The preparation and characterisation of chromatin from third instar larvae of *Drosophila melanogaster*

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ABSTRACT

A procedure is described for the isolation of large amounts of chromatin from *Drosophila* third instar larvae. This material is suitable for immunisation of rabbits, and preliminary analysis by immunodiffusion reveals little cross reaction between chromatin and cytoplasmic antigens, using antisera prepared against them. In addition to these studies, results from an examination of the exchange of radioactively labelled cytoplasmic proteins with nuclei suggest that no more than 5% of the total chromatin protein is of cytoplasmic origin. Electrophoretic analysis indicates that of this 5%, 20% migrates as a single band, in the same position as f2b histone. The techniques of radioactively labelling larvae which were used, may be suitable for achieving specific activities of up to 100 μ C/mg larval protein. Contamination of the chromatin by yeast proteins from the larval food is no more than 10%. Of this 30% is represented by one electrophoretic band. Immunological analysis reveals little cross reaction with yeast extracts.

Electrophoretic and amino acid analyses of the chromatin proteins confirm earlier reports concerning *Drosophila* histones.

INTRODUCTION

Current interest in the control of the gene activity in eukaryotes is reflected in the large number of reports concerning studies of chromatin and chromatin components¹⁻⁵. However, as in bacteria it seems likely that a combined biochemical and genetical approach will provide the most powerful method of understanding genetic control. For detailed genetic studies in the higher eukaryotes *Drosophila melanogaster* has no equal, while the excellent cytology of the larval polytene chromosomes offers the opportunity to study changes in gene activity at the microscopic level.

A number of studies of *Drosophila* histones have been made⁶⁻⁹. Helmsing *et al*¹⁰ have studied the electrophoretic pattern of non-histone proteins in different larval tissues and have studied the changes caused in these patterns by ecdysone, or by heat shock, and which may

be related to the changes in puffing which occur at the same time. Elgin and Hood¹¹ have studied changes in both histones and non-histone proteins of Drosophila embryos during development.

One approach to studying those proteins involved in the control of gene activity in eukaryotes is to use immunofluorescence techniques to study the distribution of these proteins on the larval polytene chromosomes of Drosophila and their correlation with the state of activity of specific genetic loci as assayed by puffing¹², or by incorporation of tritiated uridine¹³. For this sufficient chromatin must be available for fractionation eventually into single protein components for immunisation. None of the published techniques for isolating large amounts of chromatin have proved suitable in our hands. Nor have any authors to date considered the levels of contamination of Drosophila chromatin that might ensue from the growth of larvae on yeast medium or from the cytoplasm.

In this paper we report a procedure for the isolation of large quantities of chromatin from third instar larvae of Drosophila melanogaster, which fulfils the generally recognized criteria for chromatin¹⁴, and which is, as far as it is possible to tell, free of any large scale cytoplasmic contamination. The contamination due to food on which the larvae feed is easily characterised. This preparation is suitable for immunisation.

MATERIALS AND METHODS

Chemicals: Analar grade reagents were used when possible and were obtained mainly from British Drug Houses. Phenyl-methyl-sulphonyl fluoride was obtained from the Sigma Chemical Co., while glycerophosphoric acid (20% w/w solution) was obtained from Hopkins and Williams Ltd.

Larvae: Eggs were collected from Drosophila melanogaster (strain Oregon-R) in population cages over 24 hours, and then allowed to develop at either room temperature or at 25°C on yeast/agar medium (10% inactive dried yeast, 10% sucrose, 5% agar - autoclaved for 15 minutes). Between 500g and 1000g of larvae could be collected in this way, of which over 95% would be third instar.

Larvae were washed from the medium and suspended in 9% (w/v) NaCl. Under these conditions the larvae float, while food debris sinks. The larvae were removed from the surface, washed many times in tap water and then strained and weighed. These larvae were generally

used immediately.

Nuclei and chromatin preparation: Nuclei were isolated in Medium N⁷, but with increased sucrose concentration¹⁵ (0.25M sucrose, 2.0mM MgCl₂, 0.2mM CaCl₂, 100mM potassium glycerophosphate, pH 6.8). All operations were carried out at 4°C.

The larvae were chopped extensively with a sharp knife on a wooden board and then suspended in 2-3 volumes of Medium N. During homogenisation the medium was also made 10⁻⁴M in phenylthiourea to inhibit tyrosinase activity¹⁶. In some experiments the protease inhibitors iodoacetamide (10⁻³M), phenyl-methyl-sulphonyl fluoride (10⁻⁴M) and sodium bisulphite (5mM) were added^{17,18}. The suspension was then homogenised by two or three strokes in a Potter-Elvehjem glass homogenizer with a teflon pestle (clearance 0.13mm) rotating at 800-1000rpm. After filtering the homogenate through eight layers of muslin, the residue was suspended in an equal volume of Medium N and homogenised and filtered as before. This procedure was repeated.

The three filtrates were combined and centrifuged at 1000xg for 10 minutes. This supernatant is referred to as cytoplasm. The pellet was resuspended in 2-3 volumes of Medium N and centrifuged as before. This was repeated. The final pellet was resuspended in Medium N and made 1.67M in sucrose by mixing with a concentrated sucrose solution (2.4M sucrose, 2.0mM MgCl₂, 0.2mM CaCl₂, 100mM potassium glycerophosphate, pH 6.8)¹⁹. This suspension, having the equivalent of approximately 100g larvae per 100ml, was layered over an equal volume of concentrated sucrose solution (above) and centrifuged at 50,000xg for 10h. After removing the supernatant the gelatinous pellet was suspended in 10-15 volumes of Medium N and the nuclei sedimented by centrifuging at 1000xg for 10 minutes. This was repeated twice.

Chromatin was then extracted from the nuclei by the procedure of Huang and Huang²⁰. The nuclei were washed three times in 10-15 volumes of EDTA-saline (75mM NaCl, 24mM EDTA, pH 8.0), once in a similar volume of 50mM tris-HCl, pH 8.0, and then with successive 10-15 volumes of water, centrifuging each time at 1000xg for 10 minutes. Chromatin swelled during the 2nd and 3rd water washes when it was removed and sheared by blending in an MSE Atomix for 15 seconds, at full speed. The chromatin solution was clarified by centrifugation at 10,000xg for 10 minutes. Sonication of the pellet at this stage, in water, liberated variable, small amounts of chromatin. The chromatin

solution was concentrated by filtering through 0.2 μ Millipore filters under pressure. All stages in the extraction procedure were monitored by observing the U.V. spectra of solutions.

Fractionation of chromatin: Histones were prepared by acid extraction according to the procedure of Bonner *et al*¹⁴. Histone fractions were prepared by method 1 of Johns²¹. Total chromosomal proteins were prepared free of nucleic acids by centrifuging a solution of chromatin, containing less than 500 μ g/ml DNA in 2M NaCl, 6M urea, 0.1M tris-HCl (pH 8.0) 2mM dithiothreitol at 100,000xg for 48 hours^{22,23}. The presence of dithiothreitol increased the yields of chromosomal proteins remaining in the supernatant and not sedimenting with the DNA, although no alteration of the electrophoretic pattern was observed.

Electrophoresis: The acetic acid/urea system of Panyim and Chalkley²⁴ was used. Samples for electrophoresis were prepared in 8M urea and 1% (v/v) 2-mercaptoethanol. Pyronin Y was used as a tracking dye. After electrophoresis gels were fixed overnight in 50% trichloroacetic acid and then stained for two hours in 0.1% Coomassie Brilliant Blue R. in 50% trichloroacetic. They were destained at 37°C in 7% acetic acid.

Preparation of ³⁵S labelled cytoplasm: ³⁵S labelled yeast was prepared as described by Graham and Stanley²⁵. This was diluted to a specific activity of 0.5 μ Ci/mg wet weight of live yeast. A suspension of 50mg/ml of this yeast in 10% (w/v) sucrose, 100 I.U./ml penicillin, 100 μ g/ml streptomycin, and 1mg/ml Nipagin was prepared. About 60 well washed eggs were plated out on a 8cm circle of ashless filter paper (Whatman No. 42) soaked in 1ml of the yeast suspension. These were allowed to develop in a humidified chamber at 25°C. After 2 days a further 1ml of yeast suspension was added to the filter. The larvae were collected as late third instar on the fifth day. After washing well, the larvae were gently ground with sand in Medium N, filtered through eight layers of muslin and centrifuged for 4 minutes in a Beckman Microfuge (15,000xg). The supernatant was termed cytoplasm and had a specific activity of about 1 μ Ci/mg protein (assayed as d.p.m., precipitated by cold trichloroacetic acid). No nuclei were observed in this preparation under the phase contrast microscope.

Autoradiography of acrylamide gels: After fixing and staining, the gels were sliced into four slices which were dried down under vacuum onto filter paper. These dried gels were left in contact with x-ray film (Kodak) for up to three weeks when they were developed.

Immunology: 1. Yeast extracts were prepared from vegetative bakers yeast by sonication and by freezing and thawing, both in phosphate buffered saline (0.15M NaCl, 0.01M sodium phosphate, pH 7.2) and in water. Equal volumes of such extracts were combined, centrifuged at 10,000xg for 10 minutes and the supernatants concentrated to 15mg protein per ml.

2. Cytoplasm as prepared above was centrifuged at 12,000xg. The supernatant was collected and adjusted to a protein concentration of 15mg/ml.

3. Chromatin for injection was concentrated to 2mg DNA per ml.

Antisera to these antigens were prepared by giving rabbits intramuscular injections of 1ml of the above solutions at weekly intervals, the first being given with 1ml of complete Freund's Adjuvant (Difco) and subsequent injections being given with incomplete Freund's Adjuvant. Rabbits were bled from the external marginal vein of the ear one week after the third injection. Subsequent bleeds were preceded by one week with a booster injection as above. The designation 515-3 indicates the third bleed from rabbit 515.

Double diffusion plates were run as described by Roberts²⁶.

Analyses: Preparations of chromatin and chromosomal proteins were routinely screened by their U.V. spectra, an A_{260}/A_{230} ratio of approximately 1.0 being taken initially as the criterion for chromatin. DNA assays were carried out by the procedure of Burton²⁷, RNA assays by the orcinol reaction²⁸ and protein assays by the method of Lowry *et al*²⁹. Calf DNA, yeast RNA and bovine serum albumen were used as standards. The total DNA of third instar larvae was estimated by hot trichloroacetic extraction of a homogenate of larvae in water, according to Munro and Fleck³⁰ and assaying the extract as above.

RESULTS

Little or no cytoplasmic or cellular contamination of the nuclei was apparent after centrifugation through dense sucrose although, in addition to some fragments of cuticle, a large number of small particles was present. These were also present in the gut and in the medium on which the larvae were grown and had a similar size and shape to the yeast present in the food. However, they failed to grow on nutrient agar. Larvae grown under sterile conditions on the medium described by Nørby³¹, with the addition of 3% yeast extract and 400 units per ml of the fungicide mycostatin (Squibb), proved to be free

Figure 1. Densitometer traces of acrylamide gel electrophoretograms. a) calf thymus histone b,c,d) Drosophila histone from chromatin isolated from fresh larvae. b) total histone, c) f1 histone, d) f2b histone. e) Drosophila histone from chromatin isolated from frozen larvae f) chromatin (500µg protein) isolated from nuclei incubated in ³⁵S labelled cytoplasmic protein. g) 250µg protein isolated from particles. h) autoradiogram of f, exposed for two weeks. i and j) 250µg chromosomal proteins isolated in the presence and absence of proteolysis inhibitors respectively.

of these particles. Thus it seems that these particles are the digested remnants of yeast eaten by the larvae. We were concerned at the presence of these in the nuclei preparations, in which the particle nucleus ratio was often about 400:1 and were surprised that no authors previously working with chromatin from larval stages had observed them. Apart from growth under completely sterile conditions, we found no way to grow larvae free of yeast. Nor did it prove possible to devise procedures to separate these particles from nuclei as they have very similar sedimentation properties.

Particles have been isolated from medium on which larvae had been reared. These nuclei-free particles were purified in the same way as nuclei, by centrifugation through dense sucrose in Medium N. The same dry weight of particles, free of nuclei, and particles in the mixture with nuclei were subjected to the chromatin extraction procedure. No DNA was extracted from the particles. Some RNA and protein were extracted from the particles, the RNA:protein ratio being 0.8. The amount of protein extracted from the nuclei-free particles was 10% of the protein extracted from the particle, nuclei mixture.

Figure 1 shows a densitometer trace of a polyacrylamide gel of these particle proteins. One very strong band is apparent which is identifiable with a band in the trace of the polyacrylamide gels of chromatin. In addition a large number of faint bands were apparent only in gels containing 300-400 μ g of pure particle protein. These were much too weak, to be detected in gels of chromatin or chromosomal proteins, which usually contained 300-400 μ g of total protein.

Table 1 shows the distribution of DNA throughout four preparations of chromatin from third instar larvae. The different distribution of DNA in the preparations from frozen larvae, together with their general appearance after thawing, suggests to us that more degradation has occurred than in the preparations from fresh larvae, as a result of the release of lysosomal enzymes. This is confirmed by the electrophoretic pattern of histones isolated from such chromatin (Fig. 1). The f1 histone is absent and a number of strong bands are found migrating ahead of the f2a1 histone. Therefore we generally only use fresh larvae. Since high activities of degradative enzymes may be expected to be present in late third instar larvae, in view of the extensive histolysis which takes place following pupation, we have in general taken the precaution of incorporating the protease inhibitors phenyl-methyl-sulphonyl fluoride, iodoacetamide and sodium bisulphite³

Table 1. The characteristics of four chromatin preparations

	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>
	% total DNA			
Residue from homogenization:	7	12	17	18
Supernatants of low speed washes:	7	7	11	8
Supernatant of sucrose gradient:	61	62	26	19
Combined washes of nuclei in Medium N and in EDTA-saline:	1	1	16	22
Chromatin:	22	14	22	19
Insoluble residue:	3	5	8	14
Total DNA recovered/ 100gm wet weight larvae:	14.5mg	14.6mg	13.8mg	12.0mg
DNA recovered as chromatin/ 100gm wet weight larvae:	3.19mg	2.09mg	2.63mg	2.99mg
Protein/DNA ratio:	2.04	2.01	-	-
RNA/DNA ratio:	0.40	0.66	0.56	0.36
Spectral properties:				
A_{230}/A_{260}	1.15	1.18	1.20	1.38
A_{280}/A_{260}	0.68	0.73	0.72	0.73
A_{320}/A_{260}	0.23	0.35	0.16	0.20

A. - Chromatin isolated from fresh larvae in the absence of inhibitors.

B. - Chromatin isolated from fresh larvae in the presence of
 2×10^{-4} M PMSF
 2×10^{-3} M Iodoacetamide.

C., D. - Chromatin isolated from frozen larvae in the absence of inhibitors.

in the nuclei isolation medium. The presence of these inhibitors does not affect the yield of chromatin DNA.

The total DNA extracted from third instar larvae by hot trichloroacetic acid was estimated to be 19.6mg per 100g wet weight, while the total DNA recovered throughout eight preparations of chromatin had a mean of 16.1mg (S.D. $\pm$ 1.9) per 100g wet weight of starting material. These figures are in good agreement, although somewhat lower than other published values (25mg DNA per 100g adult flies³²; 30mg DNA per 100g third instar larvae³³). On the basis of our estimates between 10% and 20% of the larval DNA has been recovered as chromatin.

Tables 1 and 2 show the characteristics of several preparations of chromatin isolated under various conditions. The protein:DNA ratios, the histone:DNA ratios and the ultra-violet spectra are in good agreement with the general criteria of chromatin¹⁴ and with the values observed in chromatin from different tissues of *Drosophila hydei* by Helmsing et al¹⁰. In contrast, the RNA:DNA ratios are rather high, being between 0.3 and 0.5. However, at least half of this is accounted for by the RNA extracted from the particles contaminating the nuclei.

Table 2. The composition of the three chromatin preparations from third instar larvae, in the presence of 2×10^{-4} M PMSF, 2×10^{-3} M iodoacetamide, and 5×10^{-3} M sodium bisulphite.

Starting Materials	Yields					<u>RNA</u> DNA	<u>Protein</u> DNA	<u>Histone</u> DNA
	DNA	RNA	Protein	Histone	Non-			
	mg	mg	mg	mg	histone			
				(as acid extractable protein)	protein mg (by sub- traction)			
1600g	30	8.4	73	37	36	0.28	2.4	1.2
590g	12	4.2	34	17	17	0.34	2.8	1.4
260g	7.7	2.6	17	8.3	8.7	0.34	2.2	1.1

We have been concerned with establishing that the proteins isolated with the chromatin are really chromosomal proteins and not cytoplasmic proteins that have become adventitiously associated with the DNA during isolation^{14,15}. We have considered this in two ways.

The analysis of chromatin, cytoplasm and yeast preparations was carried out on double diffusion plates with antisera prepared against these preparations (Figure 2). In the two series of experiments the antigens were either dissolved in water or in 1M NaCl. The results can be summarised as follows:

A) In Water

- 1) The six antigens found in chromatin are not found in cytoplasm.
- 2) Most antigens found in cytoplasm were not found in chromatin: although three weak antigens were found in chromatin with anti-cytoplasm it was not clear whether these were also detected in cytoplasm.

Figure 2. Double-diffusion plate showing immunological comparisons between chromatin and cytoplasm from third instar larvae and yeast extract. The wells were filled as indicated. Antisera: 1) 515 (anti-chromatin); 2) 529 (anti-chromatin) 3) 520 (anti-yeast) 4) 540 (anti-cytoplasm). Antigens, all 7mg/ml protein:- A) chromatin dissolved in water; B) cytoplasm dissolved in water; C) yeast extract dissolved in water; D) chromatin dissolved in 1M NaCl; E) cytoplasm dissolved in 1M NaCl; F) yeast extract dissolved in 1M NaCl.

- 3) The antigens found in yeast are not found in chromatin.
- 4) Antisera against our first chromatin preparations (515) react with cytoplasmic antigens while those prepared against the chromatin preparations described above (529) react less readily with cytoplasmic antigens.
- 5) While 515 reacts with yeast antigens, 529 reacts only badly with those antigens.

B) In 1M NaCl

- 1) The four antigens found in chromatin are not found in cytoplasm.
- 2) As in A2 but the weak antigens detected in chromatin with anti cytoplasm were stronger. It was still not clear whether they were detected in cytoplasm.
- 3), 4) and 5) As in A. 3), 4) and 5).

However, quantification is difficult by this technique and so we have also studied the association of ^{35}S labelled cytoplasmic proteins with isolated nuclei, and their subsequent presence in the chromatin isolated from the nuclei.

In one experiment aliquots of nuclei isolated as usual by sedimentation through dense sucrose and washed twice in Medium N were incubated with labelled cytoplasm for varying times at 0°C . These were then washed three times in Medium N and the chromatin extracted as usual, the labelled cytoplasm remaining at various stages being estimated by liquid scintillation counting. Saturation is achieved after one hour. (Fig. 3a). The experiment was repeated but observing the amount of labelled cytoplasm present in chromatin isolated from nuclei which had been incubated in varying amounts of labelled cytoplasm protein for two hours. We have analysed these results in the following way.

Suppose that some fraction, F_N , of the total nuclei protein, N, is common, and exchanges, with a fraction F_C of the total cytoplasmic protein, C. Assuming all protein species of the cytoplasm initially have the same specific activity, S, then the specific activity of the exchangeable pool in the mixed nuclei and cytoplasm, at equilibrium, S' , is given by:-

$$S' = \frac{F_C CS}{F_C C + F_N N}$$

Figure 3. Incubation of nuclei with ^{35}S labelled cytoplasm. a) nuclei (1.14mg DNA; 13.5mg protein) incubated at 0°C with 3.82mg ^{35}S labelled cytoplasm (1 $\mu\text{Ci}/\text{mg}$ protein) in 1ml. Ordinate, % original label in nuclei after three washes in Medium N (●—●) and % original label in isolated chromatin (○—○), after incubation. b) ^{35}S labelled cytoplasmic protein, Cmg, (2.21×10^5 trichloroacetic acid precipitable d.p.m./mg protein) incubated with nuclei (10mg protein; 0.8mg DNA) in 2ml, at 0°C for 90 min. Chromatin isolated and the remaining label estimated (D d.p.m.). $1/D$ against $1/C$, the intercept being 0.62×10^{-5} (d.p.m.)⁻¹. Isolated chromatin had a protein content of 1.76mg ($\pm$ S.D. 0.24).

If subsequently chromatin is isolated from these nuclei and has a total protein content, A, of which a fraction, F_A , belongs to the pool of exchangeable proteins, then the total radioactivity, D, in the isolated chromatin is given by:-

$$D = F_A A S' = \frac{F_A A F_C^F S}{F_C^F + F_N^N}$$

A reciprocal plot of $1/D$ against $1/C$ will be linear and the intercept will allow calculation of F_A :-

$$\frac{1}{D} = \frac{1}{F_A A S} + \frac{F_N^N}{F_A A F_C^F S} \cdot \frac{1}{C}$$

Fig. 3b shows the results of such an experiment.

From the intercepts we have calculated that about 5% of the chromatin protein will exchange with cytoplasmic proteins under these conditions. Fig. 1 shows a trace of an autoradiogram of a polyacrylamide gel of chromatin isolated from nuclei mixed with ^{35}S labelled cytoplasm. By integration 20% of the label associated with the chromatin is in one band; the remainder is distributed throughout the gel. This band migrates in same position as the f2b histone but we have not yet determined whether it is, in fact, histone.

Total chromosomal proteins separated from nucleic acid by centrifugation in 2M NaCl and 6M urea show a complex pattern on electrophoresis (Fig. 1). It is clear that the presence of proteolytic inhibitors alters the pattern of proteins. Electrophoresis of unfractionated chromatin dissolved in 8M urea prior to electrophoresis presents a substantially different pattern.

We have considered the histones in more detail. Fig. 2 shows the trace of histones extracted with acid from chromatin. The f1 and f2b fractions are identified by comparison with fractions isolated from Drosophila chromatin by Method 1 of Johns²¹. By this method we were unable to extract soluble preparations of f2a and f3 histones. We have identified the f2a1 fraction by comparison with calf thymus histone since the f2a1 histone has been reported to have the same mobility in calf and Drosophila⁸. Histones prepared from chromatin isolated in the absence of proteolytic inhibitors show a similar pattern.

DISCUSSION

A procedure for the isolation of large quantities of chromatin from third instar larvae of Drosophila melanogaster has been developed. This procedure permits the isolation of sufficient chromatin and chromatin components for the successful immunization of rabbits against these antigens. By incorporation of proteolysis inhibitors into the medium for nuclei isolation much degradation is prevented, as indicated by the electrophoretic pattern of the chromosomal proteins.

We have had no success with the procedure of chromatin isolation from Drosophila larvae described by Oliver and Chalkley⁸. However, a possible reason for this may be that the conditions for homogenization of larvae are critical. Thus many procedures, and notably blending, in our hands, give rise to small pieces of fibrous material which co-sediment with the nuclei and which swell extensively in water, masking any chromatin which may be present. The resultant material has a strong absorption peak at 280nm and contains negligible DNA compared with protein. We have not identified this material but have considered that the fibrous particles may be sheared pieces of the body wall musculature and that the proteinaceous gel extracted could be myosin. The procedure of Dick and Johns⁶ for isolating chromatin from Drosophila without prior isolation of nuclei could lead to greater cytoplasmic contamination than our procedure. In our hands the product had an A_{230} considerably in excess of A_{260} , indicating a relatively high protein content compared with our procedure in which the A_{230}/A_{260} is approximately 1. Thus, although similar yields of chromatin DNA were achieved with this method (24% in one preparation) we have not generally used it.

We have considered the contamination of our preparation of chromatin, both from the yeast food of the larvae and from cytoplasm. The former contamination is one peculiar to our source of chromatin. It would not generally be expected to affect preparations from embryos or from larval tissues, except perhaps gut. To our knowledge no other authors have considered this problem although preparations of chromatin have been reported from whole larvae^{6,8} and from mid-gut¹⁰. We find that a major fraction (about 30%) of the protein contamination from particles is accounted for by one band on electrophoresis, while the remainder is contained in a very large number of minor, and

individually, negligible bands. Moreover, no yeast antigens were found in the chromatin.

We feel that this characterisation of the contaminating protein is sufficient for present purposes, although it may be desirable in the future to look again for ways of removing the contaminants.

The contamination of the chromatin by adventitiously associated cytoplasmic proteins is difficult to determine without any functional criteria for supposing that a given protein is cytoplasmic rather than nuclear, or without any procedure for identifying its location in the intact cell. Some proteins may well function in vivo in both the nucleus and the cytoplasm. In view of the control exercised on the nucleus by the cytoplasm, this is a real possibility. We have shown that about 5% of the chromatin protein is exchangeable with cytoplasm in vitro, and that a large part of this exchange is due to one species. For the reasons outlined above, this is an upper limit, although it is not easy to rule out the possibility that some cytoplasmic proteins have become irreversibly associated with the nucleus during isolation and that because exchange is slow, for example, their presence in chromatin is not detected by our technique. However, we feel that this is not likely to be the case for many true cytoplasmic proteins.

In addition the analysis by immunodiffusion indicates that chromatin has few cytoplasmic antigens and that the chromatin antigens are not found in the cytoplasm. We have analysed the antigens dissolved both in water and sodium chloride. Chromatin dissociates less in water than in salt hence one might expect to find more antigens under the latter conditions. However at high sodium chloride concentrations rabbit antibody-antigen complexes are solubilised. Shaw and Huang²¹ used 3M NaCl for complete dissociation of chromatin. In these initial studies we used a compromise value of 1M NaCl. Under these conditions we detected a change in the antigen pattern with anti-chromatin and an increase in the strength of the chromatin antigens detected with anti-cytoplasm. Apparently no new antigens appeared. A study of the behaviour of chromatin on double diffusion plates and the conditions required for optimal precipitation is under way. To overcome the problem of high salt concentrations avian antisera are being studied.

Table 3. Amino acid composition of chromosomal proteins from third instar larvae of Drosophila melanogaster - Moles % of recovered amino-acids

	A	B	C	D	E	F	G
Lys	11.0	10.1	8.3	13.9	16.2	12.4	14.9
His	2.1	2.2	2.9	2.1	1.8	1.8	2.3
Arg	8.2	9.5	6.7	8.6	5.8	7.3	8.9
Asx	9.0	8.7	8.5	7.3	8.8	8.2	4.9
Thr	4.9	5.1	4.1	4.8	5.4	6.0	5.3
Ser	4.9	5.9	5.1	4.1	7.0	6.4	5.0
Glx	12.2	11.3	10.9	9.4	12.4	11.1	8.4
Pro	3.3	3.6	4.2	4.5	4.7	4.6	4.8
Gly	11.6	11.0	16.9	9.2	8.5	8.5	8.7
Ala	9.1	8.8	8.2	11.5	11.7	12.0	13.6
Cys	n.d.	n.d.	n.d.	n.d.	1.0	0	0.2
Val	5.8	5.4	5.4	6.7	5.2	5.5	6.2
Met	0.5	0.8	1.0	0.7	0.2	0.4	0.6
Ile	4.9	4.1	6.2	5.2	3.3	4.3	4.4
Leu	7.7	8.3	8.3	7.7	5.0	7.4	7.7
Tyr	2.3	2.5	2.3	2.3	1.2	1.9	2.3
Phe	2.4	2.8	2.7	2.0	2.0	2.2	1.7
Lys/Arg Ratio	1.34	1.07	1.24	1.63	2.8	1.70	1.7
Basic Amino Acids (Lys + His + Arg)	21.3	21.8	17.5	24.5	23.8	21.5	26.1
Acidic Amino Acids	21.2	20.0	19.4	16.7	21.2	19.3	13.3

Notes:

1. A and B are analyses of total chromatins, prepared in the absence and in the presence of proteolytic inhibitors, respectively. (see text).
2. C is the analysis of the protein-DNA complex remaining after acid extraction of histones.
3. D is the mean of analyses of total histone from two different preparations of chromatin, both prepared in the presence of proteolytic inhibitors. For comparison we include amino acid analyses of total Drosophila histones from adult flies (E) published by Dick and Johns⁶, and larvae (F) published by Oliver and Chalkley⁸, and also of total calf thymus histones, published by Dick and Johns⁶(G).
4. Tryptophan and cysteine were not determined; no corrections were made for hydrolytic losses, nor was glycine corrected for the presence of nucleic acid in A, B and C. n.d. - not determined

From the electrophoretic analysis of chromosomal proteins the addition of proteolysis inhibitors during chromatin isolation is shown to be necessary.

The f1, f2b and f2a1 have been identified as described in the results. The f1 has a lower mobility than calf thymus f1 confirming previous reports. However we find it to be composed of three fractions all of which are extracted by 5% perchloric acid, and none of which correspond to the larval-specific protein reported by Oliver and Chalkley⁹.

There is broad agreement between our analyses of Drosophila histones and those earlier reported for Drosophila^{6,8} and for calf thymus⁶. The content of basic amino acids is slightly higher than previously reported, while the acidic amino acid content is significantly higher than previously reported for calf but not as high as previously reported for Drosophila. However, it is notable that the high aspartic acid content of Drosophila histone agrees well with previous reports, contrasting with a comparatively low value for calf thymus histone. (Table 3).

The non-histone proteins show a slight excess of acidic over basic amino acids, while total chromosomal proteins show a slight excess of basic amino acids. The lysine content is much reduced in these fractions compared with histones although the arginine content is similar in all classes. The amino acid analyses show very little difference between the chromatins extracted in the presence and in the absence of proteolysis inhibitors.

The technique for the purification of chromatin described here and the preparation of antibodies against that chromatin now permits us to study the association of those antigens with the salivary gland chromosomes of Drosophila. These studies are in progress. The same technique for chromatin purification has been applied to other larval and embryonic stages (unpublished results). We are currently studying changes in chromatin proteins during development.

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