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R. K. Mishra [a c] , A. De Vries [a] , Glenn H. Brown [a] & W. H. Falor [b]

[a] Liquid Crystal Institute Kent State University, Kent, Ohio, 44242

[b] Lymph Research Laboratory Akron City Hospital, Akron, Ohio, 4439

[c] Department of Biophysics, All-India Institute of Medical Sciences, New Delhi, 16, India

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Influence of a Carcinogenic Fraction from Tobacco Smoke Condensate on the Aggregation of Histones: A Preliminary Study†

R. K. MISHRA,‡ A. DE VRIES and GLENN H. BROWN

Liquid Crystal Institute
Kent State University
Kent, Ohio 44242,

and

W. H. FALOR

Lymph Research Laboratory
Akron City Hospital
Akron, Ohio 4439:

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Abstract—X-ray diffraction studies are made of samples of arginine-rich histones, lysine-rich histones, and total histones. Samples are first studied when dry, next after the addition of a buffer solution, and finally after a subsequent addition of a carcinogenic fraction of tobacco smoke condensate.§ The changes in the diffraction pattern are discussed and it is shown that in two out of three samples studied the addition of the carcinogen makes the diffraction pattern more similar to that of the buffer solution itself. Thus, it appears that the carcinogen can cause a breakdown in the structure of histone-buffer mixtures. This may provide a key to the explanation of its carcinogenic activity.

Histones are basic proteins derived from cell nuclei. They are believed to be located in the chromosomes. They are a heterogeneous group of proteins, of rather low molecular weights. Butler *et al.*[1] found values for molecular weights in the range of 17,000 to

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‡ Permanent address: Department of Biophysics, All-India Institute of Medical Sciences, New Delhi-16, India.

§ Referred to hereafter as carcinogen.

51,000, and molecular weights as low as 8,100 have sometimes been ascribed to certain preparations.[2]

Depending on the method of fractionation, a sizeable number of fractions can be obtained. For example, Cruft[3] in his study of electrophoresis and gel filtration of histones, found from the unfractionated histones four or more fast moving bands associated with lysine-rich α-histones, followed by a heavy band containing γ-histones, closely followed by four fainter bands. Working with histone fractions under certain conditions there may be six or more bands in the α-group and two in the γ-group. β-histones may show three major bands, and in the presence of lanthanum ions as many as nine very sharp bands are found near the origin. Study of N-terminals of various fractions confirms that a variety of histone fractions exist,[4,5,6] implying structural differences in various fractions.

A number of functions have been ascribed to the histones. Some speculations suggest structural functions: cementing together and "glueing" of DNA molecules, enhancing their coiling or a supercoiling, unfolding of DNA, stabilizing DNA, and serving as the backbone of chromosomes. Neutralization of charges on DNA phosphates and stabilizing the RNA have also been suggested.

Still other functions of histones are genetic functions. These include suppressing or enhancing gene function (RNA synthesis), blocking or accelerating the DNA synthesis, acting as a ribonuclease, or helping the release of RNA from DNA.

Stedman and Stedman[7] first suggested that histones may have an important influence in the control of gene expression. It is relevant to inquire if abnormality of control, as occurring in cancer, is related to abnormality in histone structure or its association. The occurrence of a radioactive peak, RP2L, in histones from Walker tumor, which is not found in normal tissues,[8] lead to the expectation that structurally abnormal histones occur in cancer tissues. This has been later questioned,[9] and Butler *et al.*[1] believe that histones of the tumor were essentially the same as those of other tissues. Perhaps there are indeed only few differences between histones from tumors and from a number of normal tissues including calf thymus in regard to amino acid content, NH_2 terminals and "peptide" maps.[8,10] This view is not altered by recent studies.[11,12]

While the structure of histones in cancer and normal cells may not show important differences, it has not yet been examined whether, in the presence of a chemical carcinogen, histones may associate in a different manner, and in doing so, influence the DNA uncoiling in such a manner that carcinogenesis may be initiated. A study of this possible change in self-association or aggregation is the subject matter of this paper. DNA-histone complexes and histones show variation in aggregation as a function of dispersing medium: intramolecular and intermolecular association is influenced by solvents. X-ray diffraction of nucleohistones under 98% relative humidity gives characteristically the diffraction pattern found for DNA molecules. One sees a 3.4 Å meridional diffraction spot indicating regular translation of base pairs along the helix axis, and a layerline pattern arising from the helically disposed sugar-phosphate backbone. The strong first order equatorial reflection due to the distance between the centers of adjacent double helices may vary between 22 Å and 35 Å depending upon the degree of hydration. In addition, one may get a 60 Å reflection due probably to a lipid impurity, possible sphingomyelin. A 35 Å semi-meridional arc, not found in DNA alone or histone alone, has been ascribed to the bridges of histones between roughly parallel DNA macromolecules! At low humidity (32%) the diffraction pattern due to DNA fades out and most of the diffraction is due to histones.[13] The histone structure in a consolidated form now gives diffuse 4.7 Å and 9.7 Å diffraction rings. Isolated histone, when undenatured, gives diffuse rings in the region of 4–5 and 10 Å. In some patterns a sharp 4.7 Å reflection is visible.[14] Small angle diffraction on nucleohistones at various stages of drying again shows variation in aggregation as one proceeds from 98% relative humidity to 35%.[15] One may recall solvent induced phase changes in lyotropic liquid crystals; they may be caused by variations in intermolecular force fields.[16] Similarly, in the present case too, it may be relevant to inquire if the interaction with a carcinogen would influence in a similar manner the aggregation of histone. One may recall that Cruft *et al.*[17,18] obtained data which may be interpreted to mean differences in association of histones derived from the cancer cells as compared with normal cells. Histones of cancer cells had lower solubility, an anomalous pH mobility curve and greater association in acid solution. Some differences were found in " ar-

ginine-rich " histones of different tumors, but tumors were more like one another in this respect than like normal tissues. They concluded that the disease caused somatic mutation resulting in abnormal histones.

To examine histone-carcinogen interaction directly, in specific cases, we recorded first the X-ray diffraction patterns of dry samples, next the diffraction patterns obtained after the addition of a buffer solution, and finally the diffraction patterns after the subsequent addition of a carcinogen. It was found that the diffraction patterns obtained after adding the buffer solution may be considered as lying between the diffraction pattern of the dry histone and that of the buffer solution alone. The subsequent addition of the carcinogen shifted the pattern more toward that of the buffer solution. This will be discussed in more detail in the following sections.

1. Experimental Data

Arginine-rich and lysine-rich histones prepared by Butler's procedure[19] were obtained from the Nutritional Biological Laboratories, Cleveland, Ohio. Total histones from rat liver and calf thymus were obtained using a procedure essentially based on that of Steele and Busch.[20] The histones in the supernatant were most often not precipitated but dried in a flash evaporator at 37 °C. The buffer solution used was 0.001 M sodium bicarbonate, pH7. The carcinogen used was the heptane soluble fraction of tobacco smoke condensate, which is regarded as a very active carcinogen.[21]

The samples were contained in thin walled glass capillaries of 0.5 or 1.0 mm diameter. The X-ray diffraction patterns were recorded on flat film, in a modified Laue camera[22] using Ni-filtered Cu radiation; all photographs were made at room temperature (23–26 °C).

We shall present first the data on the arginine-rich histones (ARH) and the lysine-rich histones (LRH). Samples were studied in the dry form (dry ARH, dry LRH), after adding the buffer solution (wet ARH, wet LRH), and after the subsequent addition of the carcinogen (wet ARH + C, wet LRH + C). The diffraction maxima observed can be classified into four angular ranges (Table 1), and for the purpose of this discussion we shall designate them as " scattering around the beamstop ", " inner ring " (8–12° 2θ), " middle ring " (18.5–19.5° 2θ),

TABLE 1 Diffraction Data from ARH and LRH Samples[a]

	2θ ranges			
Sample	<8°[b]	8–12°	18.5–19.5°	25–28°
Dry ARH	—	8.7°	19.3°	—
	—	12.6 Å	5.64 Å	
	VS;[c]	S;S	S;S	
Wet ARH	—	8.0°	19.2°	25.9°
	—	13.6 Å	5.67 Å	4.22 Å
	S;	W;MS	W;S	M;B
Wet ARH + C	—	(10.3°)[d]	—[e]	27.2°
	—	(10.6 Å)	—	4.03 Å
	MS;	MW;B	VW;S	S;B
Dry LRH	—	(9.5°)	19.4°	—
	—	(11.5 Å)	5.63 Å	
	VS;	W;S	M;MS	
Wet LRH	—	(11.1°)	—	27.4°
	—	(9.8 Å)		4.01 Å
	MS;	MW;B		S;B
Wet LRH + C	—	11.3°	—	27.2°
	—	9.6 Å		4.03 Å
	MS;	MW;B		S;B
Buffer	—	11.5°	—	27.3°
	—	9.5 Å		4.02 Å
	MW;	M;B		S;B

[a] Data on the various diffraction maxima are listed as follows (if available): on the first line, the position of the maximum; on the second line, the interatomic distance calculated from the position; on the third line, before the semicolon the intensity, after the semicolon the width of the maximum.

[b] This column contains information about the scattering around the beamstop.

[c] V = very, M = medium; before the semicolon S = strong, W = weak; after the semicolon S = sharp, B = broad.

[d] Brackets indicate less reliable data.

[e] Diffraction ring present, but too weak to be measured.

and " outer ring " (25–28° 2θ). The positions of the maxima are recorded in Table 1, together with the corresponding distances (calculated using Keesom's formula,[22] $2d \sin \theta = 1.229\lambda$) and with information about the intensity and the shape of the maxima. No 2θ values could be given for the scattering around the beamstop, because the intensity of this scattering kept increasing until it was cut off by the beamstop. For comparison, we have also included in the table

data from the buffer solution used in preparing the wet ARH and wet LRH samples.

Generally only one sample was used in each case, but from the dry ARH and dry LRH two samples of each were studied. The two samples of dry ARH, obtained on two different occasions from the supply house, did not give identical patterns. Both samples gave a fairly strong and sharp ring at about 8.5°, but whereas one sample gave only a moderately strong diffuse ring at about 20°, the other sample gave superimposed on this ring a strong and sharp ring at 19.3° (the data given in the tables for dry ARH are the data obtained from this second sample; only the positions of the sharp rings are used in the tables, since the diffuse ring at about 20° was masked by the sharp ring at 19.3°). The wet ARH and the wet ARH + C were obtained from the dry ARH sample that did not show the sharp ring at 19.3°. The two samples of dry LRH gave essentially identical diffraction patterns.

We have also studied the influence of adding buffer solution and carcinogen to a sample of total histones. Position, corresponding distance,† relative intensity, and shape of the diffraction maxima are

TABLE 2 Diffraction Data from a Sample of Total Histones[a]

	2θ ranges				
Sample	8–12°	18.5–19.5°	21.5°	23.9°	25–28°
Dry	8.9° 12.2 Å S;MB[b]	19.2° 5.69 Å S;MS	21.5° 4.13 Å W;VS	23.9° 3.72 Å VW;VS	—
Wet	10.0° 10.9 Å S;B	19.1° 5.72 Å W;MS	—[c]	—	26.5° 4.14 Å MS;B
Wet + C	11.0° 9.9 Å S;B	18.9° 5.76 Å VW;MB	—	—	27.0° 4.06 Å S;B

[a] See Table 1.

[b] For abbreviations see Table 1.

[c] Possibly present, but too weak to be measured.

† For the rings at 21.5° and 23.9° Bragg's formula ($2d \sin\theta = \lambda$) was used, because of the obviously crystalline character of these rings. For the other rings we used again Keesom's formula.

listed in Table 2. In addition to maxima in the 2θ ranges mentioned above, two very sharp but weak rings were observed for the dry sample, indicating that the material had crystallized to a certain extent.

2. Discussion

The data in Table 1 show a considerable difference between the diffraction patterns of dry ARH and dry LRH, in particular with respect to the inner ring. They also show differences between the patterns of wet ARH and wet LRH. The wet LRH pattern is essentially identical to that of the buffer solution, which leads to the conclusion that in wet LRH the association or structure of the histone has become very irregular. The wet ARH gives a pattern which is significantly different from that of the buffer solution: the two sharp maxima shown by the dry ARH are still present in about the same positions (although much weaker), and the maximum due mainly to the buffer (the outer ring) occurs at a position significantly different from that for the pure buffer solution and also has a lower intensity. This indicates, on the one hand, that in wet ARH the histone retains to a considerable degree the same distances found in the dry histone, and, on the other hand, that the histone somewhat modifies the structure of the water in the buffer solution. A much more extreme example of the latter effect has been encountered by us in one of our samples of total histones: a water extract of histones, containing only 1% histones, gave a diffraction pattern in which the intensity of the outer ring was much less than for water itself, whereas the intensity of the inner ring was about the same as for water.

Our data on wet ARH + C (Table 1) show that the addition of the carcinogen destroyed most of the structural features which made the diffraction pattern of wet ARH differ from that of the buffer solution: the sharpness of the inner ring disappears and its position and intensity become much closer to those for the buffer, the middle ring disappears almost completely, and the outer ring attains the same intensity and position as found for the buffer.

The data on the total histones, presented in Table 2, show a great deal of similarity to those on the ARH and LRH samples listed in Table 1. This suggests that, although the data presented in this

paper are obtained from only a limited number of samples, they may well represent a general pattern.

A comparison of the data on the wet and the dry sample (Table 2) shows that adding the buffer solution had the following effects: the inner ring broadens and moves toward larger angles, the middle ring decreases greatly in intensity, the sharp rings at 21.5° and 23.9° virtually disappear completely, and there appears an outer ring. Upon the subsequent addition of the carcinogen the inner ring moves out still further, the middle ring decreases still more in intensity and broadens somewhat, and the outer ring increases in intensity and moves to a slightly larger angle. So, as in the case of the ARH, the addition of the carcinogen has reduced the differences between the diffraction pattern of the wet histone and that of the buffer solution. These changes may be regarded as indications that the carcinogen causes a certain breakdown in the association of structure of the histone.

Before closing this section we should like to make some additional comments about the middle ring, shown by most of our histone samples. The position of this ring is remarkably constant, varying only from 18.9° to 19.4°. Also, in some of the samples this ring is fairly sharp and looks rather much like a diffraction ring from a fine crystalline powder. These characteristics seem to point to some kind of regular molecular arrangement, which varies only slightly for the samples reported on here. Another interesting point is that the position of this ring is remarkably close to the position of the so-called " outer ring " found for several liquid crystalline substances: for a series of bis-(4′-*n*-alkoxybenzal)-2-chloro-1,4-phenylenediamines[22] diffraction angles of 19.4-20.5° were measured, and for ethyl-*p*-ethoxybenzal-*p*-aminobenzoate[23] values of 20.0–20.3°. These diffraction maxima were interpreted as being caused by the interaction of neighboring parallel molecules. It may very well be that for the histone samples the presence of the middle ring also should be regarded as indicative of a parallel arrangement of neighboring molecular segments. The " regular molecular arrangement ", referred to earlier in this paragraph, would then point to a regular kind of parallel packing, e.g. like that in a smectic B phase.

It may be mentioned at this point that these data throw light from a physical angle on the hypothesis of Stedman and Stedman[7] that histones interact with DNA in specific manner to control genetic

expression. Consistent with this view, very lysine-rich histones were found to be the most effective inhibitor of DNA synthesis [24,25,26] and arginine-rich Fraction 3 has been the most effective inhibitor of RNA synthesis.[27,28] The interest in this approach was lessened somewhat when it was realized that the composition of various histone fractions from different tissues, or in different species, is not too dissimilar and the diversity necessary for specificity was not found. However, it is now realized that some subtle modification like N-terminal acetylation will reduce the repression due to the histone.[29] DNA dependent RNA synthesis is increased by phosphorylation[30] or by decreased disulfide in F3 arginine-rich histone.[31] Phosphorylation of the serine,[32] methylation of the ϵ-amino groups of lysine[33] and disulfide interchange of arginine-rich F3 histone have all been observed.[34,35] Phosphorylation of histone appeared to be associated with DNA synthesis in regenerating rat liver.[36] The disulfide form is the predominant form in inactive unfertilized sea urchin eggs.[35] Briefly, it may be stated that relatively fine alteration of the histone molecule may effect RNA polymerase or other activities, while a gross change in histone content may not have been found. The information in this field is liable to increase further since significance of some phenomena like methylation are not yet determined.

Two other lines of investigations need be briefly referred to here: concurrent changes in biochemical processes involving DNA and histone in carcinoma and the effect of exogenously administered histones. Chi-Bom Chae *et al.*[37] studied phosphorylation of thymidine and the effects of various inhibitors on the phosphorylation of thymidine and on the DNA synthesis in Ehrlich ascites carcinoma cells *in vitro*. Many compounds inhibited incorporation of thymidine $2^{14}c$ into DNA and 1-β-D-arabinofuranosylcytosine while doing this also depressed incorporation of labelled glycine into slightly lysine-rich histones, lysine-rich histones and arginine-rich histones, in the latter two cases only slightly. Sadgopal and Bonner[38] had reported similar results with fluorodeoxyuridine. Chae *et al.* suggest the possibility that the continued synthesis of the lysine-rich and arginine-rich histones, while DNA synthesis is blocked, would cause imbalance of these compounds and this may be responsible for their lethal action. This has bearing in general to the inhibition of Ehrlich ascites

carcinoma cells by various compounds like derivatives of 5-fluoro-6-pyrimidine carboxaldehyde.

Exogenous histone may affect enzyme activity in cancer cells. Morris hepatoma 5123tc cells in rats, intact or adrenalectomized, have a low basal level activity of tryptophane pyrrolase and high of tyrosine transaminase. These enzymes are inducible by hydrocortisone. Intraperitoneal histones or poly-L-lysine increase the former but not the latter.[39]

These evidences and all data involving the effects of histones in various modifications on the DNA-dependent syntheses beg the question: do the histones act primarily and directly on the DNA? Association of molecules in the net result produced by a balance of fields of various molecules gathered together. A genome, a carcinogen/physiologically active molecule and a given histone may thus constitute a specific enough combination to bring about modulation of activity in some particular genome resulting in carcinogenesis or an altered biochemical and subsequent physiological function. The present preliminary data hint this possibility and the question is further to be explored.

3. Summary

In conclusion we can say that the X-ray diffraction patterns of some mixtures of histones with a buffer solution are significantly different from the diffraction pattern of the buffer solution itself. Important differences are: the position, intensity and/or shape of the inner ring, the presence of a middle ring, and the position and intensity of the outer ring. In the cases in which these differences were observed, they were considerably reduced by the addition of a carcinogen. This indicates that the carcinogen markedly changes the structure of the histone-buffer mixtures. This change in structure apparently has two possible aspects: a degradation in the association or structure of the histone itself, and a loss of the influence of the histone on the structure of the water. These observed effects of the carcinogen may provide a key to the explanation of its carcinogenic activity.

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