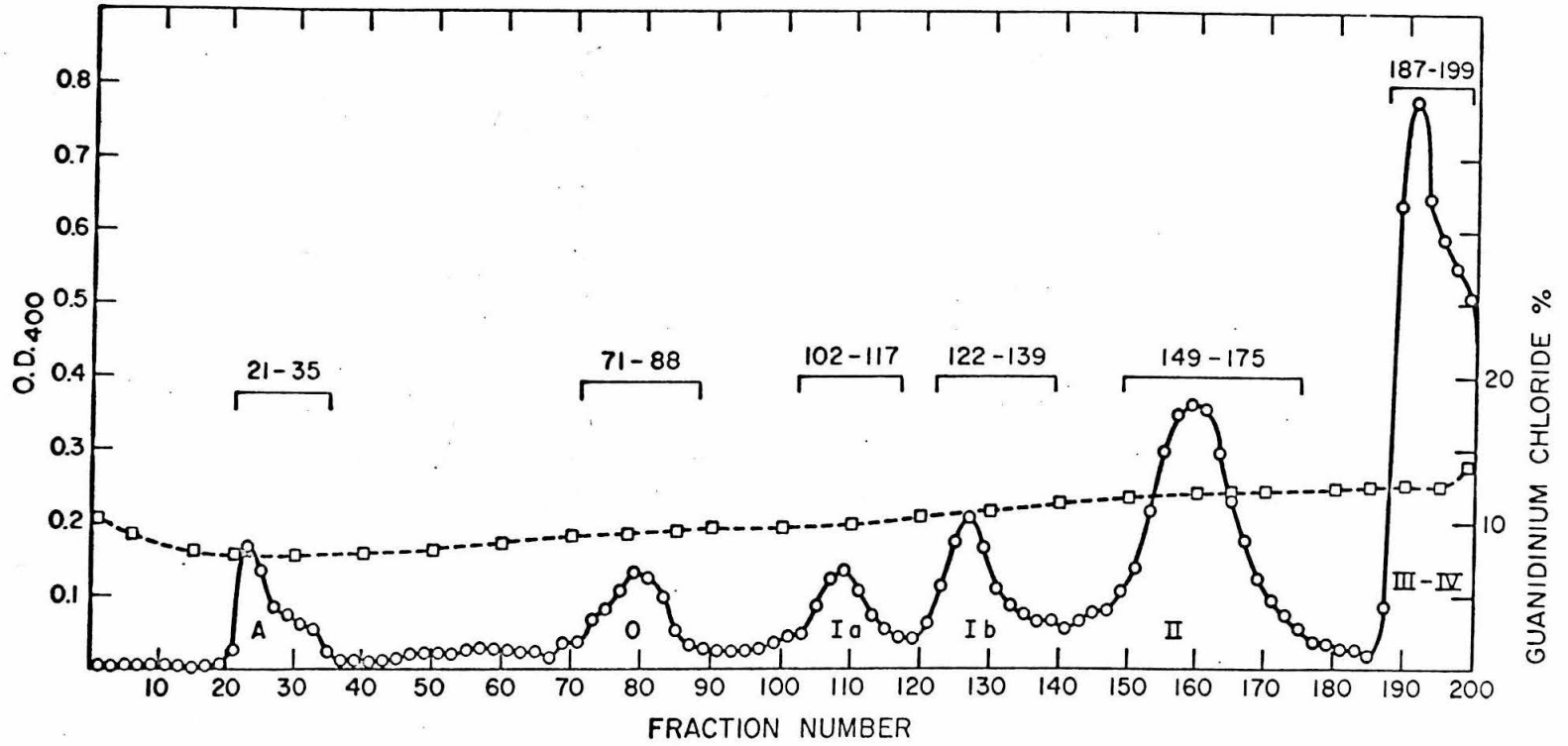

O.D.400
0.30
0.25
0.20
0.15
0.10
0.05
A
O
Ia
Ib
II
III-IV
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
FRACTION NUMBER
40
30
20
10
GUANIDINIUM CHLORIDE %

Figure 4. Fractionation of interphase histones of HeLa cells on Amberlite CG-50 (preparative column). Histones were eluted by a gradient of guanidinium chloride as described in Materials and Methods. Protein concentration (—○—○—) and guanidinium chloride concentration (-□--□-). For amino acid analysis and disc electrophoretic studies, the fractions were pooled as indicated in the figure by fraction numbers above the horizontal lines covering various peaks. The names assigned to various peaks are indicated.


O.D.400
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
21-35
71-88
102-117
122-139
149-175
187-199
A
O
Ia
Ib
II
III-IV
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
FRACTION NUMBER
GUANIDINIUM CHLORIDE %
20
10

the lysine-rich species (Ia and Ib) are better separated from each other and from the slightly lysine-rich species (O and II) on a preparative column than on an analytical column. Fractions used for amino acid analysis and examination of electrophoretic behavior were obtained by fractionation on such a preparative column. For routine experiments, however, the analytical column was found quite satisfactory since the purity of the fractions as judged by their electrophoretic behavior was comparable to that obtained for various chromatographic fractions of pea bud and calf thymus histones similarly fractionated by Fambrough and Bonner (1966).

The names assigned to various histone fractions are given in Figs. 3 and 4. Except for histone O, these names are based on the nomenclature[1] used for calf thymus and pea bud histones fractionated in an analogous manner by previous workers (Rasmussen et al., 1962; Fambrough and Bonner, 1966). Data to be presented later in this Chapter on the electrophoretic behavior and amino acid composition of HeLa histones will confirm this identification. It will be appropriate here, however, to briefly outline certain important features of the various chromatographic fractions. The first chromatographic peak A (also called the run-off peak) consists of proteins

[1] Throughout this Chapter the alternative nomenclature based on the fractionation procedure of Johns and Butler (1962) and Johns (1964a) will be used wherever necessary. The names of various histones in this nomenclature are parenthetically indicated below along with corresponding components fractionated according to the chromatographic method (used here) of Rasmussen et al. (1962): Ia and Ib (f1); IIb1 (f2a2); IIb2 (f2b); III (f3); IV (f2a1). This relationship between the corresponding fractions prepared by the two methods has been recently pointed out by Bonner et al. (1967b). The chromatographic peak A is also referred to as the run-off peak.

not retained by Amberlite CG-50 under the presently employed chromatographic conditions and is eluted with one hold-up volume of the effluent. Although proteins of peak A have been categorized as non-histone proteins, their chemical nature is still a matter of debate. The possibility that the proteins in this run-off peak have an overall basic character has not been ruled out as yet. The second peak (O) comprises a slightly lysine-rich fraction. A component chromatographing at this position has not been recorded in the histones of pea bud (Fambrough and Bonner, 1966) or calf thymus (Rasmussen et al., 1962; Fambrough and Bonner, 1966) and therefore, has so far eluded final chemical identification. The third and fourth peaks (Ia and Ib) consist of lysine-rich species and the fifth (II) of slightly lysine-rich components. The last peak (III-IV) is made up of arginine-rich histones. The names III and IV in the original nomenclature of Rasmussen et al. (1962) referred to separate chromatographic peaks. However, by the use of electrophoretic analysis it has been repeatedly observed in this study that the two arginine-rich components of HeLa histones are distributed throughout the chromatographic peak III-IV without any evidence of resolution. The resolution of two peaks in the III-IV region, only infrequently observed during the present studies, appears to be an artifact of the guanidinium chloride gradient employed for elution. Similar observations have been recorded for pea bud and calf thymus histones by Fambrough (1968). Therefore, according to Fambrough's proposal (1968), the names III and IV have been assigned to the two electrophoretically separable

bands of peak III-IV in the order of increasing mobility.

It may be noted here that the histones extracted from chromatin prepared by Methods A and B as well as from purified nuclei were similar by both chromatographic and electrophoretic criteria.

2. The Purity of Arginine-Rich Peak III-IV

There has been some question in recent years in our laboratory as well as in others (e.g. see Hnilica and Bess, 1965) about the purity of the arginine-rich histone fraction. At one time it was suspected that the peak III-IV eluted from the Amberlite CG-50 column might be highly contaminated with proteins of peak A. The possibility of contamination of peak III-IV has an important bearing upon at least three biologically significant questions discussed below: a) Since a part of chromosomal RNA has been shown by Bonner and Huang (1966) to co-chromatograph with peak III-IV, the nature of the protein covalently linked with chromosomal RNA poses an interesting question; b) The observation that the synthesis of peak III-IV (measured by incorporation of radioactive amino acids) can take place even in the absence of DNA synthesis (Chalkley and Maurer, 1965; author's own work reported in the next Chapter) can not be unambiguously interpreted without a knowledge of the extent of its contamination by peak A proteins; c) The presence of cysteine in peak III-IV has been demonstrated in the course of present studies as will be seen later. Investigation of the problem of contamination of peak III-IV will permit us to decide whether the observed cysteine in this material is due to the

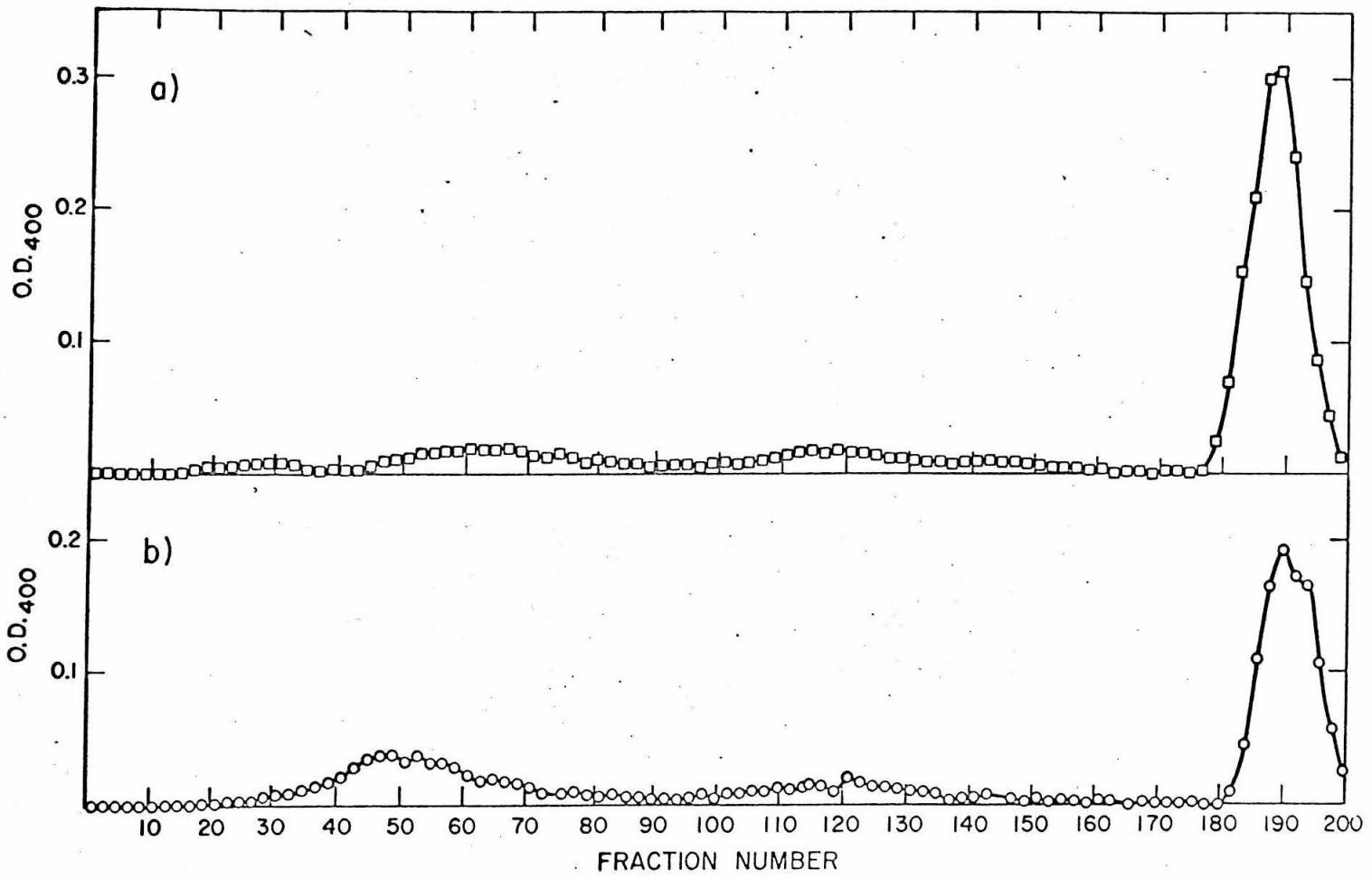
presence of non-histone proteins or not.

With these objectives in mind, two separate preparations of peak III-IV, obtained by elution from the Amberlite CG-50 column, were rechromatographed in an analytical column. The results are presented in Figs. 5a and b. It is clear that either none or less then 5% of the material elutes in the region of peak A (fraction number 20 to 30). In contrast, a varying but small proportion of material does elute in the region of slightly lysine-rich histone O (fraction number 40 to 65) and II (fraction number 105 to 145). The above data argue strongly against the possibility of significant contamination of peak III-IV by material of peak A because of ionic or hydrogen bond interactions. However, this experiment still fails to rule out the possibility of contamination by peak A proteins resulting from two other kinds of associations:

a) There is some evidence indicating that the protein covalently linked to chromosomal RNA may be associated with histones through divalent cations, such as Mg^{++} (Douglas Brutlag, personal communication). If this is the case, contamination of peak III-IV could be checked only by rechromatographing this material after dialysis against EDTA. Although such an experiment needs to be done, it may be pointed out that during the experiments presented in Figs. 5a and b the peak III-IV material was sufficiently diluted in water so as to allow the majority of divalent cations to dissociate from proteins;

b) The presence of cysteine in histone III (f3) as well as in

Figure 5. Rechromatography of arginine-rich peak III-IV on Amberlite CG-50 (analytical column). Gradient of guanidinium chloride was same as described in Figure 3. Rechromatography of two separate preparations of peak III-IV is shown in (a) □ and (b) ○ respectively.


a)
b)
O.D.400
0.3
0.2
0.1
0.2
0.1
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
FRACTION NUMBER

peak A proteins has been confirmed through many independent lines of evidence. Therefore, it is likely that peak A proteins may be linked with histone III through intermolecular disulfide bonds. A contamination of peak III-IV with peak A proteins due to such disulfide bonds will not be revealed by the rechromatography experiment described above since the experimental conditions do not favor reduction of S-S bonds. The problem of such a contamination can be resolved by rechromatographing peak III-IV material after reduction with a reducing agent (e.g. β-mercaptoethanol). Such an experiment has not been done so far, but the consequence of reduction of peak III-IV has been examined by gel electrophoresis. As will be noted later (Figs. 9c and d), the major result of this reduction is conversion of polymerized histone III to monomers. After reduction, only a very small proportion (approximately 5%) of the total material was observed to electrophorese with mobilities characteristic of peak A proteins.

3. Amino Acid Composition of Chromatographic Fractions

The histones of HeLa cells were fractionated on a preparative column and the chromatogram was partitioned in six distinct sections as indicated in Fig. 4. Fractions within each section were pooled and the amino acid composition determined according to the procedures described in Materials and Methods. The amino acid composition of various HeLa histone fractions is given in Table 2. For purposes of comparison, Table 2 also presents the composition of corresponding calf thymus and pea bud fractions obtained in an analogous manner by previous workers. The composition is expressed as moles of each

Table 2. The Amino Acid Composition of Chromatographic Histone Fractions

Amino Acids	HeLa[‡] A	Pea[a] A	HeLa[†] O	HeLa[†] Ia	Calf Thymus[b] Ia	Pea[a] I	HeLa[†] Ib	Calf Thymus[b] Ib	HeLa[‡] II	Calf Thymus[b] IIb	Pea[a] IIa	HeLa[‡] III-IV	Calf Thymus[b] III-IV	Pea[a] III-IV
Lysine	11.4	8.2	14.1	23.6	25.3	22.9	20.7	26.2	13.0	13.5	16.8	9.3	9.7	9.7
Histidine	1.7	1.5	1.9	0.4	0.4	0.9	0.7	0.2	2.8	2.8	1.6	2.0	1.9	1.9
Arginine	5.2	3.2	6.5	2.8	3.0	2.7	4.8	2.6	8.2	7.9	7.2	9.5	11.9	10.8
Aspartic Acid	7.4	7.0	5.4	2.7	2.5	3.0	3.5	2.5	5.7	5.6	6.7	6.3	5.0	6.1
Threonine	5.2	5.2	6.4	5.4	5.8	4.6	5.2	5.4	5.1	5.2	4.7	6.0	6.7	6.1
Serine	8.3	7.8	9.5	6.7	6.4	5.6	5.9	6.5	6.7	7.0	7.3	4.6	4.6	4.4
Glutamic Acid	12.4	9.7	8.7	4.6	4.5	7.8	5.8	4.3	8.7	8.7	8.6	10.0	10.4	8.8
Proline	7.7	6.8	5.1	9.1	8.6	10.0	7.5	9.1	3.9	4.7	6.9	4.2	4.2	3.9
Glycine	9.4	11.8	6.4	8.0	6.7	3.7	7.9	7.3	8.5	8.2	10.5	9.8	8.6	9.8
Alanine	9.7	12.9	11.5	21.2	24.0	22.9	21.3	24.2	11.7	11.5	7.9	10.2	11.6	9.7
Half-cystine	1.5*	n.r.	0*	0*	n.r.	n.r.	trace*	n.r.	trace*	n.r.	n.r.	1.0*	n.r.	n.r.
Valine	5.2	7.3	6.6	6.5	4.9	6.2	4.7	4.1	7.1	6.7	4.5	6.3	5.9	6.7
Methionine	1.9*	1.0	1.9*	0.6*	0.1	trace	0.9*	0.1	1.1*	0.8	0.7	1.4*	1.3	0.4
Isoleucine	3.0	4.8	4.9	1.8	1.3	2.9	2.6	1.2	4.3	4.5	3.8	4.8	5.3	5.9
Leucine	5.9	8.5	5.6	4.9	5.3	4.6	6.0	5.0	8.4	8.6	7.9	9.2	8.9	10.5
Tyrosine	1.8	2.3	3.3	0.9	0.7	0.9	1.3	0.7	3.0	3.0	2.4	2.8	2.2	2.4
Phenylalanine	2.4	2.2	2.0	0.8	0.6	1.3	1.0	0.6	1.4	1.3	2.7	2.6	2.5	3.1

See notes on the following page.

Table 2 (Continued)

[a]From Fambrough and Bonner (1966); [b]From Rasmussen et al. (1962).
All values are reported as moles/100 moles of amino acids found.
Amides, tryptophan and ϵ-N-methyllysine were not determined.
The underlined values signify a difference of 2 or more mole % among the corresponding histone fractions.
n.r. - not reported; † - average of two determinations carried out on one sample; ‡ - average of two determinations carried out on each of two samples obtained from separate histone preparations.
*To obtain accurate analyses of cysteine and methionine, protein samples were oxidized with performic acid before acid hydrolysis. In such cases, cysteine (and/or cystine) and methionine were estimated as cysteic acid and methionine sulfone respectively.

amino acid per 100 moles of total amino acids. Serine and threonine are partially decomposed during acid hydrolysis but these losses are of a relatively small magnitude (approximately 10% and 5% respectively during 24 hour acid hydrolysis; Joyce Bullock, personal communication). The data for HeLa and pea bud histones have not been corrected for these losses whereas those of calf thymus have taken them into account. To obtain accurate analysis of cysteine and methionine, each HeLa histone fraction was at least once subjected to performic acid oxidation before acid hydrolysis. Tryptophan was not determined in these studies. A difference of 2 or more mole % among the fractions of HeLa cells, calf thymus and pea bud is indicated in Table 2 by underlining of the values for the relevent amino acid.

It is important to recognize at the outset that none of the chromatographic fractions used for analysis consists of homogeneous protein molecules. As will be demonstrated by gel electrophoretic studies to be presented later (Fig. 8), each fraction is actually made up of 2 or more distinct components. In view of this, the data of Table 2 represent a kind of weighted average of the composition of various proteins comprising each fraction and can be used for only rough comparisons.

Table 3 presents certain distinguishing features in the compositions of various HeLa fractions and compares them with those of calf thymus and pea bud fractions. The main characteristics and striking similarities in the compositions of corresponding fractions

Table 3. Comparison of Certain Notable Features in the Amino Acid Composition of Histone Fractions from Different Species

	Peak A		Histone O	Histone Ia			Histone Ib		Histone II			Histone III-IV		
	HeLa	Pea	HeLa	HeLa	Calf	Pea†	HeLa	Calf	HeLa	Calf*	Pea†	HeLa	Calf	Pea
1. Total basic amino acids	18.3	12.9	22.5	26.8	28.7	26.5	26.2	29.0	24.0	24.2	25.6	20.8	23.5	22.4
2. Total acidic amino acids‡	19.8	16.7	14.1	7.3	7.0	10.8	9.3	6.8	14.4	14.3	15.3	16.3	15.4	14.9
3. Basic/acidic‡	0.9	0.8	1.6	3.7	4.1	2.5	2.8	4.3	1.7	1.7	1.7	1.3	1.5	1.5
4. Lysine/Arginine	2.2	2.6	2.2	8.4	8.4	8.5	4.3	10.1	1.6	1.7	2.3	1.0	0.8	0.9

† Pea bud histone. I and IIa used for comparison.

* Calf thymus histone IIb compared.

‡ The estimated acidic amino acid content does not take into account the fractions of glutamic and aspartic acids which exist as glutamine and asparagine respectively.

Data on calf thymus and pea bud histones taken from Rasmussen et al. (1962) and Fambrough and Bonner (1966) respectively.

of HeLa cells, calf thymus and pea bud revealed by the data of Tables 2 and 3 are discussed below. Peak A (run-off peak) of HeLa cells has a high total basic amino acid content which is, however, slightly lower than total acidic amino acid content. In this connection, it should be recognized that the estimated acidic amino acid content in such analyses fails to take into consideration the fractions of glutamic and aspartic acids which exist as glutamine and asparagine respectively. In view of this fact, no conclusion can be drawn regarding the overall charge ratio of the proteins in peak A on the basis of amino acid composition reported in Table 2 alone.

The lysine-rich histones of HeLa cells, calf thymus and pea bud are characterized by very high content of lysine, alanine and proline which alone constitute 50 to 60% of the total amino acids in these histones. They are further distinguished by very low tyrosine and phenylalanine content, relatively low content of leucine, isoleucine, glutamic acid and aspartic acid, and the absence of cysteine. Fambrough (1968) separated faster electrophoresing minor components from calf thymus and pea bud lysine-rich fractions by the use of preparative disc electrophoresis and demonstrated an absence of methionine in lysine-rich histones. The use of such electrophoretically purified lysine-rich histones also revealed certain differences between the compositions of calf thymus Ia and pea bud I which were not obvious by examination of chromatographic fractions. For instance, it was found that calf thymus histone Ia contains somewhat less arginine than does pea bud histone I and lacks histidine (Fambrough,

1968). In view of this finding, it will be of great interest to compare the composition of lysine-rich histones of calf thymus and pea bud with that of HeLa lysine-rich histones purified by preparative disc electrophoresis. Such a comparison would allow us to determine the extent of relatedness in the compositions of two mammalian lysine-rich histones and to explore the pattern of their divergence from the corresponding plant histones. However, certain conclusions can be drawn from the available data. In general, HeLa and calf thymus lysine-rich histones exhibit greater similarity to each other than to those of pea bud. For instance, the two mammalian lysine-rich histones contain significantly less glutamic acid and more glycine than the plant lysine-rich species. Compared to corresponding calf thymus and pea bud components, the higher arginine content of HeLa histone Ib may be noted. It is not yet possible to determine whether this shows a greater contamination by the arginine-rich fraction or truly more arginine in histone Ib of HeLa cells.

The lysine/arginine ratios presented in Table 3 suggest that HeLa histones contain two chromatographically separable slightly lysine-rich fractions, histone O and histone II. Calf thymus histone II has been shown to consist of two components, IIb1 (f2a2) and IIb2 (f2b), which can be resolved either by starch gel electrophoresis (Rasmussen et al., 1962) or by the method of Johns (1964a). Similarly, the presence of two or more components of histone II has also been demonstrated in pea bud histones (Fambrough, 1968). It is very likely that histone II of HeLa cells is also made up of at least two

components, respectively similar to IIb1 and IIb2. Again, it is observed that calf thymus and HeLa slightly lysine-rich fractions (IIb and II respectively) are more similar to each other than to pea bud histone IIa (the major slightly lysine-rich fraction of pea bud). Thus, both mammalian fractions are poorer in proline and glycine and richer in alanine and valine than the slightly lysine-rich histone IIa of pea bud. The heterogeneity of the chromatographic histone II fraction greatly limits more meaningful comparison of the compositions of mammalian and plant slightly lysine-rich histones.

Regarding histone O, it is sufficient to point out here that its amino acid composition is similar to that of calf thymus histone IIb2 (f2b) described by Rasmussen et al. (1962) and Hnilica et al. (1966). Histone f2b with a composition identical to that of calf thymus f2b has also been demonstrated in several rat tissues, chicken erythrocytes and a few lines of neoplastic cells (Hnilica, 1965, 1966). Both HeLa histone O and calf thymus histone IIb2 are distinguished from calf thymus IIb1 (f2a2) by higher lysine/arginine ratios, higher content of serine and tyrosine and lower content of glycine and leucine. Of all the histone fractions, histone O and IIb2 alone are characterized by essentially equal content of leucine and isoleucine (cf. Hnilica, 1965 also). Further observations on the properties of this peculiar component of HeLa cells will be presented later.

Arginine-rich peak III-IV consists of two distinct components, histones III (f3) and IV (f2a1), easily distinguishable by their amino acid composition, N-terminal and C-terminal amino acids and peptide

maps (Fambrough, 1968). HeLa histones III and IV, like the corresponding components of calf thymus and pea bud, can be resolved electrophoretically (Fig. 8g). In contrast to lysine-rich and slightly lysine-rich histones, the histone peak III-IV of mammalian and plant sources is extremely similar in composition, indicating a high degree of homology.

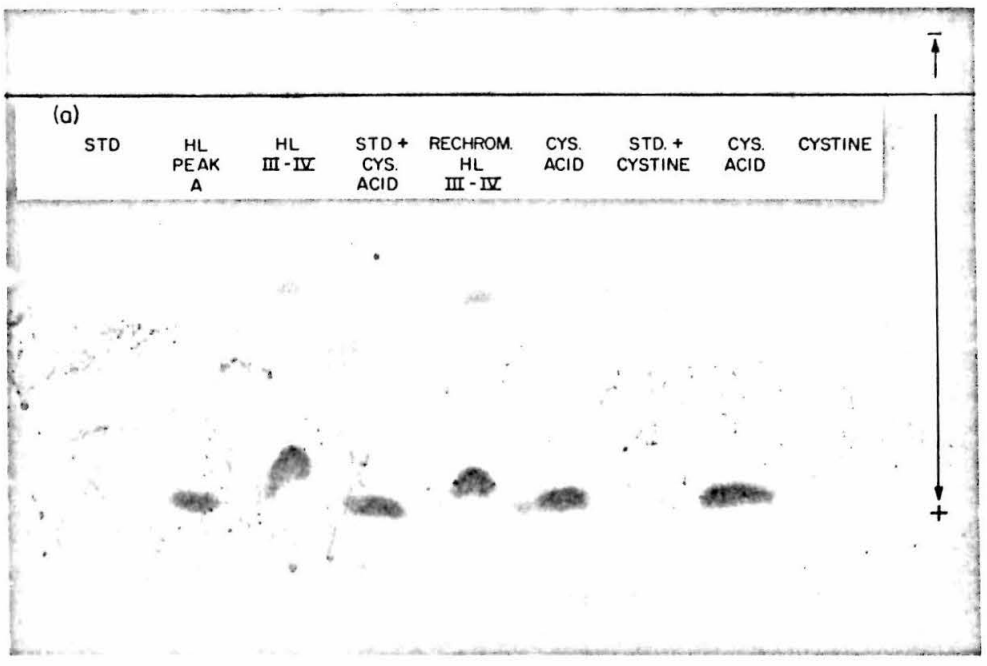
4. The Presence of Cysteine in Arginine-rich Histones

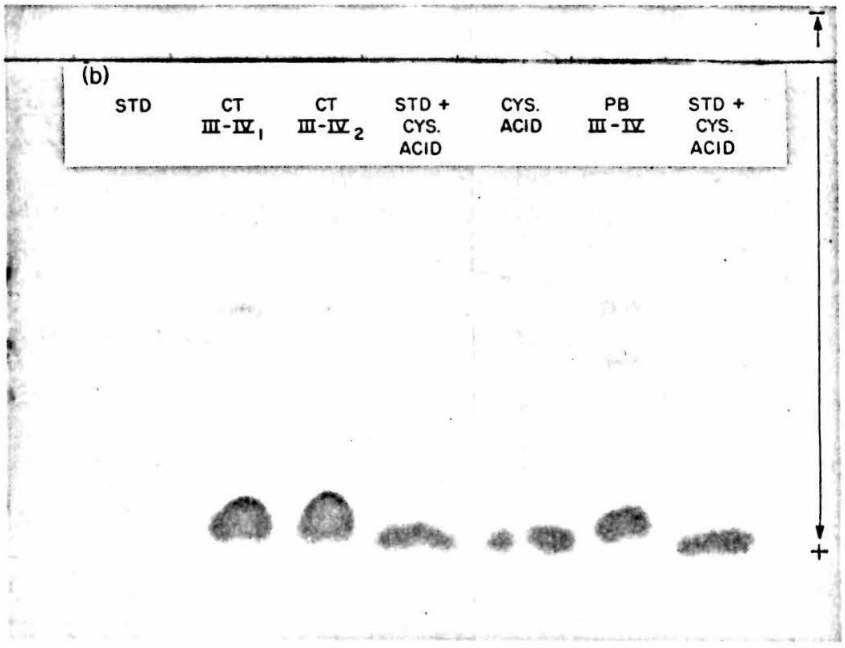
Daly and Mirsky (1954-55) observed the presence of cysteine in arginine-rich histones prepared by precipitation from aqueous solution at pH 10.6 but did not find it in lysine-rich histones. Since then a number of workers have reported cysteine to be present in appreciable quantities only in fraction f3 (histone III) isolated from several sources, such as, rat thymus (Deakin et al., 1963), chicken erythrocytes (Blazsek and Bukaresti, 1964) and calf thymus (Phillips, 1965). Lysine-rich histones in all these cases contain very little, if any, cysteine. In contrast, other workers failed to find any cysteine in fraction f3 (prepared as above) and considered histones to be free of this amino acid (Hnilica and Bess, 1965; Hnilica, 1965). Further, the arginine-rich peak III-IV, eluted from Amberlite CG-50 column has been reported to lack cysteine (Murray, 1962b; Fambrough and Bonner, 1966) and it was once proposed that the presence of cysteine in arginine-rich histones was probably due to non-histone contaminants (Murray, 1962a). The question of the presence of cysteine in peak III-IV was, therefore, reinvestigated.

It has been shown earlier in this Chapter that peak III-IV is

essentially free of any contamination by the proteins of peak A (run-off peak) due to ionic and hydrogen bond interactions, and it was argued that contamination due to links through divalent cations can not be appreciable. In the initial studies, high voltage paper electrophoresis at pH 1.62 was employed to analyze hydrolysates of peak III-IV because it provides an unambiguous identification of cysteic acid. In this method, cysteic acid alone migrates towards the positive electrode whereas all other amino acids found in protein hydrolysates migrate towards the negative electrode. Peak III-IV obtained from the chromatin of HeLa cells, calf thymus and pea bud was subjected to oxidation with performic acid to convert all cystine and cysteine to cysteic acid and then acid hydrolyzed. The results of analysis of these hydrolysates by paper electrophoresis are presented in Figs. 6a and b which show only the portion of electrophoregram extending from the origin towards the positive electrode. Fig. 6a also includes the results of electrophoresing hydrolysates of the chromatographic peak A and rechromatographed peak III-IV (obtained from the experiments described in Fig. 5) of HeLa cells. These results clearly demonstrate the presence of cysteine in the arginine-rich peak III-IV of HeLa cells, calf thymus and pea bud as well as in peak A of HeLa cells. Paper electrophoresis revealed the presence of only small traces (< 0.05 mole %) of cysteine in HeLa histone Ib and II. This was most likely due to contamination of these histones by arginine-rich histones, as shown by gel electrophoretic studies presented in Fig. 8 here and other lines of evidence reported by Fambrough (1968).

Figure 6. High voltage paper electrophoresis of protein hydrolysates in 6.7% formic acid (pH 1.62). See Materials and Methods for details. In both (a) and (b), only the portion of electrophoregram extending from origin towards the positive electrode is shown. All protein samples were oxidized by performic acid before acid hydrolysis. Following abbreviations are used: HL - HeLa cells; CT - calf thymus; PB - pea bud; III-IV - arginine-rich peak III-IV eluted from Amberlite CG-50; Rechrom. - rechromatographed (cf. Figure 5); STD - mixture of all amino acids found in proteins except cysteine (and cystine); Cys. acid - cysteic acid. CT III-IV_1 and CT III-IV_2 in (b) refer to two separate preparations.


(a)
STD
HL PEAK A
HL III-IV
STD + CYS. ACID
RECHROM. HL III-IV
CYS. ACID
STD. + CYSTINE
CYS. ACID
CYSTINE
−
+


(b)
STD
CT III-IV1
CT III-IV2
STD + CYS. ACID
CYS. ACID
PB III-IV
STD + CYS. ACID
−
+

An amino acid analyzer was used for quantitative determination of cysteine content in various histone fractions which also had been oxidized by performic acid before acid hydrolysis. The data are given in Table 2. Studies to be presented later in this Chapter will demonstrate the presence of cysteine only in histone III (f3) and will further clarify the role of cysteine in the structure of histones.

5. Fractionation of Histones by Disc Electrophoresis

Fig. 7 shows a typical polyacrylamide gel electrophoretic pattern of HeLa histone sulfates and its densitometer tracing. It may be noted that no anidoschwarz-stainable proteins are left behind at the origin and essentially all of the strainable proteins migrate towards the negative electrode. As discussed later (pages 156-159), this observation has bearing on the question of the presence of aggregated non-histone proteins in the H_2SO_4-extract of interphase chromosomes.

The various histone species appear as four major electrophoretic bands which will be referred to as A, B, C and D in order of increasing electrophoretic mobilities as shown in Fig. 8a. These bands have been correlated with the several chromatographic fractions by comparing the electrophoretic behavior of each fraction (tubes pooled as indicated in Figs. 3 and 4) with that of whole histones. The results of this study are shown in Fig. 8. The run-off peak (peak A)[1] is a highly heterogeneous fraction which yields a large number of

[1] The chromatographic peak A (or run-off peak) is not to be confused with the electrophoretic band A in Figs. 8, 15 and 16.

Figure 7. Photograph (above) and densitometer tracing (below) of polyacrylamide gel electrophoretic pattern of H_2SO_4-extracted proteins from interphase chromosomes. Peaks at the far left represent the origin of the gel, and not protein.

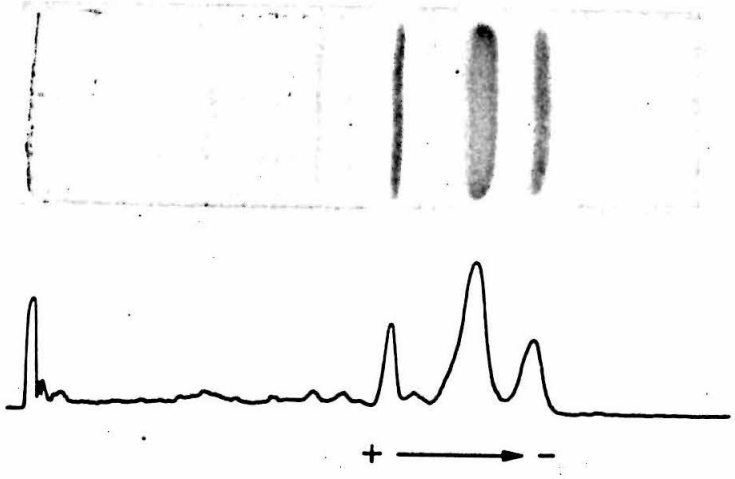
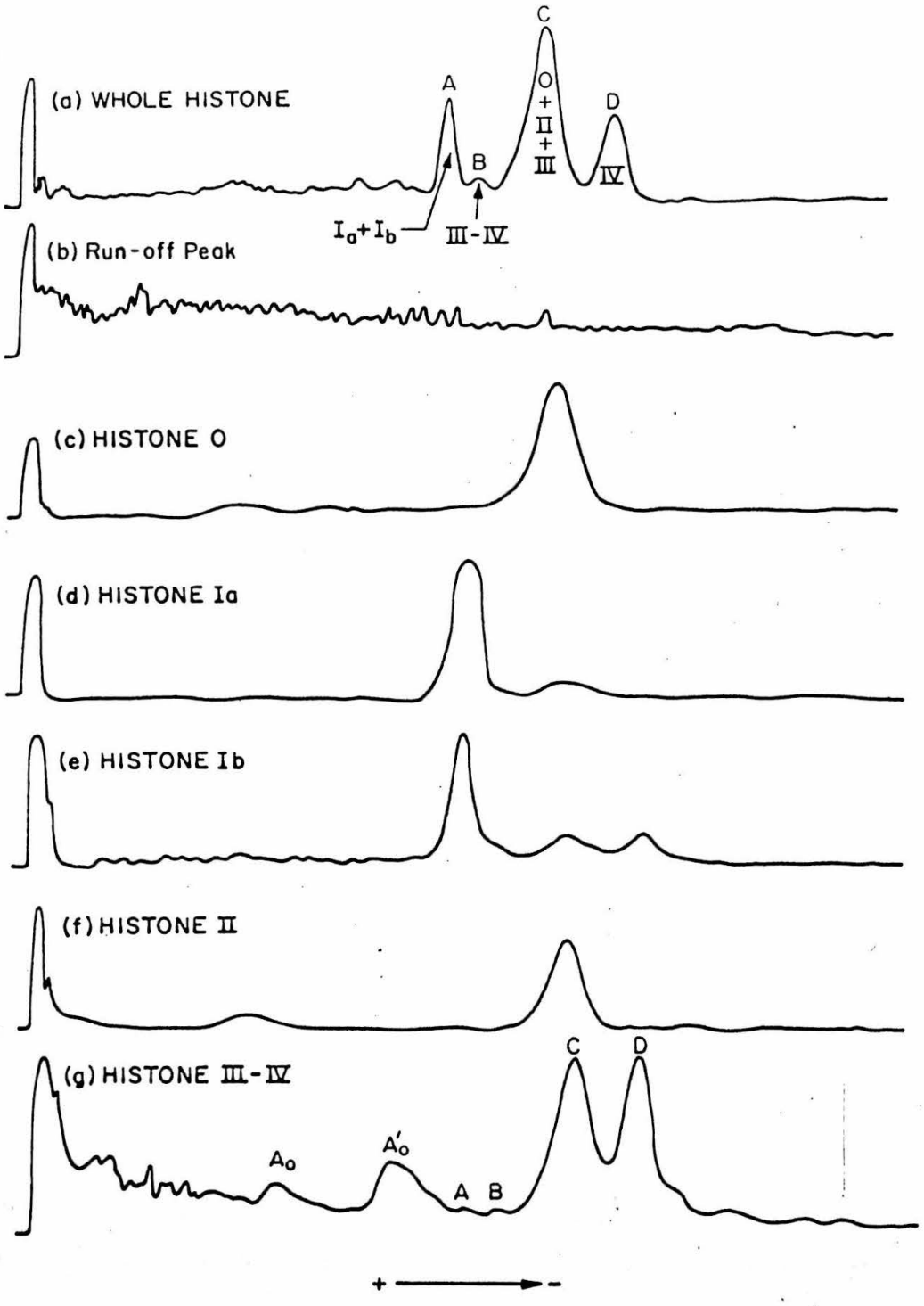


Figure 8. Densitometer tracings of disc electrophoretic patterns of interphase chromosomal histone sulfates. (a) Whole histone (H_2SO_4-extracted); (b) to (g) various chromatographic fractions from Figure 3 or 4. Note that the chromatographic peak A is referred to in this figure as the run-off peak to avoid confusion with the electrophoretic band A.


(a) WHOLE HISTONE
A
B
C
O + II + III
D
IV
I_a+I_b
III-IV
(b) Run-off Peak
(c) HISTONE O
(d) HISTONE Ia
(e) HISTONE Ib
(f) HISTONE II
(g) HISTONE III-IV
A_o
A'_o
A
B
C
D
+
-

electrophoretic bands with different mobilities (Fig. 8b). Some of these bands migrate as rapidly as one of the major histone bands (band C).

The major component in the case of both lysine-rich histones (Ia and Ib) migrates with a mobility equal to that of the slowest of the main electrophoretic histone bands, band A (Figs. 8d and e). Histone Ia also contains a minor component (comprising 10 to 20% of the total mass of this fraction) which has the same mobility as band C. Similarly, histone Ib has two minor components which migrate as fast as bands C and D respectively. These two minor components may comprise as much as 35 to 40% of the total material in this fraction. Although there is no explicit evidence, it is likely that these two components are identical to histone II (or O or III) and IV respectively and thus should be treated as contaminants of the chromatographic lysine-rich fractions, Ia and Ib. However, the possibility that these minor components may be genuine histone species distinct from other identifiable species can not be eliminated on the basis of data presented here. Strictly speaking, a comparison of amino acid composition, N- and C-terminal groups and peptide maps of these minor components (purified by preparative disc electrophoresis) with those of other histones can alone settle the question of their identity.

The slightly lysine-rich histones O and II migrate with identical electrophoretic mobilities, equal to that of band C (Figs. 8c and f). A component of varying proportion (5 to 10%) in both histones moves

much more slowly and is difficult to correlate with any particular chromatographic component. The existence of two components of histone II (IIb1 and IIb2) is indicated by the two differently stained amidoschwarz bands in the major electrophoretic band yielded by histone II; a somewhat brownish band moves slower than the more bluish band.

The arginine-rich peak III-IV yields two major electrophoretic bands together with several minor bands (Fig. 8g). The slower of the two major bands appears to consist of histone III (the cysteine containing arginine-rich histone) along with possibly some form of histone II. Evidence indicating the presence of some component other than histone III in the slower band will be presented in the next section. The faster of the two bands, termed histone IV, is most probably identical to the fastest electrophoresing histone IV of pea bud and calf thymus which lacks cysteine, and, relative to histone III, has a high glycine content and poor glutamic acid, alanine and proline content (Mauritzen et al., 1967; Fambrough, 1968). The identity of the minor electrophoretic bands (i.e., A_o, A_o'; A and B in Fig. 8g) belonging to the chromatographic peak III-IV is discussed in the next two sections.

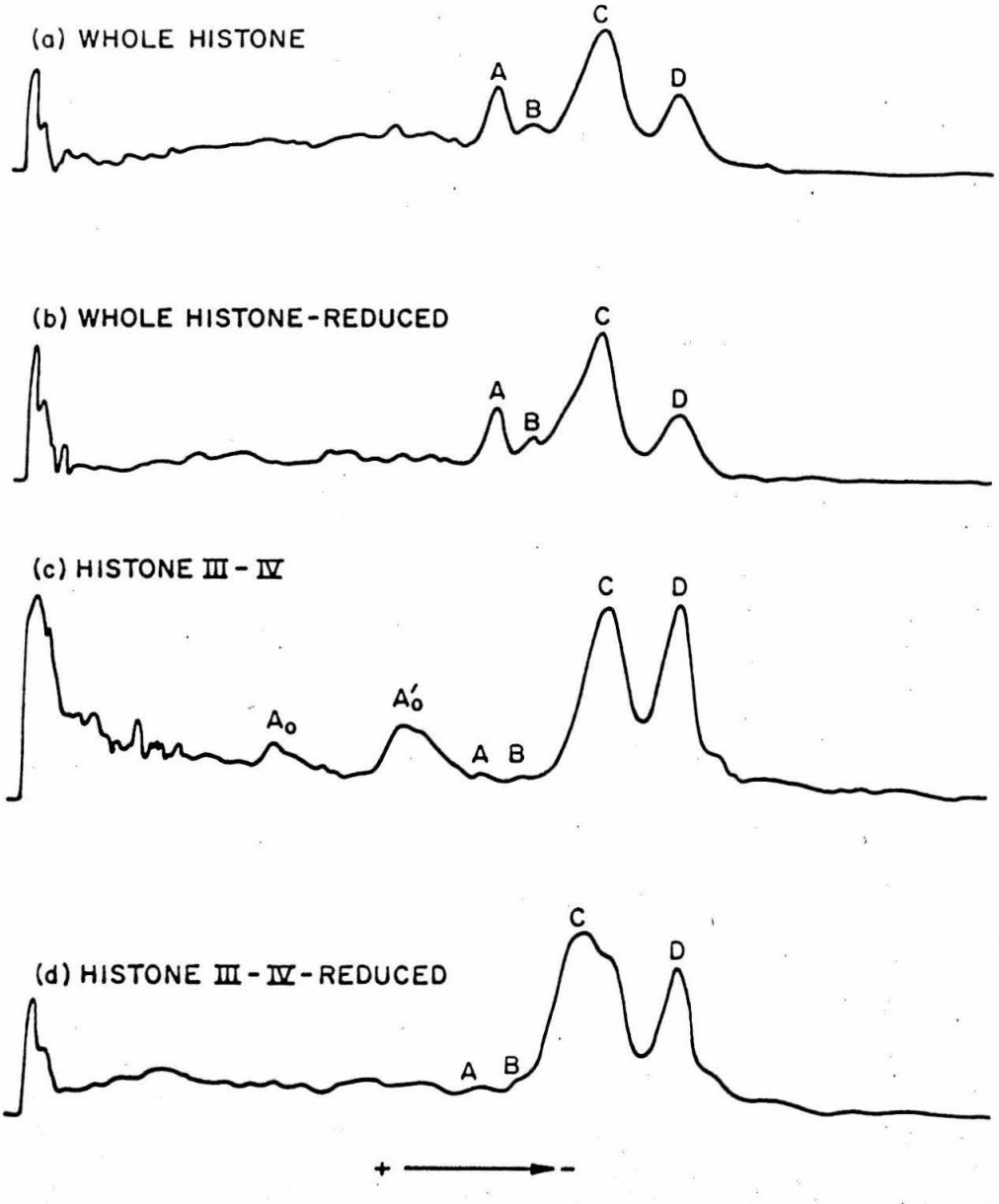
6. Role of Cysteine in the Structure of Arginine-rich Histones

The observation in previous studies of several slowly electrophoresing components in the arginine-rich fraction has been interpreted as indicating a marked heterogeneity of these histones. Such slow-moving components have also been observed in the present gel

electrophoretic studies (e.g. bands A_o and A'_o in Fig. 8g). These components have mobilities ranging from zero to 0.60 relative to histone IV (see Table 6 for the measured electrophoretic mobilities of bands A_o and A'_o). However, when the material of chromatographic peak III-IV is treated with β-mercaptoethanol, most of the slow-moving components disappear and the material therein is converted to a single component with an electrophoretic mobility identical to that of band C (Figs.9c and d). Similar observations on pea bud and calf thymus arginine-rich histones have also been reported by Fambrough (1968). It is important to note that this freshly reduced component is easily distinguishable from at least part of the material electrophoresing with a similar mobility before treatment with β-mercaptoethanol (the actual gels show even clearer separation of these two bands). The presence of two bands in this position is presumably due to the presence of some histone component other than histone III and IV in the peak III-IV.

The above results have been interpreted to mean that the slowly moving components (such as bands A_o and A'_o) consist of aggregates of histone III molecules linked together through intermolecular disulfide bands. On reduction, these disulfide bridges are broken and histone III appears as a faster electrophoresing monomer. It is, therefore, inferred that histone III in the arginine-rich peak III-IV eluted from Amberlite CG-50 exists as a mixture of monomers, dimers, trimers and multimers. These aggregates of histone III are stable in 10 M urea, 0.1 N acetic acid, 20% TCA and 40% guanidinium

Figure 9. Densitometer tracings of disc electrophoretic patterns of interphase whole histone (a, b) and chromatographic peak III-IV (c, d) both before and after reduction with β-mercaptoethanol. These histones were extracted by sulfuric acid.


(a) WHOLE HISTONE
A
B
C
D
(b) WHOLE HISTONE-REDUCED
A
B
C
D
(c) HISTONE III - IV
A_o
A'_o
A B
C
D
(d) HISTONE III - IV - REDUCED
A B
C
D
+
-

chloride. This capacity of histone III to form polymers may explain the heterogeneity of arginine-rich histones observed by earlier workers (Bellair and Mauritzen, 1965; Mauritzen et al., 1967). It is interesting to note that many of the cysteine-containing arginine-rich fractions obtained by exclusion chromatography by Mauritzen et al. (1967) have quite indistinguishable amino acid compositions and thus indeed may be polymers of histone III of the type indicated above. Hnilica and Bess (1965) have also presented evidence indicating that various components of f3 are aggregates of the same basic molecule.

Figs. 9a and b show the result of reducing whole histone sulfates of interphase chromosomes. Table 9 compares the proportional areas of different bands in unreduced and reduced whole histones. Such a comparison reveals that both unreduced and reduced interphase histones have identical proportions of band C, the band containing histone III. Considered together with the fact that no stainable proteins remain at the origin of the gel, these data suggest that unchromatographed whole histone preparation, freshly isolated from interphase chromosomes, contains essentially all of its histone III in a reduced form. The polymerized forms of histone III in peak III-IV must have resulted from subsequent oxidation during chromatography on Amberlite CG-50 at pH 6.8. In light of this conclusion, it seems likely that very little of histone III, if any, exists in the oxidized form in interphase chromosomes in vivo. A similar conclusion has also been drawn by Fambrough (1968) in the case of histone III of pea

bud and calf thymus.

7. A Minor Component in the Arginine-rich Histone Peak III-IV

Although reduction of the chromatographic peak III-IV has allowed us to identify most of the slowly electrophoresing bands in this fractions as polymers of histone III, two other minor bands of this peak apparently are not oxidized forms of histone III. These bands, referred to as A and B in Fig. 8g, are unaffected by reduction with β-mercaptoethanol (see Figs. 9c and d) and have repeatedly been observed in peak III-IV. The mobilities of these bands are identical to those of bands A and B respectively of the whole histones (cf. Figs. 8a and g, Table 6). However, it is not yet possible to decide whether band A of the chromatographic peak III-IV signifies a bona fide new histone species or whether it is identical to one of the lysine-rich histones (Ia and Ib) with which it coelectrophoreses and hence a contaminant of peak III-IV. This question can be answered only by isolation of this minor band in large quantities by preparative disc electrophoreses of peak III-IV so that chemical studies could be carried out. In contrast, the electrophoretic band B yielded by whole histones (Fig. 8a) can now be unequivocally identified as a minor component of the chromatographic peak III-IV. A comparable component has not been demonstrated in the calf thymus and pea bud histones.

C. HISTONES AND OTHER ACID-SOLUBLE PROTEINS OF METAPHASE CHROMOSOMES

1. Quantitative Estimation of Histones in Acid Extracts of Chromosomes

Earlier in this Chapter it was shown that 0.2 N HCl extracts twice as much protein from metaphase chromosomes as does 0.2 N H_2SO_4 whereas both 0.2 N HCl and 0.2 N H_2SO_4 extract the same amount of protein from interphase chromosomes (Table 1). This suggests that metaphase chromosomes possess an HCl-soluble protein fraction absent from interphase chromosomes. The following experiment was designed to answer the question: Is this HCl-soluble material, specifically associated with metaphase chromosomes, histone or some other acid-soluble non-histone protein? This experiment also proposes a general method for estimating the amount of histone in a given mixture of proteins.

It was assumed that if all of the proteins in an acid extract are identical to the major interphase histones (O, Ia, Ib, II, III and IV) they should coelectrophorese in polyacrylamide gel with one of the four major histone bands (A, B, C and D of Fig. 8a). Further, any protein unlike these major histones will be characterized by a different mobility (in general less than that of histones, as will be seen below) and thus will not contribute to the four electrophoretic bands characteristic of HeLa histones. Therefore, the total area under the electrophoretic bands A, B, C and D yielded by a given amount of acid-extracted protein should be proportional to as well as a measure of the fraction of this material which is histone. The results of such an

experiment are given in Table 4. In this experiment, the chromatographic histone fraction II from H_2SO_4-extracted interphase histones was chosen as a reference standard for correlating the histone mass with the area under an electrophoretic band determined in arbitrary units from densitometer tracings. The choice of histone II as a standard is valid because approximately 90 to 95% of the material in this fraction electrophoreses as a homogeneous band (the remaining material appears in a second minor band; cf. Fig. 8f), leaving behind no stainable protein at the origin of the gel (see Fig. 10). In addition, all histone components have identical staining constants (optical density of amidoschwarz dye bound per unit weight protein) within the concentration ranges used in the present studies (Fambrough et al., in press).

The data presented in Table 4 show that about 80% of the proteins extracted by 0.2 N H_2SO_4 from interphase chromosomes are histones, the remaining being non-histones. Among the proteins extracted from metaphase chromosomes by 0.2 N H_2SO_4, approximately 62% are histones, whereas only 32% of the proteins extracted by 0.2 N HCl from these chromosomes behave as histones. From these facts it is concluded that in contrast to interphase chromosomes, significantly larger amounts of acid-soluble non-histone proteins are associated with metaphase chromosomes; a fraction of these additional proteins is soluble in 0.2 N HCl but not in 0.2 N H_2SO_4. It is also possible to estimate the 'true' histone/DNA ratios in chromosomes from the data reported in Tables 1 and 4. The last column of Table 4

Figure 10. Photograph of polyacrylamide gels showing disc electrophoretic patterns of three preparations of chromatographic histone fraction II extracted from interphase chromosomes by 0.4 N sulfuric acid. Note the absence of amidoschwarz-stainable protein at the origins of the gels. The preparation which was employed as a standard in the analysis of data in Table 4 is shown in the polyacrylamide gel on the far left. Of all the available preparations of fraction II, this was least contaminated by the slower electrophoresing minor component.

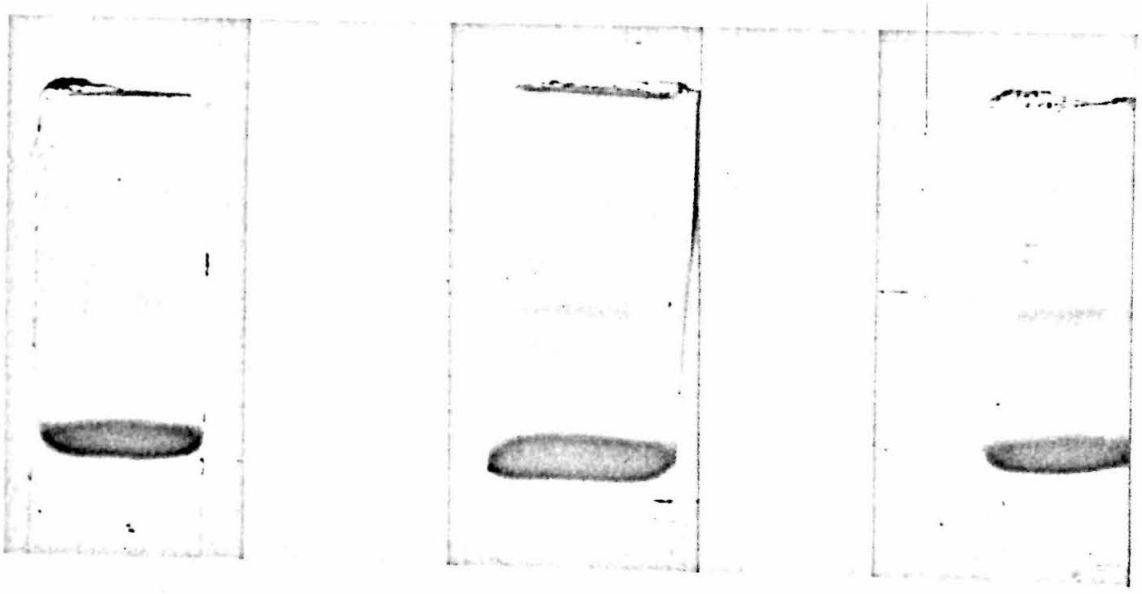


Table 4. Quantitative Estimation of Histones in Chromosomal Acid-Soluble Proteins by Disc Electrophoresis

Protein Electrophoresed	Area[a] of Histone Bands/ μg Protein Placed on Gels	Fraction of Total Proteins as Histones	Fraction of Total Proteins as Non-histones	Histone/DNA (mass ratio)[b]
Interphase H_2SO_4-extracted Histone II[c]	3.00	1.00	-	-
H_2SO_4 Extract of Interphase Chromosomes[d]	2.40	0.80	0.20	0.82
H_2SO_4 Extract of Metaphase Chromosomes[e]	1.85	0.62	0.38	0.82
HCl Extract of Metaphase Chromosomes[e]	0.97	0.32	0.68	0.82

[a]Measured by transferring the densitometer tracing to a tracing paper of uniform weight, cutting the tracing and weighing. Expressed in arbitrary units.

[b]Computed from the data presented in Table 1.

[c]See the polyacrylamide gel on the far left in Fig. 10 for its electrophoretic pattern.

[d]Sheared chromatin prepared by the Sucrose Sedimentation Method.

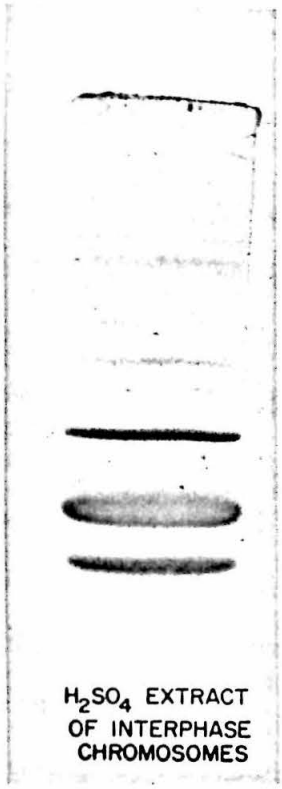
[e]Prepared at neutral pH.

demonstrates that the ratio of histone to DNA is identical in interphase and metaphase chromosomes despite the fact that the latter have much more acid-soluble proteins than do interphase chromosomes.

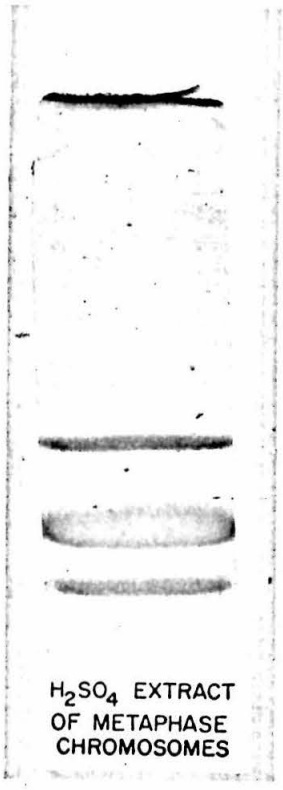
2. The Nature of Acid-soluble Non-histone Proteins

The inferences drawn above lead us to the next question: What is the nature and origin of these acid-soluble non-histone proteins of interphase and metaphase chromosomes? Although we are far from answering this important question, an interesting clue regarding one of their characteristics has been obtained. In Fig. 11, it is seen that very little, if any, amidoschwarz-stainable proteins are left behind at the origin of the gel when H_2SO_4-extracted proteins of interphase chromosomes are electrophoresed. In contrast, a considerable amount of protein remains at the origin of the gel when H_2SO_4-extracted proteins of metaphase chromosomes are electrophoresed. Even a greater fraction of the proteins extracted by HCl from metaphase chromosomes does not migrate and stays at the origin; certain other proteins in the HCl-extract of metaphase chromosomes do migrate slowly as multiple bands and are visible near the origin of the gel (Fig. 11). The slowly electrophoresing material near the gel origin can also be seen in the densitometer tracing of this gel in Fig. 14. A possible interpretation of the above observations is that the metaphase chromosomal proteins remaining at the origin are larger than the gel pores and, therefore, fail to migrate. In this case, large size of proteins may be a result of polymerization caused by

Figure 11. Photograph of polyacrylamide gels showing disc electrophoretic patterns of acid-extracted proteins of interphase and metaphase chromosomes. Note the presence of amidoschwarz-stainable proteins at the origins of the gels when acid-extracted metaphase chromosomal proteins are electrophoresed. See text for further explanation.



H_2SO_4 EXTRACT
OF INTERPHASE
CHROMOSOMES



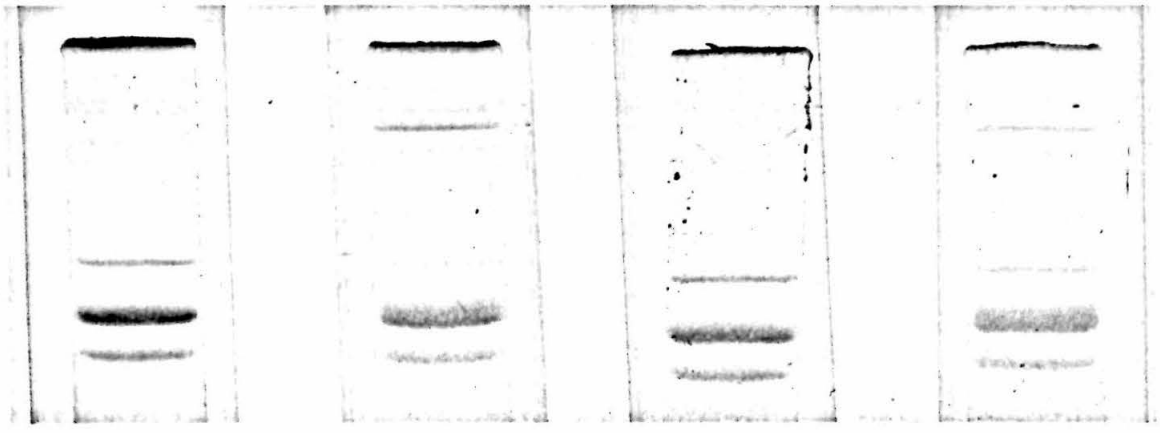
H_2SO_4 EXTRACT
OF METAPHASE
CHROMOSOMES

HCl EXTRACT
OF METAPHASE
CHROMOSOMES

intermolecular disulfide bridges. The results presented in Fig. 12 provide evidence in favor of this interpretation. It is seen that the proteins remaining at the origin can be caused to migrate toward the negative electrode if the HCl- or H_2SO_4-extracted proteins of metaphase chromosomes are treated with β-mercaptoethanol before electrophoresis. Following reduction of metaphase chromosomal acid-soluble proteins, much of the protein material disappears from the gel origin and slowly electrophoreses as multiple bands, some fairly prominent. It is possible that a longer treatment with β-mercaptoethanol would make all of the proteins at the origin to migrate. In view of these findings, we propose that a large but yet unknown proportion of acid-soluble non-histone proteins associated with metaphase chromosomes is aggregated by intermolecular disulfide (S-S) bonds. Available evidence further suggests that most of the acid-soluble non-histone proteins of interphase chromosomes lack such disulfide bridges. At a later stage in this Chapter, data will be presented which indicate that a fraction of histone III in metaphase chromosomes is also polymerized through S-S bonds. Nevertheless, quantitative considerations allow us to infer that the bulk of reducible non-electrophoresing acid-soluble material of metaphase chromosomes is not histone III.

A large amount of RNA is found in metaphase chromosomes as shown in Table 1. It has been reported that over 80% of this RNA is similar to ribosomal RNA (Huberman and Attardi, 1966; Maio and Schildkraut, 1967). Since ribosomes are known to possess acid-

Figure 12. Photograph of polyacrylamide gels showing disc electrophoretic patterns of acid-extracted proteins from metaphase chromosomes before and after reduction with β-mercaptoethanol. From left to right these are: unreduced HCl-extracted proteins; reduced HCl-extracted proteins unreduced H_2SO_4-extracted proteins; reduced H_2SO_4-extracted proteins. Note the disappearance of much of the amidoschwarz-stainable proteins from the gel origins following treatment with β-mercaptoethanol.



soluble proteins, it is conceivable that the additional acid-soluble proteins found in metaphase chromosomes could have a ribosomal origin. However, we found that only a small fraction of the proteins in deoxycholate-treated human liver ribosomes[1] is acid-soluble; 0.2 N H_2SO_4 extracted 9.5% of the total ribosomal proteins whereas 16.8% were extractable with 0.2 N HCl. Cohn and Simson (1963) observed that generally 25 to 30% of the proteins in rat liver ribosomes were extractable with 0.2 N HCl depending upon the method and the type of detergent employed for preparation of ribosomes. Assuming an RNA/protein mass ratio of 1.0 in ribosomes and all of the RNA in metaphase chromosomes to be of ribosomal origin, it can be argued that ribosomal proteins could constitute no more than 5 to 6% of the proteins in the acid (H_2SO_4 or HCl) extracts of these chromosomes. This suggests that a majority of the acid-soluble non-histone proteins of metaphase chromosomes are non-ribosomal. Nevertheless, it must be recognized that none of the available evidence rules out the possibility that acid-soluble ribosomal proteins <u>not</u> attached to ribosomal RNA may be selectively associated with metaphase chromosomes. In this way, ribosomal proteins could constitute a much greater proportion of the acid-extracted material than that just estimated on the assumption that the only source of ribosomal proteins is intact ribosomes.

It is appropriate here to draw attention to another related

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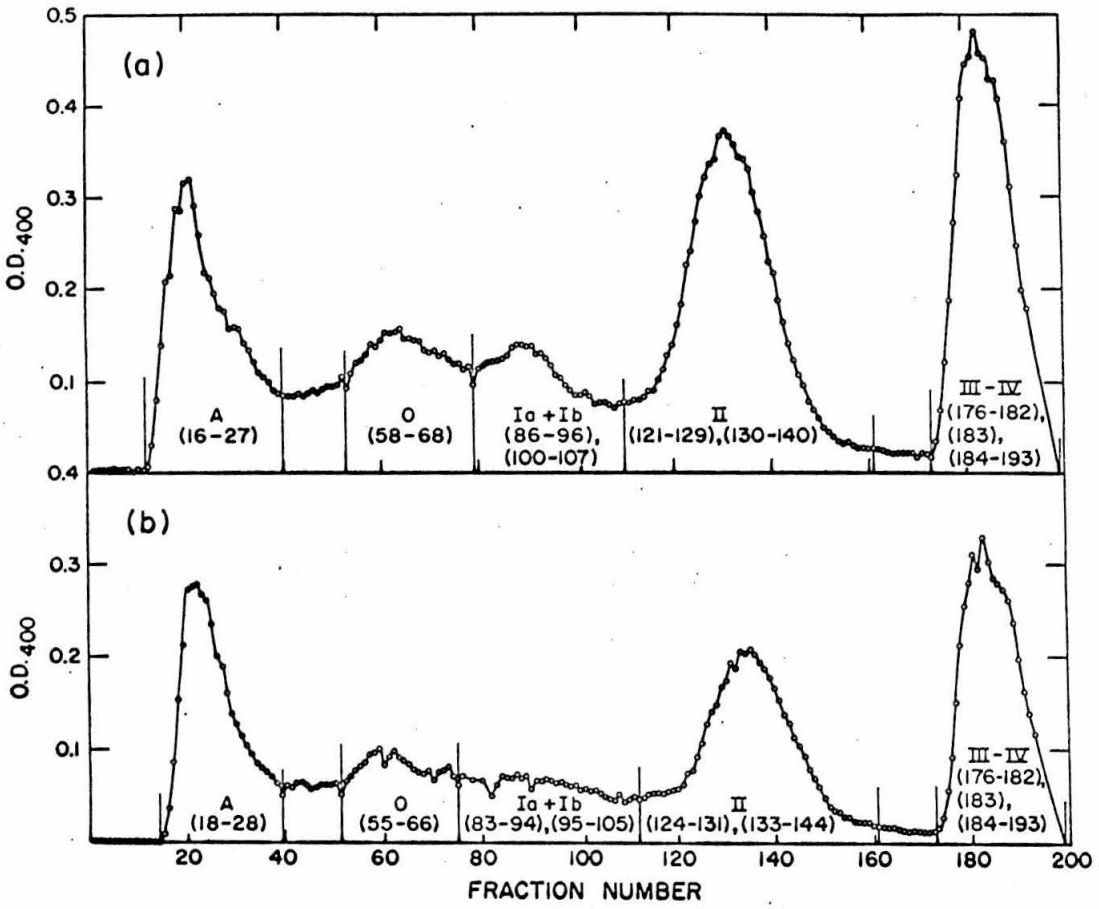
[1] The ribosomal preparation was kindly given to this author by Dr. Francessco Amaldi.

matter. The presence of such large amounts of acid-soluble non-histone proteins in metaphase chromosomes suggests that these proteins will most likely appear in chromatographic run-off peak eluted from the Amberlite CG-50 column. However, examination of the data presented in Figs. 13a and b and Table 8 reveals that only a fraction of these proteins appears in run-off peak. This poses the puzzle: Where have these acid-soluble non-histone proteins gone during chromatography? Although quantitative data were not obtained in this study, the question can be qualitatively answered by the following observation: it was repeatedly observed that a considerable proportion of the acid-extracted proteins of metaphase chromosomes did not dissolve in 8% GuCl-PO_4 and was, therefore, removed by centrifugation before chromatographing the clear supernatant. In all probability, the proteins insoluble in 8% GuCl-PO_4 represent the chromatographically unaccountable acid-soluble non-histone proteins because histones are known to be completely soluble under this condition.

3. Fractionation of Metaphase Histones by Column Chromatography

Fractionation of metaphase histones extracted with H_2SO_4 or HCl on an analytical column of Amberlite CG-50 is shown in Fig. 13. The various histone fractions have been named according to the nomenclature employed and discussed earlier for interphase histones (see Fig. 3). The identification of various histone components in each fraction has been confirmed by gel electrophoresis as will be shown in the next section. The results demonstrate the presence of similar histone components in both extracts (H_2SO_4 and HCl) of metaphase

Figure 13. Fractionation of metaphase histones on Amberlite CG-50 (analytical column). Gradient of guanidinium chloride was same as described in Figure 3. The fractions pooled for disc electrophoretic studies are indicated by numbers in parentheses (cf. Figures 15 and 16). Vertical lines mark off the areas for estimation of relative proportions of various fractions as shown in Table 8. (a) H_2SO_4-extracted histones, (b) HCl-extracted histones.

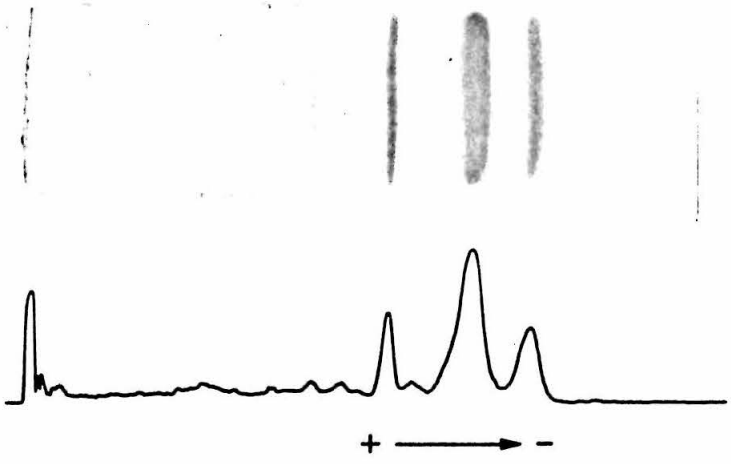

(a)
O.D.400
0.5
0.4
0.3
0.2
0.1
0.4
A
(16-27)
O
(58-68)
Ia + Ib
(86-96),
(100-107)
II
(121-129),(130-140)
III-IV
(176-182),
(183),
(184-193)
(b)
O.D.400
0.3
0.2
0.1
A
(18-28)
O
(55-66)
Ia + Ib
(83-94),(95-105)
II
(124-131),(133-144)
III-IV
(176-182),
(183),
(184-193)
20
40
60
80
100
120
140
160
180
200
FRACTION NUMBER

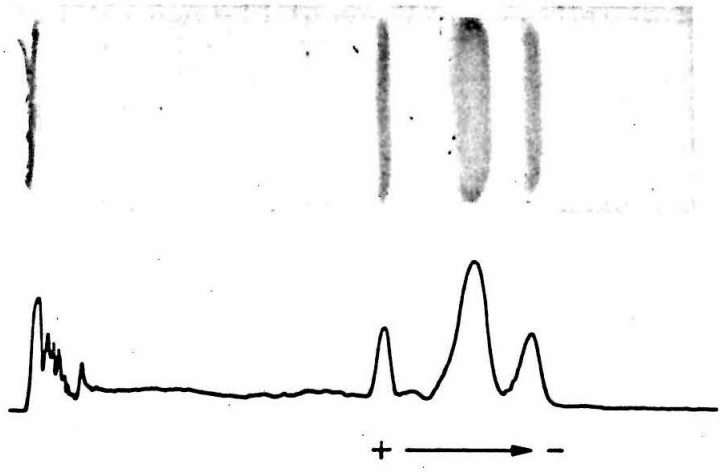
chromosomes and that these components are similar to those found in H_2SO_4-extract of interphase chromosomes (cf. Figs. 3, 13a, 13b). However, certain differences may be noted of which the most notable qualitative difference is in regard to the histone I fraction. In contrast to the resolution of interphase histone Ia and Ib fractions, no such resolution of these histone components isolated from metaphase chromosomes is detectable. The cause of this lack of resolution is not yet clear. In addition, for some obscure reason HCl-extracted histones of metaphase chromosomes do not even reveal a peak for the lysine-rich fractions, whereas the peak is definitely obtained in the case of the H_2SO_4-extract of the same chromosomes. This last difference could conceivably reflect a difference in the configuration of histone chlorides and sulfates; a chromatographic fractionation of histone chlorides of interphase chromosomes should provide an interesting comparison. Important quantitative differences, determined chromatographically, among interphase and metaphase histones will be described later.

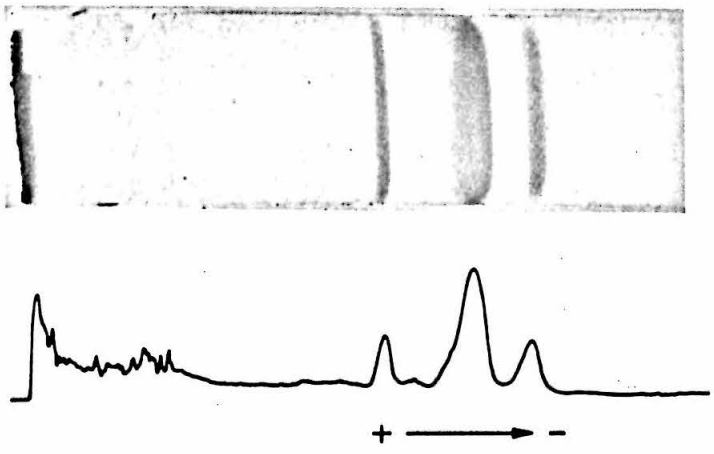
4. Fractionation of Metaphase Histones by Disc Electrophoresis

Fig. 14 compares the photographs and densitometer tracings of disc electrophoretic patterns of H_2SO_4-extract of interphase chromosomes with those of H_2SO_4- and HCl-extracts of metaphase chromosomes. A striking similarity is revealed among all three cases with respect to the four major histone bands (A, B, C and D of Figs. 8a, 15a and 16a). The differences related to the acid-soluble non-histone proteins of interphase and metaphase chromo-

Figure 14. Photographs and densitometer tracings of polyacrylamide gel electrophoretic patterns of acid-extracted proteins from interphase and metaphase chromosomes. From top to bottom each pair of photograph and densitometer tracing is H_2SO_4-extracted proteins of interphase chromosomes; H_2SO_4-extracted proteins of metaphase chromosomes; HCl-extracted proteins of metaphase chromosomes.







somes have been discussed earlier. Further evidence in support of the similarity of interphase and metaphase histones was obtained by studying the electrophoretic behavior of the various chromatographic fractions described in Figs. 13a and b. The fractions pooled for disc electrophoresis are indicated in parentheses in the figures referred to above. The electrophoretic properties of various chromatographic fractions of H_2SO_4- and HCl-extracted metaphase histones are shown in Figs. 15 and 16 respectively and should be compared with those of H_2SO_4-extracted interphase histones shown earlier in Fig. 8. This study demonstrates that the four major histone bands of whole metaphase histones are comprised of the same components as those comprising the corresponding bands of whole interphase histones. Once again, like the different fractions of interphase histones, each chromatographic fraction of metaphase histones is made up of two or more components which are electrophoretically indistinguishable from the corresponding interphase components. Therefore, it may be concluded that no new major histones appear in metaphase chromosomes. However, one may note the presence of two minor electrophoretic bands in the histone fraction Ib of metaphase chromosomes (Figs. 15e and 16e) which have not been observed in the comparable chromatographic fraction of interphase histones. It is not yet known whether these are non-histone contaminants or not.

As already demonstrated for the chromatographic peak III-IV of interphase histones, this peak as isolated from metaphase histones also yields several electrophoretic bands (A_o, A'_o, A and B) which

Figure 15. Densitometer tracings of disc electrophoretic patterns of metaphase chromosomal histone sulfates. (a) Whole histone (H_2SO_4-extracted); (b) to (g) various chromatographic fractions from Figure 13a as indicated parenthetically. Note that the chromatographic peak A is referred to in this figure as the run-off peak to avoid confusion with the electrophoretic band A.

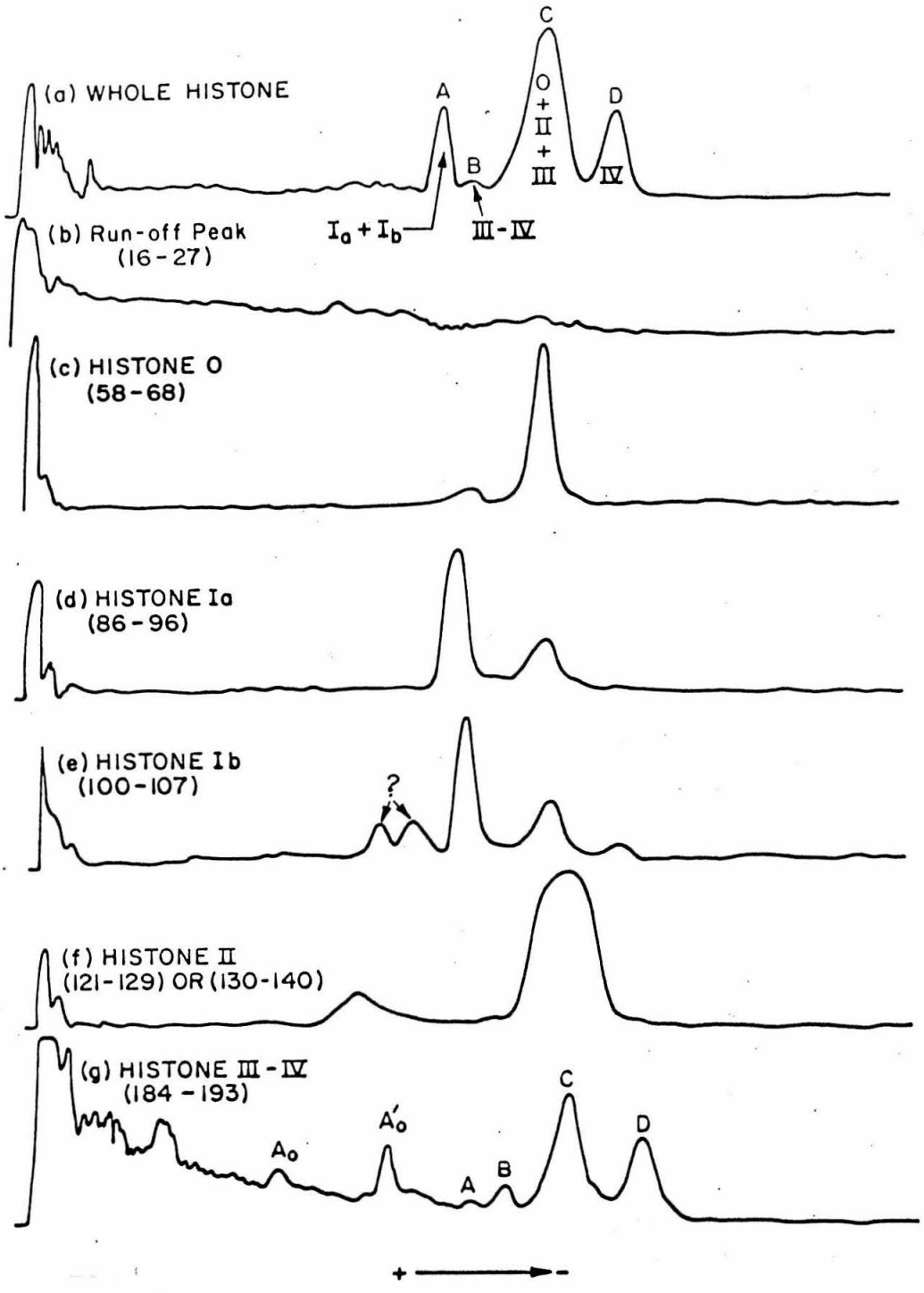
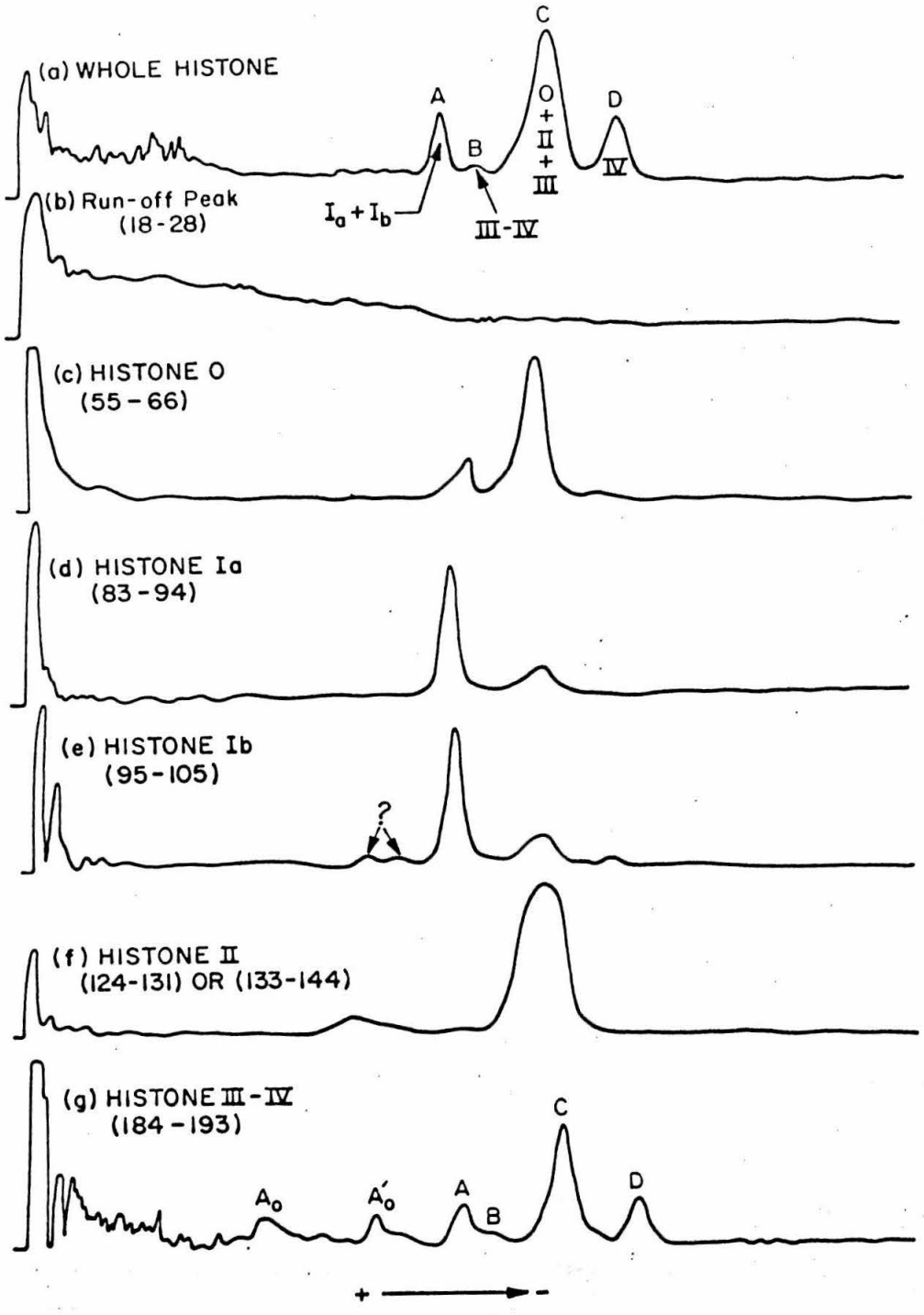

(a) WHOLE HISTONE
A
B
C
O + II + III
D
IV
(b) Run-off Peak
(16 - 27)
$I_a + I_b$
III - IV
(c) HISTONE O
(58 - 68)
(d) HISTONE Ia
(86 - 96)
(e) HISTONE Ib
(100 - 107)
?
(f) HISTONE II
(121 - 129) OR (130 - 140)
(g) HISTONE III - IV
(184 - 193)
A_o
A'_o
A
B
C
D
+
-

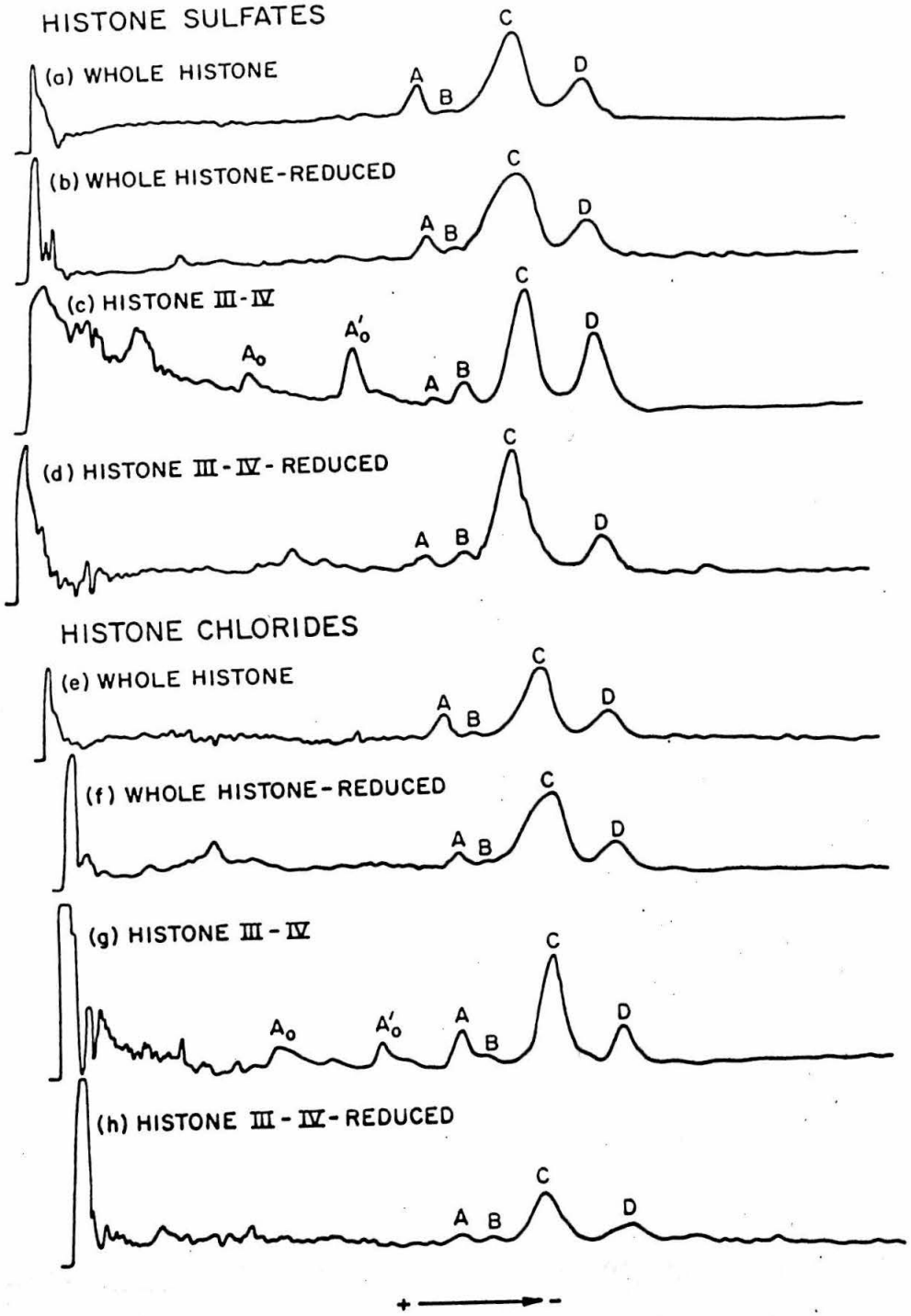
Figure 16. Densitometer tracings of disc electrophoretic patterns of metaphase chromosomal histone chlorides. (a) Whole histone (HCl-extracted); (b) to (g) various chromatographic fractions from Figure 13b as indicated parenthetically. Note that the chromatographic peak A is referred to in this figure as the run-off peak to avoid confusion with the electrophoretic band A.


(a) WHOLE HISTONE
A
B
C
O + II + III
D
IV
(b) Run-off Peak (18-28)
$I_a + I_b$
III-IV
(c) HISTONE O (55-66)
(d) HISTONE Ia (83-94)
(e) HISTONE Ib (95-105)
?
(f) HISTONE II (124-131) OR (133-144)
(g) HISTONE III-IV (184-193)
A_o
A'_o
A
B
C
D
+
-

electrophorese with mobilities less than those of the major bands (C and D) of peak III-IV (Figs. 15g and 16g). In addition, several other bands with mobilities ranging from zero to 0.6 of the mobility of the histone IV band can also be seen (cf. Table 6). Once again, as with interphase chromatographic peak III-IV, treatment of H_2SO_4- or HCl-extracted peak III-IV of metaphase histones with β-mercaptoethanol causes most of these slowly moving bands (but not bands A and B) to disappear (Figs. 17c-d and g-h). After reduction, the slowly electrophoresing material coelectrophoreses with band C and is thus identified as histone III. Bands A_o and A'_o, together with other reducible slower bands, have been interpreted to consist of histone III polymerized through intermolecular disulfide links. However, bands A and B in the chromatographic peak III-IV material of metaphase chromosomes are not affected by reduction and are thus not a result of polymerization of histone III due to disulfide bridges (Figs. 17d and h). Thus the properties of bands A and B isolated from peak III-IV material appear to be similar in interphase and metaphase histones. The question of whether electrophoretic bands A and B of metaphase peak III-IV can be regarded as additional bona fide components of this chromatographic peak will not be discussed here since the comments made earlier on the corresponding bands of interphase peak III-IV also apply in this case (see page 150).

In spite of the almost complete similarity of interphase and metaphase histones as shown by electrophoretic studies presented above, it is conceivable that these histones may differ in some more

Figure 17. Densitometer tracings of disc electrophoretic patterns of metaphase whole histone and the chromatographic peak III-IV both before and after reduction with β-mercaptoethanol. (a) to (d) H_2SO_4-extracted; (e) to (h) HCl-extracted.


HISTONE SULFATES
(a) WHOLE HISTONE
(b) WHOLE HISTONE-REDUCED
(c) HISTONE III-IV
(d) HISTONE III-IV- REDUCED
HISTONE CHLORIDES
(e) WHOLE HISTONE
(f) WHOLE HISTONE-REDUCED
(g) HISTONE III-IV
(h) HISTONE III-IV- REDUCED
A
B
C
D
A_o
A'_o
+
-

subtle manner, such as in their degree of phosphorylation or in some other aspect of their configuration. It was recognized that such subtle differences may not have been revealed by the qualitative comparisons made so far, but might be detectable if more detailed quantitative comparison of electrophoretic mobilities is carried out. Initial experiments showed that mixtures of H_2SO_4-extracted interphase and metaphase whole histones as well as of H_2SO_4- and HCl-extracted metaphase whole histones yielded electrophoretic patterns indistinguishable from those shown in Fig. 7 or Fig. 14. This indicates that the electrophoretic mobilities of various components of interphase and metaphase histones are essentially identical. In order to further substantiate the above conclusion, the electrophoretic mobilities (measured as distance from the origin of the gel to the center of a band on a densitometer tracing) of interphase and metaphase histone IV extracted with H_2SO_4 were compared. The results presented in Table 5 reveal that the differences observed in the mobility of interphase and metaphase histone IV are within the experimental error. Next, the electrophoretic mobilities of all major bands of whole histones and of bands A_o, A'_o, A, B, C and D yielded by chromatographic peak III-IV from interphase and metaphase chromosomes were measured. These mobilities, expressed as relative to the mobility of the histone IV band in the same gel, are given in Table 6. In some cases, the samples were electrophoresed for 135 minutes in addition to the standard period of 90 minutes in order to increase the possibility of detecting differences. The results of this detailed

Table 5. Comparison of Electrophoretic Mobilities of H_2SO_4-extracted Histone IV from Interphase and Metaphase Chromosomes

Experiment Number	Interphase Histone IV	Metaphase Histone IV	Standard Error Between Gels
1	1.000 (12.04)	0.991 (11.93)	1.7 to 1.8%
2	1.000 (12.13)	1.014 (12.30)	
3	1.000 (12.20)	0.992 (12.10)	
4	1.000 (11.60)	0.991 (11.50)	

Each value in parentheses indicates the distance (in cm) between the origin of the gel and the center of histone IV band. The measurements were made on densitometer tracings of the gels scanned at the same chart speed by a Canalco Model E Microdensitometer. Each value is a mean of mobilities of histone IV obtained by electrophoresing unfractionated histones on three separate gels in the same run.

Table 6. Relative Electrophoretic Mobilities of Major Histone Bands of Interphase and Metaphase Chromosomes

Bands[b]	H_2SO_4-extracted Interphase Histones				H_2SO_4-extracted Metaphase Histones				HCl-extracted Metaphase Histones			
	Whole Acid Extract		Histones III-IV[a]		Whole Acid Extract		Histones III-IV[a]		Whole Acid Extract		Histones III-IV[a]	
	90 min	135 min	90 min	135 min	90 min	135 min	90 min	135 min	90 min	135 min	90 min	135 min
A_o	-	-	0.39(3)	0.41(2)	-	-	0.39(1)	0.40(1)	-	-	0.36(1)	0.41(1)
A'_o	-	-	0.58(3)	0.59(2)	-	-	0.57(2)	0.58(2)	-	-	0.56(2)	0.59(1)
A	0.72(5)	-	0.70(3)	0.70(1)	0.71(7)	0.71(1)	0.71(3)	0.72(2)	0.71(11)	0.72(1)	0.71(4)	0.72(2)
B	0.77(4)	-	0.76(3)	-	0.77(5)	0.77(1)	0.77(2)	0.78(1)	0.77(8)	-	0.77(1)	0.78(1)
C	0.89(5)	-	0.89(4)	0.89(2)	0.88(7)	0.89(1)	0.88(4)	0.88(2)	0.89(11)	0.88(1)	0.87(4)	0.88(2)
D	1.00(5)	-	1.00(4)	1.00(2)	1.00(7)	1.00(1)	1.00(4)	1.00(2)	1.00(11)	1.00(1)	1.00(4)	1.00(2)

[a]Chromatographic peak III-IV.

[b]See Figs. 8, 15 and 16 for the names assigned to electrophoretic bands. The bands, yielded by whole histones, have been correlated with various chromatographic fractions as follows: A(Ia + Ib), B(III-IV), C(0 + II + III) and D(IV). A_o and A'_o of peak III-IV refer to polymerized forms of histone III.

All values reported in this table are relative to the mobility of histone IV in the same gel. The figures in parentheses indicate the number of gels used to compute the mean values. Several of the samples were electrophoresed for both 90 and 135 minute durations.

comparison in Table 6 failed to show any significant difference among histones of interphase and metaphase with respect to the mobilities of various components. Even the relative mobilities of polymerized histone III bands (A_o and A'_o) are identical in interphase and metaphase histones.

5. Comparison of Proportions of Various Histones of Interphase and Metaphase Chromosomes

Although, as demonstrated above, interphase and metaphase histones are comprised of the same components, it is possible that they may differ in the relative proportions of various components. Such a difference, if present, could be responsible for the coiled configuration of metaphase chromosomes. With this in mind, the proportions of electrophoretic bands A (Ia + Ib), C (O + II + III) and D (IV) were measured in different histone preparations. Band B (III-IV) was excluded from these computations since it constitutes only 3 to 4% of total histone and its amount is difficult to estimate as precisely as is possible for other histone bands. Table 7 summarizes the results of this study and shows that, within the limits of precision of this method, the proportion of each histone band is identical in interphase and metaphase histones. In contrast to disc electrophoresis, Amberlite CG-50 chromatography allows us to estimate the proportions of some additional components (e.g. histones O and II which electrophorese together as band C). Results of this comparison are presented in Table 8. These data show that the proportions of histone O and peak III-IV are higher in metaphase histones than in

Table 7. Comparison of Proportions of Major Electrophoretic Histone Bands in Acid Extracts of Interphase and Metaphase Chromosomes

Electrophoretic Histone Bands	Chromatographic Fractions	H_2SO_4-Extracted Interphase Histones (%)	H_2SO_4-Extracted Metaphase Histones (%)	HCl-Extracted Metaphase Histones (%)
A	Ia + Ib	16.0 (5)	14.0 (5)	15.0 (8)
C	O + II + III	61.0 (5)	63.0 (5)	64.0 (8)
D	IV	23.0 (5)	23.0 (5)	21.0 (8)
B(III-IV)/ A+B+C+D		0.04	0.04	0.03

Area of each electrophoretic band was measured by transferring the densitometer tracing to a tracing paper of uniform weight, cutting the tracing and weighing. The figures in parentheses indicate the number of gels used to compute the mean values reported in this table.

Table 8. Comparison of Proportions of Various Chromatographic Histone Fractions in Acid Extracts of Interphase and Metaphase Chromosomes

Histone Fractions	H_2SO_4-Extracted Interphase Histones (%)	H_2SO_4-Extracted Metaphase Histones (%)	HCl-Extracted Metaphase Histones (%)
O	9.4 ± 4.7	16.0	13.1
Ia + Ib	25.9 ± 2.2	14.6	14.3
II	41.0 ± 2.6	38.3	38.2
III-IV	23.6 ± 3.6	31.1	34.2
Peak A/ Total Proteins	0.077 ± 0.020	0.184	0.217

Amount of each fraction was estimated by transferring the chromatographic peak to a tracing paper of uniform weight, cutting the peak and weighing. All values for interphase histones represent averages of six separate preparations while those for metaphase histones of two preparations.

interphase histones. It may be tentatively argued that since the proportion of histone IV (represented by the electrophoretic band D) is identical in interphase and metaphase histones (Table 7), the increase in the proportion of chromatographic peak III-IV is possibly caused by an increase in the proportion of histone III. However, the validity of this argument is doubtful in view of the fact that peak III-IV consists of components in addition to histone III and IV as pointed out earlier in this Chapter. Also, the significance of the increase in the proportion of histone O is not clear due to the large standard error involved in its estimation. Table 8 further shows a decrease in the proportion of lysine-rich histones (Ia and Ib) in metaphase histones. This finding is in apparent conflict with the data obtained from electrophoretic analysis (Table 7) because the latter indicated an identical proportion of band A (consisting of histones Ia and Ib) in interphase and metaphase histones. This contradiction in the chromatographic and electrophoretic data is resolved by the following analysis.

It must be recognized that disc electrophoretic analysis of whole histones does not take into consideration any histone III present in a polymerized state since such forms of histone III electrophorese with mobilities slower than band A (Ia and Ib). On the other hand, the polymerized histone III will chromatograph with peak III-IV and thus will be included in chromatographic analysis. Therefore, we estimated the proportions of various electrophoretic histone bands obtained by electrophoresing both unreduced and reduced (β-mercapto-

ethanol-treated) histone preparations. The results of this study are given in Table 9. These data were obtained from densitometer tracings similar to those presented in Figs. 9a and b, and Figs. 17a, b, e and f. As discussed earlier in this Chapter, reduction does not detectably alter the proportion of band C (containing monomeric histone III) in whole interphase histones. This suggests that essentially all of histone III is present in interphase histones in the reduced state. In contrast, Table 9 shows that the proportion of band C in metaphase histones increases from 60-62% to 69-71% as a result of reduction. This is interpreted as indicative of the presence of a certain fraction of histone III in metaphase histones in the polymerized form containing intermolecular disulfide bridges. It is further observed that the proportion of band A (histones Ia and Ib) is lower in reduced metaphase histones than in reduced interphase histones. This result resolves the apparent contradiction pointed out above between the electrophoretic and chromatographic data (Tables 7 and 8) and shows that lysine-rich histones are indeed present in metaphase chromosomes in lower proportion than in interphase chromosomes if both forms of histone III (polymeric and monomeric) are taken into consideration.

D. ACID-INSOLUBLE NON-HISTONE PROTEINS OF INTERPHASE CHROMOSOMES

In the case of unsheared interphase chromosomes prepared by the Sucrose Sedimentation Method, the ratio of acid-insoluble non-histone proteins[1] to DNA is approximately 1.5 to 1.6 (Table 1).

[1] Proteins remaining associated with DNA after exhaustive extraction with 0.4 N sulfuric acid.

Table 9. Proportions of Major Electrophoretic Histone Bands‡ in Total Unreduced and Reduced Histones of Interphase and Metaphase Chromosomes

Sample Electrophoresed	H_2SO_4-extracted Interphase Histones			H_2SO_4-extracted Metaphase Histones			HCl-extracted Metaphase Histones		
	A	C	D	A	C	D	A	C	D
Whole Histone	0.15	0.63	0.22	0.14	0.60	0.26	0.16	0.61	0.24
	(0.17)*	(0.58)*	(0.25)*	(0.15)†	(0.60)†	(0.25)†	(0.15)†	(0.62)†	(0.23)†
Whole Histone-Reduced	0.17	0.63	0.21	0.10	0.69	0.21	0.09	0.71	0.20
	(0.16)*	(0.61)*	(0.23)*	(0.10)†	(0.69)†	(0.21)†	(0.10)†	(0.70)†	(0.20)†

*The figures in parentheses represent a separate preparation of histones.

†The figures in parentheses represent a replicate experiment on the same preparation.

‡The various electrophoretic bands are comprised of different chromatographic fractions as follows: Band A - Ia + Ib; Band C - 0 + II + III; Band D - IV.

Area of each electrophoretic band was measured by transferring the densitometer tracing to a tracing paper of uniform weight, cutting the tracing and weighing. Reduction of histones was accomplished by treatment with 0.1 M β-mercaptoethanol for 1 hour at 37°C.

Nearly half of these non-histone proteins are lost when unsheared interphase chromosomes are sheared and the non-histone protein/DNA ratio decreases to 0.72. It was observed that essentially all of the acid-insoluble non-histone proteins of sheared interphase chromosomes can be dissolved in 0.05 M Tris, pH 8.0 containing 1% sodium dodecyl sulfate (SDS) by incubation at 37°C for 12 hours, whereas only half of these proteins associated with unsheared interphase chromosomes are soluble in the Tris-SDS solvent. A similar observation has been made with sheared and unsheared rat liver chromatin (K. Marushige, personal communication). This suggests that the acid-insoluble non-histone proteins lost during shearing are of a different class than those associated with sheared chromosomes. With this in mind, the amino acid composition of Tris-SDS soluble and insoluble non-histone proteins of unsheared interphase chromosomes was studied. The results of these analysis are presented in Table 10 together with the composition of the sulfuric acid-soluble peak A (run-off) for comparison. The two fractions of acid-insoluble non-histone proteins are indistinguishable from each other on the basis of their amino acid compositions. In contrast, the composition of these non-histone proteins is strikingly different from that of acid-soluble peak A proteins. Compared to peak A, acid-insoluble non-histone proteins have less lysine, proline and alanine, but more leucine, tyrosine and phenylalanine. This suggests that the acid-soluble non-histone (peak A) proteins should be treated as a class different from that of acid-insoluble non-histone proteins even though

Table 10. The Amino Acid Composition of Interphase Chromosomal Non-histone Proteins

Amino Acid	H_2SO_4-soluble Peak A	H_2SO_4-insoluble Protein	
		Fraction Soluble in 0.05 M Tris + 1% SDS, pH 8	Fraction Not Soluble in 0.05 M Tris + 1% SDS, ph 8
Lysine	11.4	6.4	6.9
Histidine	1.7	2.9	3.4
Arginine	5.2	5.4	5.6
Aspartic Acid	7.4	8.9	8.7
Threonine	5.2	5.9	5.8
Serine	8.3	7.2	7.0
Glutamic Acid	12.4	11.2	10.9
Proline	7.7	4.9	4.6
Glycine	9.4	7.7	6.8
Alanine	9.7	7.6	6.9
Half-cystine	1.5*	1.7*	2.3*
Valine	5.2	5.9	7.0
Methionine	1.9*	2.1*	2.2*
Isoleucine	3.0	4.6	4.3
Leucine	5.9	8.8	8.9
Tyrosine	1.8	3.5	3.8
Phenylalanine	2.4	4.8	4.6

*To obtain accurate analyses of cysteine and methionine, protein samples were oxidized with performic acid before acid hydrolysis. Therefore, cysteine (and/or cystine) and methionine were estimated as cysteic acid and methionine sulfone respectively.

No correction for hydrolytic losses of serine and threonine has been made.

The underlined values signify a difference of 2 or more mole % among the proteins being compared.

both are distinguished from histones by their high and identical content of acidic amino acids.

DISCUSSION

Heterogeneity and Evolution of Histones

Evidence presented in this Chapter reveals the existence of only a limited number of molecular species of histones in HeLa cells. These histone species are: O[1], Ia and Ib (f1), IIb1 (f2a2), IIb2 (f2b), III (f3) and IV (f2a1). A variety of animals and plants also have been demonstrated to possess only a small number of identifiable histone components (Crampton et al., 1957; Hnilica, 1965; Hnilica et al., 1962, 1966; Palau and Butler, 1966; Johns, 1967; Sheridan and Stern, 1967; Fambrough, 1968). However, on the basis of the kinetics of release of C-terminal amino acids by carboxypeptidase, Hnilica (1965) has proposed that the fraction f2b of calf thymus, rat thymus and chicken erythrocytes is composed of at least two major components. Phillips (1966) came to the same conclusion on the basis of identification of two distinguishable N-terminal peptides in f2b. The presence of two components in pea bud histone IIa has also been indicated (Fambrough, 1968). It is conceivable that some of the other chromatographically and electrophoretically homogeneous histones may be composed of two or more components of very similar but not identical primary structures. For instance, Kinkade and Cole (1966a, b) have raised the possibility of four closely related components in the lysine-rich histones of calf thymus. Even if the possibility of such 'microheterogeneity' is taken into account, the

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[1] This component of HeLa cells appears to be similar to IIb2 (f2b).

number of histone species which exist in vivo still appears to be limited.

The tremendous heterogeneity of histones reported in some of the previous studies (e.g. Neelin and Neelin, 1960; Rasmussen et al., 1962; Bellair and Mauritzen, 1965; Mauritzen et al., 1967) can now be explained on the basis of the data presented by this author and others. A few possible causes for these cases of extensive heterogeneity are suggested below.

1) Contamination by acid-soluble non-histone proteins often occurs. Even in highly purified preparations of nuclei or chromatin, the presence of chromatographic peak A (run-off) proteins in the acid extracts (H_2SO_4 or HCl) has always been noted. Several of the fractions prepared by the method of Johns (1964a) have been shown to be contaminated by peak A proteins (Fambrough, 1968). Electrophoretic data reported in this thesis indicate that peak A proteins are extremely heterogeneous. Therefore, failure to remove such proteins from various histone fractions probably accounts for many reports of apparently extensive heterogeneity of histones. In addition, the present studies have revealed that the possibility of contamination by acid-soluble non-histone proteins is greater when HCl, instead of H_2SO_4, is employed for histone extraction.

2) Lysine-rich histones are known to be extremely sensitive to proteolysis (Johns, 1964b; Reid and Cole, 1964; Kinkade and Cole, 1966a).

3) Histones have a great tendency to aggregate. In particular,

the pH-dependent aggregation of arginine-rich histones in salt solutions has long been a well-known phenomenon which has even been used for preparation of these histones (Cruft et al., 1954, 1958; Mauritzen et al., 1967). The possibility that some non-histone proteins aggregate with arginine-rich histones in the above process is not excluded. The contamination of the chromatographic histone Ib fraction of HeLa cells by a component electrophoretically similar to arginine-rich histones has been demonstrated here. This could be due to aggregation of lysine-rich and arginine-rich histones. However, as already noted, the possibility that some of the minor components of various chromatographic fractions represent distinct bona fide histones can not be ruled out on the basis of available data.

Another great source of heterogeneity of the arginine-rich histones is the formation of histone III polymers due to intermolecular disulfide bridges. Such polymerization can occur under conditions quite commonly employed for fractionation of histones, as has been discussed at length in Results.

There is now a large amount of data in the literature demonstrating the presence of chemically similar components in the histones of rather diverse organisms. Thus, histones of a variety of animals have been shown to share homologous components (Crampton et al., 1957; Hnilica et al., 1962, 1963; Hnilica, 1966; Neidle and Waelsch, 1964; Laurence et al., 1963, 1966; Palau and Butler, 1966). The work of Fambrough (1968) has similarly demonstrated extensive homology between the histones of calf thymus and pea bud. In addition,

histones of various tissues and organs within the same animal or plant also seem to lack differences (Crampton et al., 1957; Hnilica et al., 1962, 1963; Hnilica, 1966; Neidle and Waelsch, 1964; Lindsay, 1964; Fambrough, 1968), although quantitative differences in the proportions of various histones have often been noted. Data presented in this thesis also show the presence in HeLa cells of components similar to those of calf thymus and pea bud. Thus even markedly different metabolic and growth conditions are not associated with appearance or disappearance of any major histone component. In this connection, it may be noted that a peak, coded as RP2-L, was once considered specific to histones of neoplastic cells (Davis and Busch, 1959; Busch et al., 1962) but could not be detected in more purified histone preparations from neoplastic tissues in later studies (Busch et al., 1963; Hnilica, 1965). The only well-established case of a species-specific histone seems to be that of the fraction F2c (or fraction 5) in chicken erythrocytes (Neelin et al., 1964; Hnilica, 1965) which probably represents a general feature of nucleated erythrocytes (Hnilica, 1965). The biological significance of this fraction remains obscure.

The lack of species specificity among the histones suggests that the homologous histone components in various organisms are related through phylogenetic evolution. The homology of arginine-rich histones in HeLa cells, calf thymus and pea bud is particularly striking. As pointed out by Fambrough (1968), it seems that many of the sequences in the primary structures of histones are crucial to

their function and, therefore, a majority of mutations leading to changes in amino acid sequences of histones must have been lethal. However, it is important to recognize that the high degree of homology observed so far among the histones of different species does not necessarily signify identical amino acid sequences in corresponding histone components. Indeed, several important differences in the compositions and peptide maps of various histones from calf thymus and pea bud have been reported by Fambrough (1968). Table 2 in this Chapter also reveals significant differences in composition of corresponding fractions from HeLa cells, calf thymus and pea bud. On the basis of amino acid composition and chromatographic and electrophoretic behavior, the histones of the two mammalian systems (HeLa cells and calf thymus) seem to be more related to each other than to the histones of the plant system (pea bud). Therefore, it is expected that studies currently in progress in various laboratories on the primary structures of histones from different species will elucidate the pattern of mutations which was acceptable during evolution. An understanding of this pattern will clarify the relative importance of different segments in a histone molecule with respect to its function and thus will be of considerable significance in building models of nucleohistone and chromosomal structure.

It must be stressed that the question of minor histone components has not been adequately explored so far. The demonstration of extensive homology in histones from various tissues and organisms, as discussed above, is based on the comparison of major components

only. Electrophoretic band B in whole histones or chromatographic peak III-IV (Figs. 8a, 8g, 15a, 15g, 16a and 16g) of both interphase and metaphase HeLa cells is an example of such a minor component. Similarly, the undetermined chemical identity of certain other minor components of various chromatographic fractions has been pointed out in the Results. It is probable that some minor histone components may be of yet unsuspected biological significance and thus provide an interesting area for future work.

The discovery of a unique component, histone O, in HeLa cells deserves some comment. As already pointed out, histone O is electrophoretically indistinguishable from the slightly lysine-rich histone II fraction and has an amino acid composition strikingly similar to that of IIb2 (f2b) reported in several animal tissues and a few neoplastic cell strains (Rasmussen et al., 1962; Hnilica, 1965, 1966; Hnilica et al., 1966). In light of this, it is puzzling that histone O elutes from the Amberlite CG-50 column before lysine-rich histones and is thus chromatographically well resolved from the slightly lysine-rich fraction. It is unlikely that histone O is a proteolytic product of histone IIb2 (f2b). The following two reasons can be advanced in support of this contention:

a) Histone O has been found in all types of nucleoprotein preparations so far obtained from HeLa cells, such as interphase chromosomes (or nuclei) prepared by two different methods and metaphase chromosomes prepared at acid or neutral pH. In none of these preparations was there any indication of degradation of lysine-rich histones which, of all histones, are most susceptible to proteo-

lysis;

b) Two protein molecules of similar amino acid composition but different molecular size would have distinguishable electrophoretic mobilities on a polyacrylamide gel, which separates proteins on the basis of size as well as charge. The observations that histone O and IIb2 (f2b) have similar amino acid composition and coelectrophorese as band C (cf. Figs. 8c and f) suggest that they are of the same size and, therefore, can not be related to each other through proteolysis.

It may be pointed out here that any chemical modification of histone IIb2 (f2b) increasing the number of negative charges per molecule will cause it to elute from Amberlite CG-50 earlier than the unmodified molecule. Although studies on the N- and C-terminal residues and the peptide maps of histone O are yet to be conducted, it seems possible that histone O and IIb2 (f2b) are homologous components differing in some such chemically subtle manner.

Acid-Soluble Proteins of Metaphase Chromosomes and their Possible Involvement in Chromosomal Condensation

At least three possible ways can be proposed by which histones could lead to condensed configuration and inhibition of RNA synthesis as interphase chromosomes are transformed to metaphase chromosomes. We will discuss each of these separately in light of the findings presented in this Chapter.

1) A change from extended to condensed configuration may be caused by an increase in histone/DNA ratio. It was reported recently that metaphase chromosomes indeed possess almost 2.0 to 2.5-fold

as much HCl-soluble protein as is present in interphase chromosomes (Huberman and Attardi, 1966; Maio and Schildkraut, 1967). These observations are confirmed here (Table 1). However, the data shown in Table 4 clearly demonstrate that essentially all of these additional acid-soluble proteins found in metaphase chromosomes are not histones and that histone/DNA ratio is actually identical in extended and condensed chromosomes. Consistent with this conclusion, earlier workers have reported that the "histone"/DNA ratio of chromatin is unrelated to its state of condensation or capacity to synthesize RNA (Dingman and Sporn, 1964; Frenster, 1965), but in these studies no effort was made to determine the fraction of acid-soluble proteins which was actually histone. The need for such a determination is emphasized by two observations reported in this thesis. First, HCl and H_2SO_4 differ in their ability to extract proteins from chromosomes. Second, acid-extracts even from interphase chromosomes may contain as much as 20% non-histone proteins.

2) The condensation of chromosomes may be a consequence of either an appearance or disappearance of some histone component, or, alternatively, it may be caused by quantitative alteration in the relative amounts of various histones. On the basis of rather inconclusive data, many workers have previously proposed that histones of extended and condensed chromosomes are similar (Frenster, 1965; Harbers and Vogt, 1966; Grogan et al., 1966; Maio and Schildkraut, 1967; Comings, 1967). The present author has reinvestigated this problem in detail with the aid of improved chromatographic and

electrophoretic techniques. These extensive comparisons have failed to reveal any major difference in the types of molecular species comprising histones of interphase and metaphase chromosomes. The significance of finding two new minor components in the histone Ib fraction of metaphase chromosomes can not be evaluated until more data on the chemical properties of these components are obtained (see Figs. 15e and 16e). At present, it may be concluded that an alteration in the types of major histones can not be invoked to explain the properties of metaphase chromosomes.

The alternative possibility of differing relative amounts of various histones in interphase and metaphase chromosomes has also been examined in detail. It was shown that, relative to interphase chromosomes, metaphase chromosomes are poorer in lysine-rich histones and richer in arginine-rich histones. However, the relationship of this difference to the extended and condensed state of chromosomes is not obvious so far. In addition, the possibility that these differences may have resulted from some kind of artifact can not yet be eliminated. Similar quantitative studies need to be carried out on metaphase chromosomes prepared by other methods before attaching any significance to these data.

3) It is likely that a change in some specific property of existing histones leads to condensation of chromosomes. Some evidence in favor of this possibility was obtained in the present studies. An undetermined but apparently significant proportion of histone III in metaphase chromosomes was found to exist in a polymerized state as a result of the formation of intermolecular disulfide bridges

(Table 9). In contrast, essentially all of histone III in interphase chromosomes has been shown to exist in the reduced monomeric state. If this finding is not an artifact of the procedures involved in preparation of these chromosomes, it may reflect an important characteristic of the condensed configuration of metaphase chromosomes. Later in this Discussion, a possible role for such polymerized histone III in chromosomal coiling will be suggested.

The present studies have demonstrated that the most obvious difference between the acid-soluble proteins of interphase and metaphase chromosomes lies not in histones but in the acid-soluble non-histone proteins. First, compared to interphase chromosomes, metaphase chromosomes contain far greater quantities of acid-soluble non-histone proteins per given amount of DNA. Second, a considerable, but as yet unknown proportion of these proteins of metaphase chromosomes exists as large aggregates formed by intermolecular disulfide bridges; corresponding acid-soluble non-histone proteins of interphase chromosomes are not similarly aggregated. These findings immediately raise two important questions: Are these additional non-histone proteins associated with metaphase chromosomes <u>in vivo</u>, or do they become attached during isolation? Assuming that they are natively associated with metaphase chromosomes, do they contain the disulfide bridges described above or are these artifacts of the preparative procedures? Such questions can not be answered as yet but some comments may be made now in this context. Metaphase chromosomes have been prepared by two very different methods, namely the acid pH method of Huberman and Attardi (1966) and the neutral pH method

of Maio and Schildkraut (1967). Although metaphase chromosomes prepared at acid pH appear to be somewhat poorer in acid-soluble proteins than those prepared at neutral pH, both metaphase chromosomal preparations are significantly richer in acid-soluble proteins than are interphase chromosomes themselves prepared by two different methods (Table 1). Experiments showing the presence of polymerized acid-soluble non-histone proteins in metaphase chromosomes have so far been carried out only on chromosomes prepared at neutral pH, a condition which does not inhibit the formation of disulfide bridges. However, acid-soluble non-histone proteins of interphase chromosomes, also prepared at neutral pH, showed no evidence of polymerization through the formation of such disulfide links. Nevertheless, the importance of studying the corresponding proteins in metaphase chromosomes prepared under conditions unfavorable to the oxidation of thiol groups can not be minimized.

For the present, with no unambiguous way to exclude the possibility of artifacts, there may still be some value in assuming that the above interesting similarities and differences among the chromosomal proteins of interphase and metaphase represent the in vivo state and thus may be biologically significant. In the absence of explicit studies on the nature of the association of histones and acid-soluble non-histone proteins with DNA in metaphase chromosomes, it may be somewhat hazardous to consider a specific role for these proteins in chromosomal structure. Yet, a purely speculative role is proposed below for the acid-soluble non-histone proteins

in hope of generating further work on their properties. In a model of chromosomal coiling proposed by Cole (1962), it was suggested that metaphase chromosomes are formed by successive coiling (secondary, tertiary and quaternary) of DNA as a result of association with increasing amounts of histones. However, the data presented here demonstrate that no more histones appear in metaphase chromosomes when the cell traverses from interphase to mitosis. Therefore, it is expected that the proteins involved in the chromosomal coiling as proposed by Cole (1962) are other than histones. It is suggested that the acid-soluble non-histone proteins polymerized through intermolecular disulfide bonds may be involved in this additional coiling in metaphase chromosomes, whereas histones participate in the primary coiling of DNA common to both interphase and metaphase cells. We further recall that formation of histone bridges has been frequently invoked in the coiling of DNA by various workers to explain X-ray diffraction and hydrodynamic properties of necleohistone (Zubay and Doty, 1959; Wilkins et al., 1959; Ohba, 1966). Since available evidence indicates the presence of polymerized histone III only in metaphase chromosomes, it is attractive to speculate that the above-mentioned additional coiling of metaphase chromosomes is stabilized by disulfide bonds between two or more molecules of histone III on adjacent chromosomal loops.

CHAPTER 2. THE RELATIONSHIP BETWEEN HISTONE AND DNA SYNTHESIS

INTRODUCTION

The close association between histones and DNA in chromosomes of higher organisms suggests the possibility that the synthesis of these basic proteins might be coupled to replication of DNA. A number of attempts have therefore been made in the last few years to establish the temporal relationship between histone and DNA synthesis. Using cytochemical methods together with microphotometric measurements, it has been shown that in growing onion root tips (Alfert, 1955), rat liver and fibroblasts (Bloch and Godman, 1955), blood lymphocytes (Perugini et al., 1957) and in the cells of several species of plants (Rasch and Woodard, 1959; Woodard et al., 1961), the increase in histone content of nuclei closely parallels the increase in DNA content. Prescott (1966) has demonstrated that in the macronucleus of the amoeba, Euplotes, histone synthesis begins in phase with DNA synthesis, continues at a constant rate during the S phase and ends with the completion of DNA synthesis. On the other hand, incorporation studies have shown that histone synthesis is partially dissociated from DNA synthesis in regenerating livers (Holbrook et al., 1962; Evans et al., 1962; Umaña et al., 1964). Flamm and Birnstiel (1964) have presented evidence indicating that histone synthesis can occur in cultured tobacco cells in which DNA synthesis has been blocked with 5-FUdr. Further, recent reports have shown that in synchronized mouse fibroblasts (L cells) and HeLa cells, grown in vitro, histone

synthesis proceeds throughout the interphase but increases strikingly with the onset of the S phase (Littlefield and Jacobs, 1965; Spalding et al., 1966). However, in similar studies with synchronized HeLa cells other workers have concluded that there is a precise correlation between histone and DNA synthesis, although their data can be easily interpreted differently (Robbins and Borun, 1967; Borun et al., 1967). By applying chromatographic procedures for histone separation, Chalkley and Maurer (1965) have found that in several non-dividing and highly differentiated plant and animal tissues (e.g. pea cotyledons, calf endometrium), radioactive amino acids are incorporated only into arginine-rich histones but are not incorporated into lysine-rich and slightly lysine-rich histones. Thus we are faced with a whole spectrum of relationships between histone and DNA synthesis in different physiological situations. Unfortunately, unambiguous interpretation of results in many of the above-mentioned studies is not possible due to insufficient accompanying data on both the purity and the chemistry of histone preparations utilized.

The work reported so far in this thesis on synchronization of HeLa cells and on detailed chemical characterization of their histones has laid the essential groundwork for reinvestigating the problem of the metabolic connection, if any, between histone and DNA synthesis. The question will be examined below in two physiologically extreme cell types: a) non-dividing and metabolically highly specialized chicken erythrocytes, and b) undifferentiated rapidly dividing HeLa cells. It will be shown that the relationship between histone and

DNA synthesis is apparently different in these two cell types. In light of the results obtained in our studies, an attempt will be made in the Discussion to present briefly an integrated view of previous work reported in the literature concerning this question.

MATERIALS AND METHODS

A. RADIOACTIVE LABELING OF HISTONES IN HeLa CELLS

Randomly dividing or synchronized HeLa S3 cells in suspension culture were utilized in this work. The cells were cultured and synchronized by double treatment with excess thymidine according to the procedures described in the Materials and Methods (see Fig. 2b) of Part I of this thesis. Histones were labeled with radioactive amino acids in one of two possible ways as outlined below:

1) Cells growing in 800 to 900 ml of suspension cultures at a concentration of 2.0 to 2.5 $\times 10^5$ cells/ml were harvested by centrifugation at 500xg for 5 minutes at room temperature. The cell pellet was then resuspended in 210 ml of prewarmed (37°C) culture medium (supplemented with 5% calf serum) at a concentration of about 9 to 11 $\times 10^5$ cells/ml. To this suspension was added 25 μc of L-[^{14}C]-U.L.-leucine (231 mc/mM; New England Nuclear Corp.) dissolved in 1 ml of sterile isotonic salt solution at pH 7.0.

2) A mixture of five C^{14}-labeled amino acids (10 μc of each) dissolved in 5 ml of isotonic salt solution at pH 7.0 was added to 800 to 900 ml of suspension culture (cell concentration = 1.8 to 2.0 $\times 10^5$ cells/ml) under sterile conditions. These amino acids were as follows: L-[^{14}C]-U.L.-arginine (International Chemical and Nuclear Corporation; 250 mc/mM); L-[^{14}C]-U.L.-glycine (New England Nuclear Corp.; 116 mc/mM); L-[^{14}C]-U.L.-leucine (New England Nuclear Corp ; 231 mc/mM); L-[^{14}C]-U.L.-lysine (International Chemical and Nuclear Corporation; 225 mc/mM); L-[^{14}C]-U.L.-valine (New England

Nuclear Corp.; 208 mc/mM). The choice of these amino acids as labeled precursors of histones was based on two considerations: (a) The labeled amino acids were already present in the culture medium as non-radioactive nutrients in large quantities. Such a choice minimized the possibility of any difference in the specific activity of a labeled amino acid in intracellular pools of the cells undergoing different phases of the cell division cycle. (b) The combination of these five amino acids comprised a high and approximately equal molar percentage (about 40-45%) of the total number of amino acids in the various histones of HeLa cells.

Incubation of cells was continued for either 90 or 270 minutes as specified in the Results, followed by quick chilling of the cultures in crushed ice. The cells were harvested by centrifugation at 500xg for 5 minutes in International PR-2 centrifuge at 2 to 4°C and then washed thrice with precooled isotonic salt solution. The washed cells were either stored frozen at -80°C for later use or processed immediately for fractionation of cell constituents and histone extraction.

B. PREPARATION AND ANALYSIS OF RADIOACTIVE HISTONES

The methods employed for preparation of nuclei, chromatin and histones from HeLa cells have been described earlier in the Materials and Methods of Chapter 1 of Part II in this thesis. Radioactive histones (3-6 mg) were fractionated on an Amberlite CG-50 analytical column using a nonlinear gradient of guanidinium chloride (GuCl) in phosphate buffer (pH 6.8) and the histone content of every

alternate fraction was determined by measurement of optical density at 400 mμ after addition of TCA to a final concentration of 1.1 M (see the Materials and Methods of the previous Chapter for details). The distribution of radioactivity among the various histones was determined by collecting alternate fractions (not to be used for measurement of histone content) directly into scintillation counting vials. To each vial were added 15 ml of scintillation counting fluid (4 g PPO, 0.05 g POPOP, 120 g naphthalene, 980 ml dioxane, 20 g Cab-O-Sil) and the mixture shaken vigorously. Radioactivity in each vial was determined by counting in the Beckman Liquid Scintillation Spectrophotometer.

The thixotropic agent Cab-O-Sil (Cabot Corp., Boston, Mass.) was added to the scintillation fluid to prevent precipitation of GuCl from the fluid; in its absence GuCl precipitated resulting in significantly lowered counting efficiency. Counting efficiency of Carbon-14 in the presence of Cab-O-Sil remained essentially constant throughout the range of concentrations of GuCl used to elute lysine-rich and slightly lysine-rich histones, but was somewhat lower in effluent fractions containing peak III-IV (arginine-rich histones).

RESULTS

A. HISTONE SYNTHESIS IN NON-DIVIDING CHICKEN ERYTHROCYTES[1]

Fig. 1 shows the chromatographic fractionation of [^{14}C] leucine-labeled chicken erythrocyte histones on an Amberlite CG-50 column. The radioactivity appears associated primarily with peak A (run-off) and peak III-IV which contain respectively non-histone protein and arginine-rich histones (cf. Chapter 1 of Part II in this thesis). It seems improbable that this histone synthesis occurred in contaminating leucocytes or thrombocytes since the latter account for less than 0.4% of the total protein syntehsis by erythrocytes from severely anemic chickens (Kabat, 1967). In addition, as will be shown below, all histone fractions are synthesized in dividing cells. Therefore, the absence of synthesis of lysine-rich and slightly lysine-rich histones in these cells makes it unlikely that the observed incorporation of radioactivity is due to dividing cells. A comparison with the data presented by Kabat (1967) further reveals that arginine-rich histones are synthesized at approximately the same rate as are the hemoglobins in this cell population despite the fact that unfractionated whole histones are only weakly labeled compared to other nuclear proteins.

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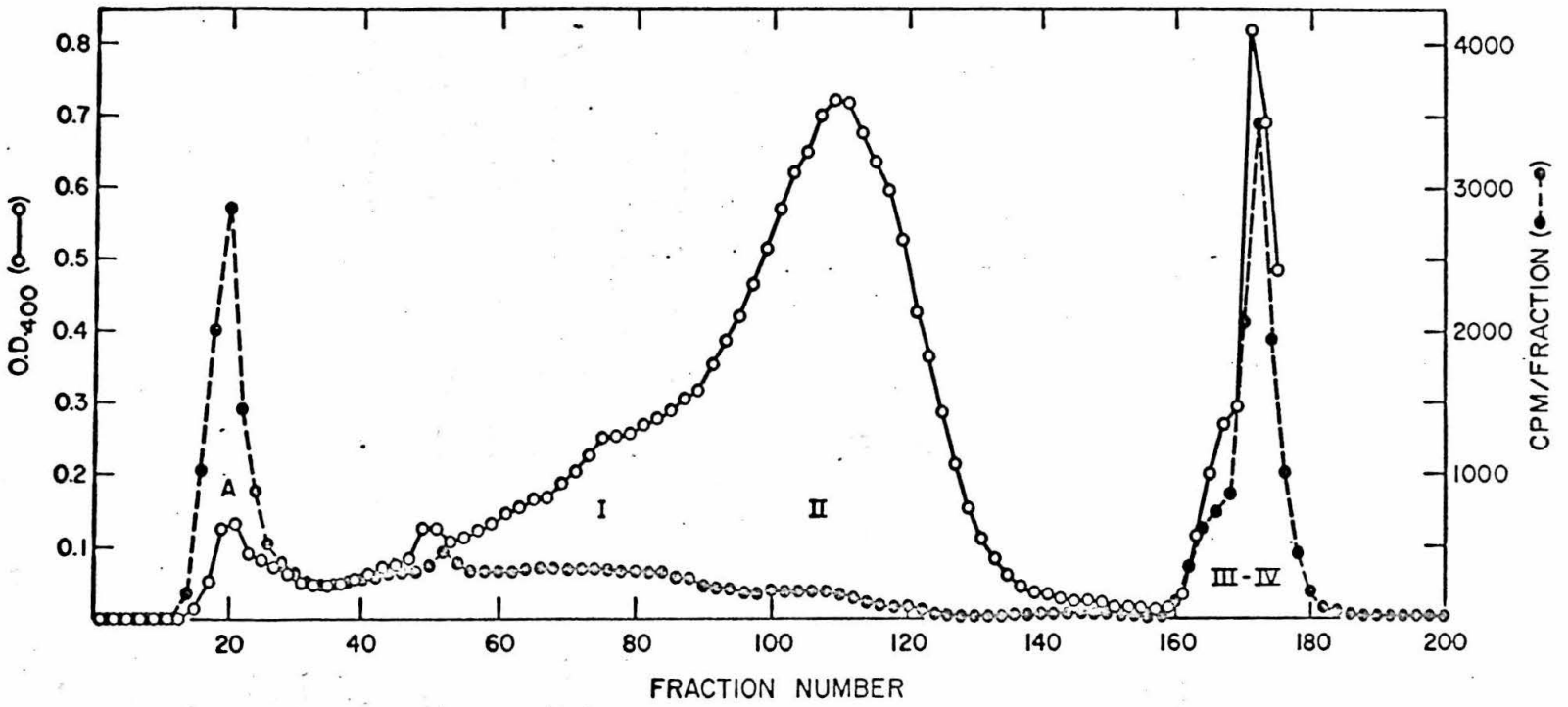
[1] Radioactively labeled histones from chicken erythrocytes were supplied by Dr. David Kabat.

Figure 1. Chromatographic fractionation of [^{14}C] leucine-labeled chicken erythrocyte histones on an Amberlite CG-50 column (analytical scale). Erythrocytes from severely anemic chickens were suspended in 33 ml of a leucine-deficient modified Eagle's medium (supplemented with 5% dialyzed chicken serum) at a concentration of 1.5×10^9 cells/ml and preincubated for 1 hour at 37°C. To this cell suspension, 100 μc of L-[^{14}C]-U.L.-leucine (273 μc/μM) was added, and incubation continued for 100 minutes at 25°C. At the end of 100 minutes of incorporation, the cells were harvested, and histones extracted from chromatin with 0.2 N HCl. Experimental details concerning preparation and incubation of erythrocytes, chromatin isolation and histone extraction have been described by Kabat (1967). See Materials and Methods of the previous and the present Chapters for the procedures involved in column chromatography of histones and in analysis of protein concentration and radioactivity in the effluent fractions.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II
III-IV
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80
100
120
140
160
180
200
FRACTION NUMBER

B. HISTONE SYNTHESIS BY LOGARITHMICALLY GROWING HeLa CELLS IN ABSENCE AND PRESENCE OF DNA SYNTHESIS

Table 1 presents absolute as well as relative rates of synthesis of various histone fractions in logarithmically growing HeLa cell cultures in the absence and presence of DNA synthesis. The specific activity of each fraction has been normalized for its leucine content, using the leucine content of the peak III-IV as a reference standard. This normalization assumes that the leucine utilized in the synthesis of all histone components is derived from a common intracellular pool. It is seen that, of all the histones, the slightly lysine-rich histone II is synthesized at the lowest rate in logarithmically growing cultures. In contrast, the other slightly lysine-rich histone fraction O and the lysine-rich fraction Ia + Ib are synthesized somewhat more rapidly than are the arginine-rich histones (peak III-IV). Leucine incorporation into all histones is significantly slower than into the non-histone peak A. However, it must be pointed out that the overall rates of synthesis of histone fractions O, Ia + Ib and III-IV are probably very similar in these cultures despite the apparent differences shown by the data of Table 1. This is because the specific activity of peak III-IV is underestimated due to slight quenching by the high concentrations of guanidinium chloride used for its elution. This quenching is difficult to quantify because the elution is carried out very rapidly by washing the resin with 40% guanidinium chloride. Consequently, the salt concentration varies significantly in the different effluent fractions in which peak III-IV is eluted (see Fig. 3 of the previous Chapter). Nevertheless, the effluent fraction of peak III-IV having the highest

Table 1. Histone Synthesis by Logarithmically Growing HeLa Cell Population in Absence and Presence of DNA Synthesis

	Control[a]			5-FUdr-Treated[b]			Excess Thymidine-Treated[c]		
	Specific Activity (cpm/mg)	Normalized Specific Activity (cpm/mg)†	Relative Normalized Specific Activity	Specific Activity (cpm/mg)	Normalized Specific Activity (cpm/mg)†	Relative Normalized Specific Activity	Specific Activity (cpm/mg)	Normalized Specific Activity (cpm/mg)†	Relative Normalized Specific Activity
Peak A	10,784	16,850	2.28	10,667	16,667	1.91	10,282	16,065	1.81
Histone O	5,370	8,804	1.19	3,739	6,130	0.70	4,874	7,989	0.90
Histone Ia + Ib	4,969	8,422	1.14	4,485	7,601	0.87	3,953	6,700	0.76
Histone II	4,393	4,828	0.65	1,520	1,670	0.19	1,809	1,988	0.22
Histone III-IV	7,395	7,395	1.00	8,705	8,705	1.00	8,874	8,874	1.00
Total Histones	5,238	6,635	-	4,816	5,792	-	4,948	5,967	-

[a] Logarithmically growing HeLa cell culture.

[b] Logirithmically growing HeLa cell culture was preincubated for 4 hours at 37°C in the presence of 10^{-6} M 5-FUdr and then incubated with [^{14}C] labeled leucine in MEP containing 5-FUdr at the same concentration.

[c] Logarithmically growing HeLa cell culture was preincubated for 4 hours at 37°C in the presence of 5 mM thymidine and then incubated with [^{14}C] labeled leucine in MEP containing thymidine at the same concentration.

† Normalized for the leucine content of each peak, using the leucine content of peak III-IV as a reference standard (see Table 2 of the previous Chapter for the leucine content of different histone fractions).

Notes continued on the following page.

Table 1 (Continued)

HeLa cells growing logarithmically (2.0 to 2.5×10^5 cells/ml) in phosphate-rich modified Eagle's medium (MEP; cf. Part I for composition) were harvested and resuspended in 210 ml of prewarmed (37°C) MEP (supplemented with 5% calf serum) at a cell concentration of 9 to 11×10^5/ml. To this continuously stirring cell suspension was added 25 μc of L-[^{14}C]-U.L.-leucine (231 mc/mM) and the incubation was continued for 90 minutes at 37°C. At the end of incubation, the cells were harvested followed by isolation of purified nuclei from which histones were extracted with 0.4 N H_2SO_4. Histones were fractionated by chromatography on an Amberlite CG-50 column (analytical scale), and the protein concentration and radioactivity in the effluent fractions were plotted on a graph. The area under each peak was estimated by transferring the peak to a tracing paper of uniform weight, cutting the peak and weighing it. The relationship described by Bonner et al. (1967a) for histone concentration and $O.D._{400}$ of a TCA-containing solution of histones was used for expressing the specific activity of each peak as counts per minute/mg. protein (ten μg histone/ml in the final TCA-containing solution yields $O.D._{400}$ of 0.093). See Materials and Methods for all experimental details.

protein concentration displays a quenching of the order of 10 to 12% relative to the effluent fractions in which histone O or histone Ia + Ib is eluted. If this rough estimate of quenching of radioactivity in peak III-IV is taken into account, the normalized specific activities of histone fractions O, Ia + Ib and III-IV become essentially similar.

As will be seen below, the 'true' synthetic rates of different histones are actually not important for the understanding of the relationship between DNA and histone synthesis. Also, it may be noted that since a logarithmic culture consists of 32% of cells in S, 16% in G_2, 4% in M and 48% in G_1, the rate of leucine incorporation into a histone fraction represents only a weighted average of synthetic rates in cells belonging to different cell division cycle stages.

Before discussing the dramatic changes brought about in the pattern of histone synthesis by addition of 5-FUdr or excess thymidine, it must be pointed out that some caution need be used in comparing absolute rates of synthesis of various histones in control and treated cultures because of the unknown effects of these inhibitors on the leucine concentration in the intracellular pool. It is also worth mentioning that the histones utilized in these experiments were extracted from purified nuclei and not from chromosomes. Most probably the results obtained here will also hold true for histones extracted from chromosomes since the histones extracted from nuclei and chromosomes are qualitatively and quantitatively indistinguishable. (cf. previous Chapter). However, the possibility should be kept in mind that some histones in the nucleus may not be bound to DNA and may have a synthetic pattern different from that of chromosomally-

associated histones.

In part I of this thesis it has been shown that 5-FUdr (10^{-6} M) and excess thymidine (5 mM), which cause deficiency of deoxyribotides by means of different biochemical mechanisms, quickly interrupt DNA synthesis. Therefore, the data of Table 1 clearly demonstrate that only a part of histone synthesis is dependent on DNA synthesis. Abolition of DNA synthesis by either inhibitor has the greatest adverse effect on the synthesis of histone II whereas the rate of leucine incorporation into histone O and Ia + Ib is relatively less affected. In contrast to the synthesis of lysine-rich and slightly lysine-rich histones, no reduction in the synthesis of arginine-rich histones (peak III-IV) or of non-histone peak A is caused by inhibition of DNA syntehsis. The significance of a slight but definite increase in leucine incorporation in peak III-IV following the addition of inhibitors is not yet clear, but it is probably a consequence of altered leucine concentration in the intracellular pool under these nonphysiological conditions. The most significant conclusions to be drawn from these data are that synthesis of all histones continues in part in complete absence of DNA synthesis and that the different histones differ considerably in their relative dependence on DNA synthesis

C. SYNTHESIS OF CHROMOSOMALLY-ASSOCIATED HISTONES BY SYNCHRONIZED HeLa CELLS DURING THE S AND G_1 PHASES

The absolute and relative specific activities of various histones and other proteins labeled with five radioactive amino acids during the S and G_1 phases are presented in Table 2. Unlike the specific activites in Table 1, the specific activities of different histone

Table 2. Synthesis of Chromosomally-Associated Histones by Synchronized HeLa Cells During S and G_1 Phases of the Cell Division Cycle

	Logarithmic Culture* (Unsynchronized)		S Phase (Synchronized)		G_1 Phase (Synchronized)		Specific Activity in S/Specific Activity in G_1
	Specific Activity (cpm/mg)	Relative Specific Activity	Specific Activity (cpm/mg)	Relative Specific Activity	Specific Activity (cpm/mg)	Relative Specific Activity	
Peak A	18,527	1.63	50,150	2.14	26,722	2.24	1.88
Histone O	10,657	0.94	33,937	1.44	6,022	0.50	5.64
Histone Ia + Ib	11,703	1.03	31,648	1.35	5,627	0.47	5.62
Histone II	9,243	0.81	24,313	1.04	6,056	0.51	4.01
Histone III-IV	11,342	1.00	23,470	1.00	11,935	1.00	1.97
Total Histones	10,450	-	26,392	-	6,959	-	3.79
Acid Insoluble† Non-Histone	-	-	7,958	-	10,799	-	0.74

*Unsychronized logarithmically growing HeLa cell culture was labeled with the mixture of five C^{14} labeled amino acids for 90 minutes only in a separate experiment.

†Protein remaining associated with DNA after exhaustive extraction with 0.4 N H_2SO_4 was incubated in Tris-SDS buffer (0.05 M Tris-HCl, 1% sodium dodecyl sulfate, pH 8.0) for 12 hours at 37°C and the soluble fraction assayed for protein concentration and radioactivity.

Notes continued on the following page.

Table 2 (Continued)

HeLa cell culture in phosphate-rich modified Eagle's medium (MEP) was synchronized by double treatment with 5 mM thymidine as described in Part I. The cell population immediately after the release of second thymidine block constituted the S phase in this experiment. (cf. Figure 6 of Part I). The G_1 phase in this experiment refers to the culture seven hours after addition of the second thymidine block, when all cells were in some part of G_1 with none in S. Histones and other proteins of synchronized and unsynchronized cell populations were labeled as follows: an amino acid mixture consisting of 10 μc each of L-[^{14}C]-U.L.-arginine (250 mc/mM), L-[^{14}C]-U.L.-glycine (116 mc/mM), L-[^{14}C]-U.L.-leucine (231 mc/mM), L-[^{14}C]-U.L.-lysine (225 mc/mM) and L-[^{14}C]-U.L.-valine (208 mc/mM) was added to 850 ml of culture in MEP (cell concentration between 1.8 to 2.0 × 10^5 cells/ml); incubation was carried out for 270 minutes (90 minutes for the unsynchronized logarithmic culture) at 37°C with continuous stirring. At the end of incorporation, the cells were harvested and purified unsheared chromatin was isolated by the Sucrose Sedimentation Method (Method B). Histones were extracted from the above chromatin with 0.4 N H_2SO_4 and fractionated on an Amberlite CG-50 column (analytical scale). Experimental details for all of the above operations have been described in the Materials and Methods of the previous and present Chapters. See the legend of Table 1 for analytical details and procedures involved in estimation of specific activities of different chromatographic peaks.

fractions given here have not been normalized. For reasons discussed earlier, such normalization of synthetic rates is not of any real importance for the present purpose. The last column in Table 2 compares the absolute specific activities of various histones in the S and G_1 phases.

The data reveal that the lysine-rich (Ia and Ib) and slightly lysine-rich (O and II) histones are synthesized during S phase at a rate approximately 4- to 5-fold greater than that found during G_1 phase. In contrast, the arginine-rich histones (peak III-IV) and the non-histone peak A are synthesized at only about a two-fold higher rate during S phase than during G_1 phase. Further, when all cells in a population are going through the S phase together, histone fractions O, Ia + Ib and II are synthesized faster than or at least as fast as the arginine-rich histones. This was not obvious by study of the synthetic rates of these histones in logarithmic cultures in which only 32% of the cells are in the S phase at any given moment. Also, the use of synchronized cultures reveals that during the G_1 phase (when no DNA synthesis occurs) the lysine-rich and slightly lysine-rich histones are synthesized only half as fast as the arginine-rich histones. Once again the unmistakable conclusion from Table 2 is that in HeLa cells at least a part of histone synthesis does not require concurrent DNA synthesis and can take place in cells not undergoing S phase. On the other hand, another part of histone synthesis occurs only simultaneously with DNA synthesis.

It is significant that the synthesis of the acid-insoluble non-

histone protein (fraction soluble in the Tris-SDS buffer; cf. legend of Table 2) appears to be much less dependent on DNA synthesis than that of either the histones or of the acid-soluble non-histone peak A and is actually somewhat faster during G_1 than in S phase. Also, this acid-insoluble non-histone protein is made slower than histones during S phase but faster during the G_1 phase. It is, however, synthesized significantly more slowly than acid-soluble non-histone peak A during both S and G_1 phases. Incidentally, this additional evidence provides further support in favor of the proposal made in the previous Chapter regarding these two groups of non-histone proteins. It can now be stressed that acid-soluble non-histone proteins are metabolically as well as chemically different from acid-insoluble non-histone proteins (cf. Table 10 of the previous Chapter). It seems likely that these two classes of non-histone proteins play different functional roles in the chromosomes of higher organisms.

DISCUSSION

Our observations on the continued synthesis of only arginine-rich histones in non-dividing differentiated chicken erythrocytes are in complete agreement with those of Chalkley and Maurer (1965). These authors also found that radioactive amino acids are incorporated only in peak III-IV in several tissues not undergoing cell division. The significance of this important and probably general feature of non-dividing and maturing tissues is not yet understood. It may be noted, however, that at least in the case of chicken erythrocytes the selective synthesis of arginine-rich histones accompanies a decline in the rate of RNA synthesis (Cameron and Prescott, 1963) and progressive chromosomal condensation (Lucas and Jamroz, 1961; Harris, 1966). This finding is especially suggestive in view of the known ability of histones to suppress DNA-dependent RNA synthesis in vitro and to maintain the superstructure of chromosomes. It is also interesting that, besides arginine-rich histones, both chromosomally-associated acid-soluble non-histone proteins (Fig. 1) and acid-insoluble non-histone proteins (Kabat, 1967) are synthesized in non-dividing chicken erythrocytes. However, no particular significance can be attached at present to this observation since information on the biological role of non-histone proteins is completely lacking.

The experiments reported above with unsynchronized and synchronized HeLa cells have clearly demonstrated that only a part of histone synthesis in these cells is dependent on concurrent DNA synthesis whereas another part of histone synthesis continues even in

G_1. These findings confirm similar observations by Spalding et al. (1966) in HeLa cells. In addition, the present studies have revealed that various histone fractions of HeLa cells show differences in degree of dependence on DNA synthesis; lysine-rich and slightly lysine-rich histones appear to be more dependent on DNA synthesis than are arginine-rich histones.

All the previous work reported in the literature (see Introduction) as well as the results presented here suggest the possibility that the syntheses of histones and DNA may be governed by different regulatory mechanisms in cells in different developmental and physiological stages. The categories of relationships between histone and DNA synthesis which can be generally recognized are discussed below.

1) Histone and DNA syntheses are strictly coupled with each other in slowly dividing and not highly differentiated or matured cell types, such as those of root tips, lymphocytes, livers and amoebae. Most of the studies which conclude that there is a precise correlation between these two processes involve the demonstration of parallel increase in nuclear histones and DNA. However Bloch and Brack (1964) have reinterpreted these observations by suggesting that histones may be synthesized in the cytoplasm in G_1 as well as in S but may be transferred to the nucleus only during DNA synthesis. Despite this interesting suggestion, it is likely that in cell types such as those mentioned above a majority of the histones are synthesized only during the S phase.

2) Only arginine-rich histones are synthesized without the

accompanying synthesis of lysine-rich and slightly lysine-rich histones in non-dividing and highly differentiated cells or tissues as pea cotyledons and calf endometrium (Chalkley and Maurer, 1965) and chicken erythrocytes (present studies).

3) In rapidly proliferating and undifferentiated cells as L cells or HeLa cells, at least a part of synthesis of all of the histones is completely independent of DNA synthesis and continues in G_1. Initiation of DNA synthesis leads, however, to marked increase in histone synthesis even in these cases. Regenerating livers apparently present a situation intermediate between strict coupling and significant uncoupling of the two processes. In this case, histone synthesis appears to be only partially dissociated from DNA synthesis. It begins just prior to the onset of DNA synthesis and continues during the S phase.

The above recognition of differences in the relationship between histone and DNA synthesis among metabolically distinguishable cells may prove to be of fundamental biological importance. It is interesting to consider the possibility that the differences in the synthetic pattern of these two chromosomal components are causes, rather than consequences, of major alterations in cell division and other physiological processes.

REFERENCES

Alfert, M. 1955. In "Symposium on Fine Structure of Cells," Leiden, 1954, Groningen, P. Noordhoff, Ltd.

Allfrey, V. G., Littau, V. C., and Mirsky, A. E. 1963. Proc. Natl. Acad. Sci. U.S. 49, 414.

Barr, G. C. and Butler, J. A. V. 1963. Nature 199, 1170.

Bellair, J. T. and Mauritzen, C. M. 1965. Austral. J. Biol. Sci. 18, 160.

Blazsek, V. A. and Bukaresti, L. 1964. Experientia 20, 369.

Bloch, D. P. and Brack, S. D. 1964. J. Cell Biol. 22, 327.

Bloch, D. P. and Godman, G. C. 1955. J. Biophysic. Biochem. Cyt., 1, 17.

Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B., and Widholm, J. 1967a. In "Methods in Enzymology," Academic Press, New York, N.Y., in press.

Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C., Marushige, K., and Tuan, D. Y. H. 1967b. Science in press.

Bonner, J. and Huang, R. C. 1963. J. Mol. Biol. 6, 169.

Bonner, J. and Huang, R. C. 1966. In "Histones," (de Reuck, A.V.S. and Knight, J., eds.), Little, Brown and Company, Boston, p. 18.

Borun, T. W., Scharff, M. D., and Robbins, E. 1967. Proc. Natl. Acad. Sci. U.S. 58, 1977.

Burton, K. 1956. Biochem. J. 62, 315.

Busch, H., Byvoet, P., and Adams, H. R. 1963. Exptl. Cell. Res., Suppl. 9, 376.

Busch, H., Hnilica, L. S., Chien, S. C., Davis, J. R., and Taylor, C. W. 1962. Cancer Res. 22 (1), 637.

Cameron, I. L. and Prescott, D. M. 1963. Exptl. Cell Res. 30, 609.

Chalkley, G. R. and Maurer, H. R. 1965. Proc. Natl. Acad. Sci. U.S. 54, 498.

Cohn, P. and Simson, P. 1963. Biochem. J. 88, 206.

Cole, A. 1962. Nature 196, 211.

Comings, D. E. 1967. J. Cell Biol. 35, 699.

Crampton, C. F., Stein, W. H. and Moore, S. 1957. J. Biol. Chem. 225, 363.

Cruft, H. J., Mauritzen, C. M., and Stedman, E. 1954. Nature 174, 580.

Cruft, H. J., Mauritzen, C. M., and Stedman, E. 1958. Proc. Roy. Soc. B, 149, 21.

Dahmus, M. and Bonner, J. 1965. Proc. Natl. Acad. Sci. U.S. 54, 1370.

Daly, M. M. and Mirsky, A. E. 1954-55. J. Gen. Physiol. 38, 405.

Davis, J. R. and Busch, H. 1959. Cancer Res. 19 (2), 1157.

Deakin, H., Ord, M. G., and Stocken, L. A. 1963. Biochem. J. 89, 296.

Dingman, C. W. and Sporn, M. B. 1964. J. Biol. Chem. 239, 3483.

Dische, Z. 1930. Mikrochemie 8, 4.

Dreyer, W. J. and Bynum, E. L. 1967. In "Methods in Enzymology," Academic Press, New York, N.Y., in press.

Eagle, H. 1955. Science 122, 501.

Eagle, H. 1959. Science 130, 432.

Evans, J. H., Holbrook, Jr., D. J. and Irvin, J. L. 1962. Exptl. Cell Res. 28, 126.

Fambrough, Jr., D. M. 1968. Ph.D. Thesis, California Institute of Technology, Pasadena, California.

Fambrough, D. M. and Bonner, J. 1966. Biochemistry 5, 2563.

Fambrough, D. M. Fujimura, F., and Bonner, J. Biochemistry in press .

Feinendegen, L. E. and Bond, V. P. 1963. Exptl. Cell Res. 30, 393.

Flamm, W. G. and Birnstiel, M. L. 1964. Biochim. Biophys. Acta, 33, 616.

Frenster, J. H. 1965. Nature, 206, 680.

Giannoni, G. and Peacocke, A. R. 1963. Biochim. Biophys. Acta 68, 157.

Grogan, D. E., Desjardins, R., and Busch, H. 1966. Cancer Res. 26, 775.

Harbers, E. and Vogt, M. 1966. In "Proceedings of International Symposium on the Cell Nucleus--Metabolism and Radiosensitivity," Taylor and Francis, Ltd., London, p. 165.

Harris, H. 1966. Proc. Roy. Soc. B, 166, 358.

Hindley, J. 1963. Biochem. Biophys. Res. Commun. 12, 175.

Hnilica, L. S. 1965. In "Developmental and Metabolic Control Mechanisms and Neoplasia," The Williams and Wilkins Company, Baltimore, p. 273.

Hnilica, L. 1966. Biochim. Biophys. Acta 117, 163.

Hnilica, L. S. and Bess, L. G. 1965. Anal. Biochem. 12, 421.

Hnilica, L. S., Edwards, L. J., and Hey, A. E. 1966. Biochim. Biophys. Acta 124, 109.

Hnilica, L., Johns, E. W., and Butler, J. A. V. 1962. Biochem. J. 82, 123.

Hnilica, L. S., Taylor, C. W., and Busch, H. 1963. Exptl. Cell. Res. Suppl. 9, 367.

Holbrook, Jr., D. J., Evans, J. H. and Irvin, J. L. 1962. Exptl. Cell. Res. 28, 120.

Holoubek, V. 1966. J. Cell Biol. 31, 49A.

Huang, R. C. and Bonner, J. 1962. Proc. Natl. Acad. Sci. U.S. 48, 1216.

Huang, R. C. and Bonner, J. 1965. Proc. Natl. Acad. Sci. U. S. 54, 960.

Huang, R. C., Bonner, J., and Murray, K. 1964. J. Mol. Biol. 8, 54.

Huberman, J. A. and Attardi, G. 1966. J. Cell Biol. 31, 95.

Izawa, M., Allfrey, V. G., and Mirsky, A. E. 1963. Proc. Natl. Acad. Sci. U. S. 49, 544.

Johns, E. W. 1964a. Biochem. J. 92, 55.

Johns, E. W. 1964b. Biochem. J. 93, 161.

Johns, E. W. 1967. Biochem. J. 104, 78.

Johns, E. W. and Butler, J. A. V. 1962. Biochem. J. 82, 15.

Kabat, D. 1967. Ph.D. Thesis, California Institute of Technology, Pasadena, California.

Kinkade, J. M. and Cole. R. D. 1966a. J. Biol. Chem. 241, 5790.

Kinkade, J. M. and Cole, R. D. 1966b. J. Biol. Chem. 241, 5798.

Konrad, C. G. 1963. J. Cell Biol. 19, 267.

Laurence, D. J. R., Phillips, D. M. P., and Butler, J. A. V. 1966. Archs. Biochem. Biophys. 113, 338.

Laurence, D. J. R., Simson, P., and Butler, J. A. V. 1963. Biochem. J. 87, 200.

Lindsay, D. T. 1964. Science 144, 420.

Littau, V. C., Burdick, C. J., Allfrey, V. G., and Mirsky, A. E. 1965. Proc. Natl. Acad. Sci. U.S. 54, 1204.

Littlefield, J. W. and Jacobs, P. S. 1965. Biochim. Biophys. Acta 108, 652.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. J. Biol. Chem. 193, 265.

Lucas, A. M. and Jamroz, C. 1961. Atlas of Avian Hematology. U.S. Dept. of Agr., Washington, D.C.

Luck, J. M., Rasmussen, P. S., Satake, K., and Tsvetikov, A. N. 1958. J. Biol. Chem. 233, 1407.

Maio, J. J., and Schildkraut, C. L. 1967. J. Mol. Biol. 24, 29.

Marushige, K. and Bonner, J. 1966. J. Mol. Biol. 15, 160.

Marushige, K., and Ozaki, H. 1967. Devel. Biol. in press.

Mauritzen, C. M., Starbuck, W. C., Saroja, I. S., Taylor, C. W. and Busch. H. 1967. J. Biol. Chem. 242, 2240.

Murray, K. 1962a. In "The Nucleohistones," (Bonner, J. and Ts'o, P.O.P., eds.), Holden-Day Inc., San Francisco, p. 15.

Murray, K. 1962b. In "The Nucleohistones," (Bonner, J. and Ts'o, P.O.P., eds.), Holden-Day Inc., San Francisco, p. 21.

Neelin, J. M., Callahan, P. X., Lamb, D. C. and Murray, K. 1964. Canad. J. Biochem. 42, 1743.

Neelin, J. M., and Neelin, E. M. 1960. Canad. J. Biochem. Physiol. 38, 354.

Neidle, A. and Waelsch, H. 1964. Science 145, 1059.

Ohba, Y. 1966. Biochim. Biophys. Acta 123, 76.

Palau, J. and Butler, J. A. V. 1966. Biochem. J. 100, 779.

Peacocke, A. R. and Preston, B. N. 1961. Nature 192, 228.

Perugini, S., Torelli, U., and Soldati, M. 1957. Experientia 13, 441.

Phillips, D. M. P. 1965. Biochem. J. 97, 669.

Phillips, D. M. P. 1966. Biochem. J. 101, 23P.

Prescott, D. M. 1966. J. Cell Biol. 31, 1.

Prescott, D. M., and Bender, M. A. 1962. Exptl. Cell Res. 26, 260.

Rasch, E. and Woodard, J. W. 1959. J. Biophysic. Biochem. Cytol. 6, 263.

Rasmussen, P. S., Murray, K., and Luck, J. M. 1962. Biochemistry 1, 79.

Reid, B. R., and Cole, R. D. 1964. Proc. Natl. Acad. Sci. U.S. 51, 1044.

Reisfeld, R. A., Lewis, U. J., and Williams, D. E. 1962. Nature 195, 281

Robbins, E. and Borun, T. W. 1967. Proc. Natl. Acad. Sci. U.S. 57, 409.

Satake, K., Rasmussen, P. S., and Luck, J. M. 1960. J. Biol. Chem. 235, 2801.

Schmidt, G. and Thannhauser, S. J. 1945. J. Biol. Chem. 161, 83.

Schneider, W. C. 1957. In "Methods in Enzymology," (Colowick, S. P. and Kaplan, N. O., eds.), Academic Press, New York, N.Y., vol. 3, p. 680.

Sheridan, W. F. and Stern, H. 1967. Exptl. Cell Res. 45, 323.

Spalding, J., Kajiwara, K., and Mueller, G. C. 1966. Proc. Natl. Acad. Sci. U.S. 56, 1535.

Taylor, J. H. 1960. Ann. N.Y. Acad. Sci. 90, 409.

Tuan, D. 1967. Ph. D. Thesis, California Institute of Technology, Pasadena, California.

Umaña, R., Updike, S., Randall, J., and Dounce, A. L. 1964. In "The Nucleohistones," (Bonner, J. and Ts'o, P.O.P., eds.), Holden-Day, Inc., San Francisco, p. 200.

Wilkins, M. H. F., Zubay, G., and Wilson, H. R. 1959. J. Mol. Biol. 1, 179.

Woodard, J., Rasch, E., and Swift, H. 1961. J. Biophysic. Biochem. Cytol. 9, 445.

Zubay, G. 1962. In "The Nucleohistones," (Bonner, J., and Ts'o, P.O.P., eds.), Holden-Day, Inc., San Francisco, p. 95.

Zubay, G. and Doty, P. 1959. J. Mol. Biol. 1, 1.