Trout-Liver Histones

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1. The histones of trout liver were examined and four main fractions (f1, f2b, f2a and f3) were isolated and characterized. 2. The amino acid analyses, *N*-terminal group analyses and starch-gel electrophoresis patterns are remarkably similar to the corresponding fractions of calf thymus. 3. The group f2a was also separated into two subfractions, f2a1 and f2a2, which are similar to those of calf thymus.

Calf-thymus histones can be fractionated into four main groups, f1, f2a, f2b and f3 (Johns & Butler, 1962*a*; Johns, 1964). The differential extraction procedures used for this tissue have also been employed to prepare histone fractions from other mammalian tissues and mammalian tumours, giving similar fractions. A comparison between homologous fractions from these tissues does not show appreciable differences, either in their electrophoretic behaviour or in their amino acid and *N*-terminal group analyses (Hnilica, Johns & Butler, 1962; Laurence, Simson & Butler, 1963; Laurence, Phillips & Butler, 1966). Phillips & Johns (1965) and Hnilica (1965) have fractionated the group f2a from calf thymus into two fractions (f2a1 and f2a2), but hitherto there has been no correlation of these fractions with histone fractions from other sources.

The study of histones from animals other than mammals would throw further light on their specificity and possibly yield some clues about the biological role of histones. Although whole histone has been prepared from a great variety of sources (Phillips, 1962), very little has been done to fractionate this material. Early histone fractionations from several species were done by Cruft, Mauritzen & Stedman (1957) and additional work has since been done in some other Laboratories. Neelin (1964) and Hnilica (1964) both found one extra fraction in chicken-erythrocyte histones with threonine as the main *N*-terminal amino acid. Johns & Butler (1962*b*) prepared histone fractions from wheat germ; they showed that the fraction corresponding to the arginine-rich histones of animal tissues (f3) appears to be missing and that there are considerable differences in amino acid and *N*-terminal amino acid analyses compared with calf-thymus histone fractions. Iwai (1964) has obtained histone fractions from rice embryos and the amino acid analyses of these differ greatly from those of wheat germ.

We have now examined trout-liver histones. Since fishes come much earlier than mammals in the evolutionary scale, it seemed desirable to know if the basic proteins of their somatic chromosomes are similar to the histones of mammalian species.

EXPERIMENTAL AND RESULTS

Preparation of nuclear material. The livers extracted from trout immediately after death were rapidly frozen by pressing between blocks of solid CO_2 and then stored for 2 days at $-18°$. In one operation 8·4g. of this material was cut into small pieces, allowed to thaw partially in the cold room, homogenized (Dounce, 1955; as modified by Laurence *et al.* 1963) with 40ml. of 0·25M-sucrose–3mM-$CaCl_2$ with two pestles of increasing diameter and the suspension was then filtered through two thicknesses of surgical gauze. The suspension was diluted with 40ml. of the same solution and centrifuged at 2500*g* in an MSE angle-head centrifuge for 15min. The supernatant was discarded, the sediment resuspended and homogenized in 40ml. of 0·25M-sucrose–3mM-$CaCl_2$ with a third tightly fitting pestle and the suspension filtered through four thicknesses of surgical gauze. The suspension of nuclei was diluted with 40ml. of the same solution and centrifuged at 1800*g* for 10min. The supernatant was discarded and the nuclei were resuspended in 40ml. of 0·9% NaCl solution and homogenized in an MSE tissue blender for 1min. at top speed. The suspension was diluted with 40ml. of 0·9% NaCl solution and centrifuged at 2500*g* for 15min. The supernatant was discarded. The sediment constituted the nuclear material to be used in further steps. All the above operations were carried out at 4°. The method described is based on that of Laurence & Butler (1965) for preparing rat-liver histones.

Preparation of trout-liver whole histone. The material obtained as described above was resuspended in 15ml. of 0·25N-HCl, transferred to a specimen bottle and rotated

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on a turn-table for 2hr. at room temperature. After centrifugation at 2500 *g* for 15min. the whole histone was precipitated from the supernatant by adding 6vol. of acetone, and the precipitate washed twice with more acetone and dried under vacuum. The yield was about 4mg./g. wet wt. of tissue.

Preparation of histone fractions. (*a*) The same procedure as above was used to prepare saline-washed nuclear material from 56·7g. of frozen livers. The last sediment was washed with 4vol. of ethanol in a Dounce homogenizer and centrifuged at 2500 *g* for 10min. A second wash was carried out with 4vol. of 80% (v/v) ethanol in the same way. The final pellet was resuspended overnight in 35ml. of ethanol–1·25N-HCl (4:1, v/v) and then centrifuged. A second extraction for 2hr. was carried out with 10ml. of the same solvent and the suspension then centrifuged. The two extracts were combined. The sediment was further extracted with 30ml. of 0·25N-HCl for 2hr. After centrifugation the pellet was re-extracted with 10ml. of 0·25N-HCl overnight and the suspension then centrifuged. The two extracts were not combined. The sediment was discarded. All the above operations were carried out at 4°.

The ethanol–HCl extract was dialysed against 67ml. of ethanol overnight in the cold room, followed by two more dialyses with 67ml. of fresh ethanol for 2hr. each time. The precipitate (fraction f3) was washed once with ethanol, twice more with acetone and dried under vacuum. The yield of fraction f3 was 9mg. Then 3vol. of acetone was added to the supernatant, and the precipitate obtained was washed twice with acetone and dried under vacuum. The yield of fraction f2a was 46mg.

The two solutions of the 0·25N-HCl extracts were treated separately. To each one 3vol. of acetone was added with vigorous stirring and the precipitates were washed once with acetone–0·25N-HCl (3:1, v/v), twice more with acetone and then dried under vacuum. The combined yield of fraction f1 was 24mg. A further 3vol. of acetone was added to each supernatant and the precipitates obtained were washed twice with acetone and dried under vacuum. The combined yield of fraction f2b was 21·5mg.

The procedure described above is based on method 2 of Johns (1964).

(*b*) To another ethanol–HCl extract, prepared as described in (*a*), 6vol. of acetone was added and the precipitate obtained was washed with acetone and dried under vacuum. In this way the slightly lysine-rich and arginine-rich histones (fractions f2a+f3) were isolated together. This material (32mg.) was dissolved in 1·86ml. of water and 1·34ml. of 3N-HCl added to make a solution 1·25N with respect to HCl. About 1mg. of the material was insoluble and was separated by centrifugation. Ethanol was now added to the clear supernatant to make this solution 80% (v/v) with respect to ethanol. A precipitate formed, which was centrifuged down, washed once with ethanol–1·25N-HCl (4:1, v/v) and twice with acetone and dried under vacuum. The yield of precipitate (fraction f2a1) was 12mg. The supernatant was dialysed at 4° for 2hr. against a large volume of ethanol. The precipitate (fraction f3), after being washed once with ethanol and twice with acetone and dried under vacuum, amounted to 1mg. Then 3vol. of acetone and 4 drops of conc. HCl were added to the solution, and the precipitate that formed was washed twice with acetone and dried under vacuum. The yield of precipitate (fraction f2a2) was 11·5mg.

Starch-gel electrophoresis. The method was that described by Johns, Phillips, Simson & Butler (1961), unbuffered 0·01N-HCl being used for the electrode vessels and for making the gel.

The whole histone of trout liver has an electrophoretic pattern similar to that of calf-thymus whole histone, although the fastest band is diffuse and two extra fine bands appear near the origin.

The relative mobilities of the main electrophoretic bands of trout-liver histone fractions correspond closely to those of calf-thymus histone fractions described by Johns & Butler (1962*a*) (Fig. 1). Fraction f1 shows a strong band

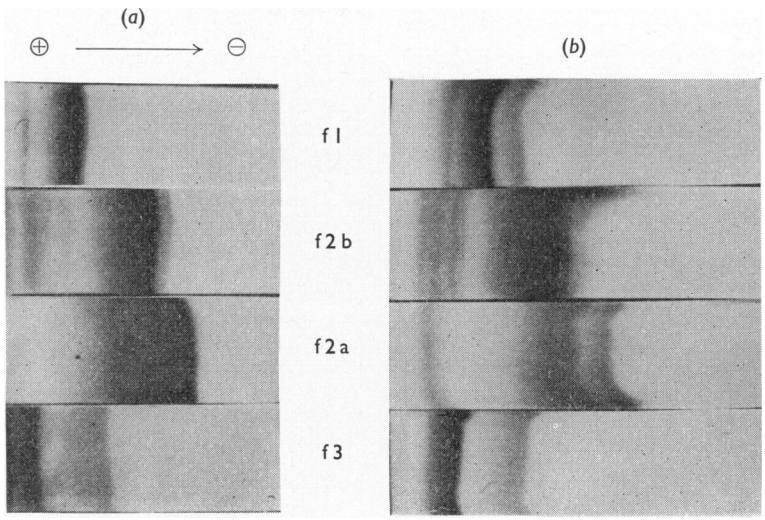

Fig. 1. Starch-gel electrophoretic pictures of histone fractions from (*a*) calf thymus and (*b*) trout liver.

with a fringe and a weak band behind them. Fraction f2b gives a strong band with a double fringe and four very fine bands near the origin. Fraction f2a shows the main band split into three and one fine band near the origin. Fraction f3 gives a strong band of low mobility and two fast-moving faint bands.

Fraction f2a2 shows two bands and fraction f2a1 one at intermediate mobility, in agreement with the pattern of fraction f2a.

Analyses. Total amino acid analyses were carried out by Miss P. Simson, using the method of Moore, Spackman & Stein (1958) with a Technicon Autoanalyzer apparatus. The samples were hydrolysed for 24hr. in 6N-HCl at 110°. The *N*-terminal amino acids were determined by the modification of Sanger's (1945) method described by Phillips (1958) and Phillips & Johns (1959). The results are shown in Tables 1 and 2. The analyses of calf-thymus histone fractions (prepared by method 2 of Johns, 1964) by Phillips & Johns (1965) are also given for comparison.

Countercurrent distribution of whole histone. The apparatus used was a fully automatic model manufactured by J. W. Towers and Co. Ltd. with 120 tubes. The capacity of each tube is 3ml. for each phase.

The procedure of Power & Butler (1965) was used to examine 21mg. of trout-liver whole histone in one experiment at room temperature. The system employed was 0·2% trichloroacetic acid–butan-2-ol. The shaking time was 50sec. and the settling time was 50min. The protein determination was carried out for each tube by the Lowry, Rosebrough, Farr & Randall (1951) method. The pattern obtained was very similar to that for calf-thymus whole histone in the same operational conditions. No isolation of the fractions was carried out.

DISCUSSION

Whole histone, four main fractions and two subfractions of trout liver have been prepared and characterized by the methods described above. They are very similar in amino acid analyses to those of calf thymus. Lower contents of lysine and higher contents of arginine have been found in all the fractions, but these differences may be too small to be significant.

The yields of *N*-terminal amino acid groups are similar to those of the same fractions for calf thymus. However, fraction f1 shows significant amounts of alanine and proline.

Table 1. *Composition of trout-liver whole histone and histone fractions, and of calf-thymus histone fractions*

The amino acid compositions are expressed as moles/100moles of all amino acids found, and no corrections have been made for hydrolytic losses. The results for calf-thymus histones (prepared by method 2 of Johns, 1964) were obtained by the fluorodinitrobenzene method by Phillips & Johns (1959). N.D., Not determined.

	Amino acid composition (moles/100moles)								
	Trout-liver histones					Calf-thymus histones			
	Whole histone	Fraction f1	Fraction f2b	Fraction f2a	Fraction f3	Fraction f1	Fraction f2b	Fraction f2a	Fraction f3
Asp	6·7	4·3	5·8	6·3	5·0	7·5	15·9	5·9	5·2
Glu	9·4	5·3	8·7	9·3	11·2			8·8	11·7
Gly	8·7	5·8	8·7	10·7	6·4	7·8	7·4	12·1	6·2
Ala	10·8	19·9	10·7	10·7	12·6	23·3	11·0	10·1	13·2
Val	6·5	7·2	6·7	7·2	5·1	5·7	6·2	7·5	4·7
Leu	7·3	4·4	6·7	10·3	9·3	5·5	10·8	14·3	14·2
Ile	5·0	2·5	5·0	4·8	5·1				
Ser	6·1	6·4	6·9	3·0	4·5	6·1	8·3	3·1	5·1
Thr	5·3	4·0	6·3	5·5	6·5	5·6	5·6	5·8	6·9
Phe	2·4	1·4	2·0	2·1	3·0	0·8	1·6	1·9	2·2
Tyr	2·7	1·8	3·3	3·0	2·5	0·6	3·1	2·6	2·5
Pro	5·0	7·3	3·7	3·5	4·6	8·1	4·7	2·8	5·1
Met	1·8	0·8	1·4	1·0	1·3	N.D.	N.D.	N.D.	N.D.
His	1·9	1·0	2·1	1·8	1·6	0·4	1·7	2·5	2·0
Lys	12·0	23·8	13·3	9·4	8·5	25·9	15·8	11·8	10·2
Arg	8·0	3·6	8·3	10·9	12·1	2·8	7·9	10·8	10·8
CyS	0·5	0·4	0·6	0·4	1·0	N.D.	N.D.	N.D.	N.D.
	N-Terminal amino acids (μmoles/g. of protein)								
Pro	13	14	41	2	0	5	39	1	0
Ala	8	10	9	7	30	3	9	5	35
Gly	1	2	2	2	0	1	3	1	0
Others	4	4	7	7	0	2	4	2	1

Table 2. *Composition of histone fractions f2a1 and f2a2 from trout liver and from calf thymus*

The fractions were prepared by the method of Phillips & Johns (1965). The amino acid compositions are expressed as moles/100 moles of all amino acids found, and no corrections have been made for hydrolytic losses.

	Amino acid composition (moles/100 moles)			
	Trout-liver histones		Calf-thymus histones	
	Fraction f2a1	Fraction f2a2	Fraction f2a1	Fraction f2a2
Asp	5·8	6·0	5·9	6·4
Glu	8·9	9·3	7·4	9·8
Gly	11·2	8·9	15·1	10·2
Ala	9·0	12·9	7·4	12·4
Val	7·2	6·4	8·4	6·1
Leu	9·5	11·5	8·3	11·5
Ile	5·4	4·2	5·7	4·3
Ser	3·2	3·3	2·4	3·2
Thr	6·0	5·1	6·4	4·0
Phe	2·5	2·2	2·2	1·2
Tyr	3·5	2·5	3·4	2·1
Pro	2·5	4·0	1·6	4·5
Met	1·3	0·6	0·7	0·2
His	2·0	1·8	2·6	2·9
Lys	9·5	10·1	10·1	11·1
Arg	12·2	10·8	12·8	9·9
CyS	0·3	0·2	—	—
Lys/Arg ratio	0·78	0·94	0·79	1·12

The starch-gel electrophoretic patterns of the whole histone and of the fractions are complex and similar to those of the calf-thymus histones.

The amino acid and *N*-terminal group analyses and the starch-gel electrophoretic pattern for fraction f1 show that it tends to be contaminated with fractions f2b and f3, but reduction of the time of extraction with hydrochloric acid to 2hr. decreases the contamination markedly.

Fraction f3 appears to be difficult to prepare from trout liver and the yields are low. This might be due either to a low content of this fraction in the chromosome or to a tendency of fraction f3, under the experimental conditions chosen, to be carried over into the other fractions. The arginine content and the *N*-terminal group analyses of the fractions seem to favour the second possibility.

The f2a group of histones is composed of several proteins. Phillips & Johns (1965) and Hnilica (1965) have separated calf-thymus fraction f2a into two subgroups, f2a1 and f2a2, which are still complex. We have found a similar fractionation of the f2a group for trout liver. Although the compositions of trout-liver fractions f2a1 and f2a2 show many features in common with those of calf-thymus fractions f2a1 and f2a2, some differences can be observed (Table 2). Since the methods of preparation are not the same as for calf thymus, these differences might be due to a different cut being taken between the two fractions. In addition, the lower contents of glycine are consistent with some contamination by fraction f3, which is likely in view of the low yield of fraction f3 obtained in this preparation (*b*). The histones of trout liver are therefore very similar to those of calf thymus.

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REFERENCES

Cruft, H. J., Mauritzen, C. M. & Stedman, E. (1957). *Phil. Trans.* **241**, 93.

Dounce, A. L. (1955). In *The Nucleic Acids*, vol. 2, p. 93. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Hnilica, L. S. (1964). *Experientia*, **20**, 13.

Hnilica, L. S. (1965). *Experientia*, **21**, 124.

Hnilica, L. S., Johns, E. W. & Butler, J. A. V. (1962). *Biochem. J.* **82**, 123.

Iwai, K. (1964). In *The Nucleohistones*, p. 59. Ed. by Bonner, J. & Ts'o, P. O. P. San Francisco: Holden–Day Inc.

Johns, E. W. (1964). *Biochem. J.* **92**, 55.
Johns, E. W. & Butler, J. A. V. (1962*a*). *Biochem. J.* **82**, 15.
Johns, E. W. & Butler, J. A. V. (1962*b*). *Biochem. J.* **84**, 436.
Johns, E. W., Phillips, D. M. P., Simson, P. & Butler, J. A. V. (1961). *Biochem. J.* **77**, 631.
Laurence, D. J. R. & Butler, J. A. V. (1965). *Biochem. J.* **96**, 53.
Laurence, D. J. R., Phillips, D. M. P. & Butler, J. A. V. (1966). *Arch. Biochem. Biophys.* **113**, 338.
Laurence, D. J. R., Simson, P. & Butler, J. A. V. (1963). *Biochem. J.* **87**, 200.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
Neelin, J. M. (1964). In *The Nucleohistones*, p. 66. Ed. by Bonner, J. & Ts'o, P. O. P. San Francisco: Holden-Day Inc.
Phillips, D. M. P. (1958). *Biochem. J.* **68**, 35.
Phillips, D. M. P. (1962). *Progr. Biophys. biophys. Chem.* **12**, 211.
Phillips, D. M. P. & Johns, E. W. (1959). *Biochem. J.* **72**, 538.
Phillips, D. M. P. & Johns, E. W. (1965). *Biochem. J.* **94**, 127.
Power, D. F. & Butler, J. A. V. (1965). *Biochem. J.* **97**, 32P.
Sanger, F. (1945). *Biochem. J.* **39**, 507.