Metabolism of Histones in Malignant Tissues and Liver of the Rat and Mouse

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1. The relative amounts of incorporation *in vivo* of L-lysine, and in one experiment L-arginine, into different histone fractions from Krebs ascites and a lymphoma ascites cells of mice and a 'solid' tumour and liver of rats have been determined.
2. No marked differences in the incorporations of the amino acids into the fractions F1, F2a, F2b and F3 from the tumours were generally observed, although in some experiments there was a greater incorporation into fraction F2b, which could be decreased by further purification.
3. In the tumours the incorporations into all cell protein fractions obtained were approximately the same, indicating that the amount of incorporation was that required for the increase of cell mass.
4. In rat liver, the incorporations into fractions F1, F2a and F3 were not greatly different. That into fraction F2b was variable. The incorporation into the histone fractions was much less than that into the acid-insoluble nuclear residue, indicating that considerable turnover of amino acids in the latter occurs.
5. The decrease in radioactivity of labelled histone and acid-insoluble nuclear protein *in vivo* during several days confirmed the relatively small turnover of the histone fraction. The time taken for liver whole histone to lose half its radioactivity was about 1 week. A histone fraction of slower metabolism was also detected.
6. It is concluded that no appreciable turnover of protein occurs in any one histone fraction, the somewhat higher values obtained in certain cases being associated with acidic impurities. The apparently high rate of incorporation into histone of resting liver is discussed in relation to recent evidence on DNA metabolism of resting liver.

Histones have been found in cells only in association with DNA and clearly may be regarded as part of the mechanism controlling the replication and function of chromosomes. A somewhat similar group of basic compounds has been found in ribosomes (cf. Butler, Cohn & Simson, 1960; Cohn & Simson, 1963), but this is characteristically different in having a higher content of dicarboxylic amino acids. It would be of interest to know if histones have functions in the cell other than those mentioned. This would be demonstrated if the amount of histone formed in each cell cycle were greater than that required for the replication of the chromosomes. Experiments to compare the rates of biosynthesis of histone in rat liver and certain tumours were made by Butler & Laurence (1960). Differences found in the rates of incorporation of a radioactive amino acid in the separate tissues were small although the rates of cell division were markedly different; and this suggested that in resting liver possibly more histone was synthesized than was needed for replication. However, the interpretation of these results was limited by lack of information about the relative pool sizes of the amino acids used.

Another approach is to study the relative rates of incorporation of an amino acid into the different histone fractions of one tissue, since in such a case it may reasonably be assumed that all fractions utilize the same pool of amino acids; and if the histones are used for purposes other than replication it might be expected that the different histone fractions would show marked differences in the additional amounts of synthesis required.

Studies have therefore been made of the incorporation of lysine into various histone fractions of certain ascites tumours and a 'solid' tumour and also of rat liver. The amino acids used were L-lysine and, in one experiment, L-arginine. To compare the rates of synthesis of the different histone fractions, which differ markedly in their lysine (arginine) content, the observed radioactivities/mg. of protein were divided by the corresponding lysine (arginine) contents.

Most of the incorporation studies were done with an interval of 1 hr. between injection of the labelled amino acid and killing the animal. Busch, Davis & Anderson (1958) showed that uptake of isotope into various cell protein fractions was linear over the

first 30 min. after injection, but during the second half hour there was a fall in rate of incorporation in certain tissues. This accords with the general observation that the specific activity of the free amino acid pool decreases appreciably 30 min. after injection (cf. Maurer, Niklas & Lehnert, 1954). In the Discussion section it is shown that the conditions under which our experiments were carried out were adequate to detect excess of turnover in one or more protein fractions.

The activity of some of the protein fractions was also examined after a period of a few hours and after several days. If there is very rapid turnover the activity of the protein-bound amino acid should follow the activity of the pool and would decrease by some orders of magnitude in the prolonged experiments. If the turnover is small, however, there should be little fall in activity, and the activity of the various protein fractions should keep in step whatever interval is chosen between injection and killing.

The long-term experiments with liver were done with [^{14}C]glycine injection to minimize the effect of recycling, which is more pronounced when essential amino acids such as lysine are used to label the whole animal (Penn, Mandeles & Anker, 1955).

EXPERIMENTAL

Tumours. Krebs and lymphoma ascites cells in (C—) mice were obtained 9–10 days after transplantation. The volume of packed cells was about 1.0–1.5 ml./mouse. Osteosarcomas, type D177, in August rats were obtained 14 days after transplantation and were of average weight 7 g. The Lissamine Green technique of Goldacre & Sylvén (1962) showed that more than 90% of the tumour mass was connected to the vascular system of the host.

Livers. These were obtained from 200 g. August rats (8 weeks old) bearing the D177 tumour, from C.B. stock rats (8 months old), average weight 550 g., that had been used for breeding, and also from hooded rats (4 months old) that had stopped growing.

Injections of labelled amino acids. These were intraperitoneal. Details are given in the respective Tables. Specific activities of the amino acids (from The Radiochemical Centre, Amersham, Bucks.) were: L-[^{14}C]lysine, 7 c/mole; [^{14}C]glycine, 6 c/mole; L-[^{14}C]arginine, 6 c/mole; DL-[^3H]lysine, 50 c/mole. The last-named compound was employed at a time when L-lysine of a suitable radioactivity was unavailable. This was justified, as it has been shown (Rothstein, Bly & Miller, 1954) that only L-lysine is incorporated into proteins. It has also been shown that L-lysine enters proteins in mammalian cells almost unexclusively unmodified. Thus Davies & Morris (1963) found that only about 5% of the radioactivity of [^{14}C]lysine appears in other amino acids (aspartic acid and glutamic acid). Some observations reported below confirm that at least 95% of the radioactivity of the proteins in the lysine experiments was due to the incorporation of L-lysine itself.

The incorporation of carbon from glycine into amino acids

other than glycine itself is probable, but this amino acid was used only in experiments where the fall in activity of labelled protein fractions was investigated. For this type of experiment the actual details of incorporation of isotope are of secondary importance. Comparisons were made between the radioactivities of the same protein at different times after injection.

Tissue fractionation. (a) Ascites cells. The ascites containing 8–20 ml. of packed cells were collected in 100 ml. of 1% (w/v) sodium citrate solution and fractionated by the method of Fisher & Harris (1962). The cells were sedimented at 250 g for 5 min. and the packed-cell volume was measured. The supernatant (P1) was removed and the cells were resuspended in 150 ml. of cold 0.14 M-NaCl to which CaCl_2 and sodium phosphate buffer were added to give final concentrations 1 mM and pH 7 (Table 2). All further operations were at 4°. The cells were again centrifuged at 250 g for 5 min. and the supernatant was removed (P2). The cells were suspended in 15–25 times the packed-cell volume of 0.1% Tween 80 solution and stirred with a 4 cm. diam. three-bladed nylon propellor (Bassett-Lowke Ltd., Holborn, London) attached by a nylon shaft to the drive unit of the MSE homogenizer. The speed of rotation of the propellor was adjusted with a variable autotransformer. A suitable speed resulted from one-third of the rated voltage to the homogenizer. An initial warming-up period was allowed to stabilize the speed. The cell suspension was stirred for 30 min. and the nuclei were then collected by centrifuging at 600 g for 15 min. The supernatant (C1) contained cytoplasmic material. The nuclei were resuspended with a fairly loose Fluon-glass Dounce homogenizer (Laurence, Simson & Butler, 1963) in the saline-calcium-phosphate solution and centrifuged at 600 g for 10 min. The supernatant (C2) was removed and the nuclei were then blended in 0.14 M-NaCl in 0.01 M-sodium citrate, pH 7, in the MSE homogenizer, fitted with stainless-steel blades, at full speed (14000 rev./min.) for 2 min. The broken nuclei were centrifuged for 15 min. at 1400 g, and used to prepare the histone fractions. The supernatant (C3) was sometimes retained.

(b) Rat liver (Expts. 11 and 12). The chilled livers were homogenized in 4 parts (w/w) of 0.25 M-sucrose containing CaCl_2 (0.1 mM) in Dounce homogenizers of various degrees of tightness and filtered through four thicknesses of surgical gauze. All operations were carried out at 4°. The nuclei were centrifuged at 1400 g for 10 min. The supernatant (Table 7) was retained (C10) and the nuclei were resuspended in sucrose solutions of density 1.32 and 1.27 to give a final density of 1.27. The nuclei were then centrifuged in the no. 21 head of the Spinco model L ultracentrifuge (60 min. at 20000 g) (Chaveau, Moulé & Rouiller, 1956). The supernatant was discarded and the nuclear pellets were resuspended with a Dounce homogenizer in saline-calcium-phosphate solution. Subsequent treatment was as for ascites nuclei after the removal of supernatant C1 [see (a) above].

(c) Liver and D177 tumour (Expt. 13). The tissues were rapidly frozen after removal from the animals by pressing between blocks of solid CO_2 and stored for a few days at -18° before use. They were then allowed to partially thaw in the cold room and homogenized in the MSE Ato-Mix blender at full speed (12000 rev./min.) for 2 min. in 4 vol. of 0.14 M-NaCl, pH 7. The nuclear material was sedimented at 600 g for 30 min. and resuspended in the same volume of 0.14 M-NaCl, pH 7. Sedimentation and resuspension were

repeated three times, the sedimentation time being reduced to 20 min.; the final resuspension was in 0.14M-NaCl in 0.01M-sodium citrate, pH7. All operations were carried out at 4°. The connective tissue was not removed and so the bulk of material was large, especially with the tumour.

(d) Liver (Expt. 14). The livers were rapidly frozen and stored as in (c) above, and treated individually according to method C of Laurence *et al.* (1963) to prepare whole histones.

Histone fractionation methods. The nuclear material from ascites cells and rat liver prepared as described in the preceding section was used to prepare histone by the following methods:

(1) Whole histones. These were isolated by acid extraction (Laurence *et al.* 1963).

(2) Method A (Hnilica, Johns & Butler, 1962) consists of extraction with aq. 80% (v/v) ethanol-0.25N-HCl to extract the F2a + F3 group followed by extraction of the insoluble residue with aq. 0.25N-HCl. This method was used with lymphoma ascites (Expt. 5), Krebs ascites (Expts. 6 and 7), and also with rat liver (Expt. 11) and in the experiment with rat liver and solid tumour (Expt. 13). To ensure that a suitable ethanol concentration was present in the first extraction in Expt. 13, the first 80%-ethanol wash was replaced by a wash with 4 times the precipitate volume of 100% ethanol.

(3) Method B (Johns, 1964) consists of extraction of the histone F1 with 0.75N-HClO₄; the F2a + F3 group is then extracted as the perchlorates with 80% ethanol and finally the histone F2b is extracted with aq. HCl. This method is not as specific as method A as many proteins are soluble as the perchlorates in 80% ethanol. When this method is applied to impure nuclear material the yield is too high and the protein is predominantly acidic in composition. The method can be used for ascites-cell nuclei or liver nuclei prepared by methods (a) and (b) of the Tissue fractionation section, but not for nucleoprotein from tumour or liver by the cruder procedure (c). The method was used for Expts. 8, 9, 10 and 12.

(4) Further fractionations. The ethanol-HCl extract of method A, and the ethanol extract of method B to which had been added a few drops of conc. HCl, were either precipitated by the addition of 5 vol. of acetone (Expts. 9, 10, 11, 12 and 13) or fractionally precipitated first with 1.75 vol. and then with a further 4 vol. of acetone (Expts. 5, 6, 7 and 8). The fractional precipitation resulted in a separation of histones F3 and F2a, but the proportions of the fractions were somewhat variable. The liver histones F2a and F3 and the histones of the D177 tumour were redissolved in 80% ethanol-0.25N-HCl, separating the small insoluble fraction (labelled insoluble fraction from F2a + F3; Table 6) and dialysed against 100% ethanol (Johns & Butler, 1962; Johns, 1964). Fraction F3 is precipitated and fraction F2a is recovered from the supernatant fluid by acetone precipitation. The same method was used in a partial fractionation of the whole histones from Expt. 14, in which the stability of liver histone over a period of 1 week was investigated.

The aq.-HCl extract from method A was fractionated by the addition of trichloroacetic acid to give a concentration of 5% (w/v) to bring down fraction F2b and then to 18% (w/v) to precipitate fraction F1. This method was satisfactory with the ascites histones but failed with liver histones from Expt. 11. In Expt. 13 a different method was tried (Johns, 1964), namely the addition of 3 vol. of acetone to

precipitate fraction F1 and then a further 4 vol. to precipitate fraction F2b. This method differs from the trichloroacetic acid precipitation in the order in which fractions F1 and F2b are precipitated.

The aq.-HCl extract from method B was shown to be fraction F2b by amino acid analysis and starch-gel electrophoresis. In view of the high activity of this fraction in liver (Expt. 12), this fraction was refractionated by dissolving in aqueous acid and dialysing against ethanol of increasing concentration (Table 7).

All histone samples were finally washed with acetone as the hydrochlorides and dried by suction on a water pump. They were finally dried over CaCl₂ and stored at -5°. Samples of supernatant protein were precipitated and washed twice with cold 5% trichloroacetic acid. The residue after extraction of the histones from the nucleus (acid-insoluble nuclear residue) was also dried with acetone.

Protein analysis. All ascites-cell products were analysed by chromatography of the dinitrophenyl derivatives (Phillips, 1958; Phillips & Johns, 1959). In most cases the completeness of coupling with fluorodinitrobenzene could be checked by comparison with the original weight of protein taken. In some cases, e.g. the acid-insoluble residue, which contained about equal quantities of protein and DNA, the completeness of coupling was assumed from experience of the method in the other cases. The liver samples from Expt. 11 were analysed, for basic amino acids only, on the EEL amino acid analyser, and the samples from Expt. 12 on the Technicon Autoanalyser. Samples from Expt. 13 were assayed for lysine content by electrophoresis in 0.5% Na₂CO₃ (Evered, 1959) by using a quantitative ninhydrin method.

Radioactivity assays. The lymphoma-ascites proteins were originally assayed at near infinite thickness on Perspex planchets of 0.16 cm.² area by using a thin-window counter of 5% efficiency with a background of 6 counts/min. The correction for lysine content was made by an independent determination of the amount of lysine in a weighed amount of the material from the planchet. If there were x μ moles of lysine in y mg. of sample, the recorded counts were multiplied by y/x . The radioactivity of these samples and also those from the liver (Expts. 11 and 12) were counted in a Packard Tri-Carb scintillation counter after hydrolysis for 24 hr. at 105° in 6N-HCl, removal of the HCl by repeated evaporation to dryness and solution of the hydrolysate in water (1 mg. of hydrolysed protein/ml. of water). The phosphor used contained toluene, dioxan and methanol in the proportions 5:5:3 (by vol.), and 80 g. of naphthalene, 5 g. of 2,5-diphenyloxazole and 50 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. of phosphor solution. To 8 ml. of phosphor solution 0.1 ml. of protein hydrolysate and 0.2 ml. of water were added. The lysine content was determined on a separate portion of the same solution of hydrolysate. The efficiency of the Packard counter was 65% for ¹⁴C and 15% for ³H, with corresponding backgrounds of 30 and 55 counts/min. respectively. Samples from Expt. 13 were measured in the same way in the IDL scintillation counter, which had similar sensitivity and background. The relative corrected counts for a single experiment were finally reduced to a convenient basis, e.g. the average histone activity taken as unity. The fractions from Expt. 14 were assayed for radioactivity by infinite-thickness counting on the polythene 1 cm.² planchets in the thin-window counter. As no precise comparisons were made between fractions of differing

amino acid content the activities observed were not in this case corrected for differing amino acid contents of different fractions.

All counts were large enough to reduce the counting error to less than 5%.

Mode of incorporation of lysine into histone. An acid hydrolysate of F2a histones (24 hr. at 105° in 6N-HCl) from Expt. 10 was freed of HCl and redissolved to give a neutral solution. A sample containing 0.2 mg. of hydrolysed protein was subjected to electrophoresis on filter paper at pH7 (Evered, 1959). The diamino and dicarboxylic amino acids were located on a test strip with ninhydrin, eluted from the main strip and dried on planchets. The following activities were observed: basic group, 47 ± 2 counts/min.; acidic group, 10.5 ± 0.3 counts/min.; clean planchet, 10.2 ± 0.35 counts/min. The results show that incorporation into the main amino acids derived from lysine, namely aspartic acid and glutamic acid, was not significantly different from background and certainly less than 5% of the activity of the incorporated lysine.

RESULTS

Whole histone from lymphoma ascites cells. In Table 1 principal features of the compositions are given of whole histones extracted from the nuclear material prepared from lymphoma ascites cells. This Table shows that a consistent yield of histones is obtained with composition similar to that of calf-thymus histone. The high proportion of *N*-terminal groups (93%) of histone that are proline or alanine would rule out any substantial attack by proteolytic enzymes. The content of the acid-insoluble residue in the nucleoprotein is similar to that obtained for liver by the Chaveau *et al.* (1956) method described above. As about half the acid-insoluble residue is DNA, there are about equal amounts of histone, DNA and acid-insoluble protein in the nuclear material.

Incorporation of L-[U-¹⁴C]arginine into lymphoma ascites cells. Table 2 gives the relative weights and radioactivities of lymphoma-ascites fractions after 1 hr. metabolism of labelled arginine. As histone contains more arginine than the other protein fractions, the specific radioactivity/mg. of protein in the absence of correction for arginine content is the highest in this fraction. The small but measurable activity in the ascites plasma and the somewhat larger activity in the cell supernatant P2 may be due to leakage of protein material from inside the cells.

Incorporation of L-[U-¹⁴C]lysine for 1 hr. into ascites histone fractions. The amino acid analysis of

Table 2. *Dry weights of lymphoma cell fractions and relative specific activities corrected for arginine content*

	L-[U- ¹⁴ C]Arginine (1 μ C) was injected into each mouse, and the animals were killed 1 hr. later.	
	Wt. of protein fraction	Specific activity of arginine in protein
Ascites plasma (P1)	5.3	0.06
First cell wash (P2)	0.9	0.12
Cytoplasmic proteins (C1)	10.5	1.09
First nuclear wash (C2)	0.8	1.23
Second nuclear wash (C3)	0.25	1.15
Whole histone	1.0*	1.0*
Acid-insoluble nuclear residue	1.84	1.45

* Histone taken as 1.0. The measured radioactivity of the histone was 710 counts/min. at infinite thickness on a planchet 0.16 cm.² in area.

Table 1. *Composition of whole histones from lymphoma ascites and calf thymus*

The amino acids are expressed as moles/100 moles found.

Expt. no. ...	1	2	3	4	Calf thymus*
Aspartic acid + glutamic acid	15.5	14.9	15.8	15.6	14.2
Alanine	11.9	12.5	11.9	12.2	13.8
Histidine	2.1	2.1	2.1	1.7	2.0
Arginine	8.9	8.6	8.0	8.8	9.2
Lysine	13.5	12.7	13.2	13.1	15.6
Histidine + arginine + lysine	24.5	23.4	23.3	23.6	26.8
<i>N</i> -Terminal groups:					
Proline		53			53-60
Alanine		40			36-39
Other		7			12
Yield:					
Histone (mg.)		82	50	39	
Acid-insoluble nuclear residue (mg.)		158	92	71	
Packed cells (ml.)		17	9.8	8.8	
Histone (mg./ml. of packed cells)		4.8	5.1	4.4	

* Results of Phillips & Johns (1959).

Table 3. *Composition of histone fractions from lymphoma ascites*

The amino acids are expressed as moles/100moles found. The amino acid values that are characteristic of the various histone fractions are in **bold** type.

Fraction...	F1		F3		F2a		F2b	
	8	5	8	5	8	5	8	5
Aspartic acid	2.3	2.5	16.1	15.2	13.4	13.2	14.3	15.9
Glutamic acid	4.6	5.6						
Glycine	6.7	7.1	9.0	7.9	14.0	14.4	7.9	8.4
Alanine	25.2	22.9	12.7	13.5	8.6	8.9	10.1	10.0
Valine	5.1	5.6	8.9	7.6	9.4	7.6	19.3	8.0
Leucine	5.6	4.9	14.0	12.5	15.0	14.3		11.3
Serine	6.7	6.7	3.8	5.6	3.9	3.3	8.0	8.0
Threonine	5.9	5.6	5.1	6.2	6.2	6.2	6.4	6.2
Phenylalanine	0.3	0.6	1.5	2.1	2.7	2.1	2.1	2.0
Tyrosine	0.3	0.6	1.8	2.5	2.3	3.4	2.6	2.6
Proline	8.9	9.2	4.0	5.2	2.5	2.3	4.8	4.8
Histidine	0.2	0.3	2.5	1.7	1.9	2.2	1.8	1.8
Lysine	25.6	25.9	9.0	10.7	8.8	9.4	12.7	12.6
Arginine	2.6	2.5	11.5	9.4	11.3	12.4	9.2	6.8

Table 4. *Yields and relative activities, corrected for lysine content, of histones from Krebs (Expts. 6 and 7) and lymphoma (Expts. 5, 8 and 9) ascites cells*

(a) Yields of fractions (mg.)

Fraction in order of extraction:	Method of fractionation ...		A			B	
	Expt. no. ...	...	5	6	7	8	9
F1			—	—	—	11.1	5.6
F3			20.6	22.5	8.8	20.2	—
F2a			7.6	25.0	26.1	7.6	7.8
F2b			13.8	18.5	17.5	9.7	8.8
F1			7.6	9.8	8.7	—	—
Acid-insoluble nuclear residue			131	190	224	144	74

(b) Relative specific radioactivities of lysine: L-[U-¹⁴C]lysine (1μc) was injected into each mouse, and the animals were killed 1 hr. later

Fraction in order of extraction:	Expt. no. ...		5		6		7		8		9
	Assay method*	...	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)
F1			—	—	—	—	—	—	1.02	0.84	1.02
F3			0.88	0.93	0.99	1.13	0.99	1.19	0.94	0.92	0.96
F2a			0.94	0.88	0.88	1.05	0.87	0.90	1.02	1.10	
F2b			1.38	1.20	1.25	1.10	1.22	1.01	1.00	1.13	1.40
F1			0.82	1.00	0.86	0.80	0.93	0.88	—	—	—
Acid-insoluble nuclear residue			1.54	1.54	1.49	1.49	1.47	1.49	1.57	1.83	1.90
Absolute counts/min./mg. of protein by method (ii):											
F2a			6355		2333		4700		10126		4300†

* Assay method (i) was infinite thickness counting on Perspex microplanets. Method (ii) was by scintillation counting.

† The value for F2a + F3 of Expt. 9 was estimated from the value obtained by using assay method (i) and the proportion between the two methods observed in the other cases.

some lymphoma-ascites histone fractions are given in Table 3. The samples from Expt. 8 were prepared by method B and from Expt. 5 by method A. The amino acids values that are characteristic of the various histone fractions (Johns, 1964) are in **bold** type. The yields and relative specific activities of lysine in a number of experiments with lymphoma and Krebs ascites are given in Table 4.

Incorporation of L-[U-¹⁴C]lysine for 48 hr. into Krebs ascites-cell fractions. In Table 5 it is shown that, in comparison with Tables 2 and 4, the variation of lysine activities after 2 days' metabolism is very small. As noted in the Experimental section, there was no detectable incorporation in this experiment as amino acids other than lysine. The products of dead cells may contribute to the activity in the extracellular fluid, which is larger than in Table 2.

Incorporation of DL-[G-³H]lysine into rat liver. The analyses of histone fractions isolated in Expt. 12 from rat-liver nuclei are given in Table 6. The amounts of the characteristic amino acids are in **bold** type. Fraction F2b was fractionated by dialysis against ethanol and fractions 1, 2 and 3 are insoluble in 80% and 90% and soluble in 90% ethanol respectively. In Table 7 are given the relative specific activities of lysine in the protein fractions isolated. In Expt. 11 the results from a fractionation of the aqueous hydrochloric acid extract were both mixtures of fractions F1 and F2b. The activities of two of the supernatant mixtures obtained during isolation of the nuclei are given together with the activity of an impurity in the F2a+F3 group isolated by failure to redissolve during fractionation of the group. The fractions of histone F2b in Expt. 12 are those also given in Table 6.

Results with livers and tumours of D177-tumour-bearing rats. The relative yields of the various histone fractions were similar for liver and tumour (Table 8). This Table also gives the specific lysine activities observed with a comparison with the results for previous experiments with liver and ascites tumours. The method used for the D177 tumour was developed to provide large quantities of the histone fractions for peptide-sequence work (D. M. P. Phillips & D. J. R. Laurence, unpublished work). This method gives satisfactory results with all fractions apart from fraction F1, which is contaminated

Table 5. *Incorporation into Krebs ascites cells during 2 days in vivo (Expt. 10)*

Radioactivities were corrected for lysine content. Fractionation was by method B. L-[U-¹⁴C]Lysine (2.5 μ C) was injected into each mouse, and the animals were killed 2 days later. The absolute radioactivity of F3+F2a was 15100 counts/min./mg. of protein by scintillation counting.

	Radioactivity (histone average activity 1.0)
Ascites plasma (P1)	0.21
(P2)	0.26
Cells cytoplasmic proteins (C1)	0.98
First nuclear wash (C2)	0.97
Second nuclear wash (C3)	1.00
Histones:	
F1	1.02
F3+F2a	1.02
F2b	0.96
Acid-insoluble residue	1.04

Table 6. *Composition of rat-liver histone fractions (Expt. 12)*

The amino acids are expressed as moles/100 moles found. The amino acid values that are characteristic of the various histone fractions are in **bold** type.

Fraction ...	F1	F3	F2a	F2b(1)	F2b(2)	F2b(3)	Acid-insoluble residue
Aspartic acid	2.6	5.5	6.2	6.8	5.5	5.3	8.7
Glutamic acid	3.9	10.2	7.9	11.5	9.5	8.1	11.4
Glycine	6.4	8.0	12.5	8.1	6.8	9.6	12.6
Alanine	23.1	12.5	9.9	9.2	11.8	10.0	7.3
Valine	4.2	5.2	7.0	5.4	5.6	7.1	5.8
Leucine	4.1	10.0	9.9	6.8	7.9	7.6	8.7
Serine	7.0	4.3	3.5	7.9	6.4	6.1	6.5
Threonine	5.9	5.3	5.0	5.6	6.2	5.6	4.8
Phenylalanine	0.6	2.2	1.6	2.6	2.3	1.7	3.6
Tyrosine	0.6	2.2	2.8	2.5	2.5	3.3	2.7
Proline	10.7	5.7	3.9	7.3	6.1	4.8	6.4
Histidine	0.2	2.2	2.6	2.2	2.1	2.6	2.3
Lysine	26.6	9.6	10.7	11.3	11.6	13.6	7.3
Arginine	2.6	10.9	10.9	7.3	10.8	8.9	6.6
Isoleucine	1.5	4.9	4.9	3.8	4.9	5.0	4.3
Methionine	0.1	0.7	0.6	1.2	0.9	0.5	—
Cysteic acid	0.1	0.5	0.2	0.5	0.3	0.1	0.9

with a more acidic protein. It is this fraction that differs most markedly from the results with the previous methods.

Results with livers of rats labelled with [1-¹⁴C]-glycine. Six rats were injected and divided into two groups. Three rats were killed 5 hr. after injection and the other three were killed 7 days after the first group. There was no significant change in body weight of the animals during the experiment, and the weight of livers and spleens in the two groups did not differ significantly. Table 9 gives the results

of determinations of the radioactivity of the histones and acidic nuclear proteins in the two groups. After determination of the activities of proteins from individual rats similar fractions in each group were pooled and refractionated. The histone fractionation has been described above. The acidic proteins were fractionated into material soluble and insoluble after 1 hr. at room temperature in 0.1N-sodium hydroxide. The soluble material was recovered by precipitation with N-acetic acid.

DISCUSSION

The incorporation of amino acids into a protein present in a growing tissue will include contributions due to: (1) increase of the amount of the protein due to growth; (2) synthesis of protein to replace protein catabolism or for transport (i.e. turnover). The absolute amounts of amino acid incorporated depend on other factors, such as the amount of labelled amino acid reaching the tissue in question and the size of amino acid pools, and as a rule no comparison can be made of different tissues. It may be expected, however, that different histone fractions and possibly other nuclear proteins share the same amino acid pools so that comparisons can be made of their relative rates of synthesis.

If x is the weight of a quantity of cells and x_i that of a particular fractional component, the increase in weight in time δt , due to growth, may be expressed as $v x \delta t$ or $v x_i \delta t$, where v is the growth rate. Similarly, if turnover is occurring in the component i at a rate w_i , this component will lose a quantity $w_i x_i \delta t$, which is replaced by new synthesis of the same amount. The total entry of new material into the fraction i in the time δt is thus $(v + w_i) x_i \delta t$. If A

Table 7. *Incorporation of lysine into rat-liver proteins (Expts. 11 and 12)*

Relative radioactivities of protein fractions were corrected for lysine content. DL-[G-³H]Lysine (500 μ C) was injected into each rat, and the animals were killed 1 hr. later. The absolute radioactivity of F2a in Expt. 11 was 1700 counts/min./mg. of protein and in Expt. 12 was 1480 counts/min./mg. of protein by scintillation counting.

	Expt. no. ... 11	12
	Method ... A	B
1400g Supernatant (C10)	4.6	—
Saline-citrate supernatant (C30)	3.4	—
Histones:		
F1	—	0.9
F3	1.0*	1.0*
F2a	0.75	0.75
Insoluble from F2a + F3	1.67	—
F2b	1.94	F2b { 4.4 (1) 1.5 (2) 1.1 (3)
+		
F1	1.60	
Acid-insoluble nuclear residue	5.1	4.1

* Fraction F3 is taken as 1.0.

Table 8. *Yields and radioactivities of histones from D177 tumour and liver of August rats (Expt. 13)*

DL-[G-³H]Lysine (100 μ C) was injected into each rat, and the animals were killed 1 hr. later. The results obtained from previous experiments are summarized. The absolute activities of histone fractions from the D177 tumours were on the average 3 times the corresponding activities of the fractions obtained from the livers of the same animals. The values for fraction F2a were 1750 counts/min./mg. of protein for the tumour and 650 counts/min./mg. of protein for the liver sample, determined by scintillation counting.

Histone fraction	Yields (mg.) (Expt. 13)		Relative specific activities			
			Liver*		Tumour†	
	Liver	D177 tumour	Expt. 13	Expts. 11 and 12 (Table 7)	Solid tumour D177	Average of ascites cells (Tables 4 and 6)
F1	25	73	1.5	0.9	1.14	0.89
F2a	30	69	0.8	0.75	0.92	0.95
F2b	37	66	1.3	1.1-1.4	0.89	1.16
F3	10	52	1.0	1.0	1.04	1.00

* The liver activities are expressed with F3=1.0.

† The tumour activities are expressed with the average of all four fractions=1.0.

Table 9. *Decrease in the radioactivity of rat-liver nuclear proteins during 1 week in vivo (Expt. 14)*

[^{14}C]Glycine ($10\mu\text{C}$) was injected per rat, and rats 1–3 were killed 5 hr. and rats 4–6 killed 7 days 5 hr. after injection. Radioactivities are given in counts/min. for a 1 cm.^2 infinitely thick layer of protein.

Time-interval	Rat no.	Histone			Acid-insoluble nuclear protein				
		Individual values	Average	Fractions insoluble in ethanol-HCl	Fractions soluble in ethanol-HCl	Individual values	Average	Fractions insoluble in NaOH	Fractions soluble* in NaOH
5 hr.	1	132	133	183	57.5	$\left\{ \begin{array}{l} 210 \\ 210 \\ 221 \end{array} \right.$	214	259	160
	2	136							
	3	130							
7 days 5 hr.	4	77	70	74.5	43.5	$\left\{ \begin{array}{l} 62.5 \\ 65 \\ 73 \end{array} \right.$	66.5	79	44.5
	5	69							
	6	68.5							
Ratio $\frac{7\text{ days } 5\text{ hr.}}{5\text{ hr.}}$			0.53	0.41	0.76		0.31	0.31	0.27
% decrease in 1 week			47	59	24		69	69	73

* The low activity of this group was probably due to dilution with associated DNA.

and a_i are the specific radioactivities of the pool and the fraction i respectively at time t (both functions of t), the change in specific activity of component i in time δt is to a first approximation:

$$\delta a_i = (A - a_i)(v + w_i)\delta t$$

$$\text{or} \quad \frac{da_i}{dt} = (A - a_i)(v + w_i) \quad (1)$$

A number of cases can be considered:

(1) If A suddenly assumes a value A^0 and then remains constant, a_i will increase initially linearly with time at a rate that is proportional to the sum of the growth and turnover rates. For a number of components using the same pool, the initial slope will increase with increasing turnover but the final specific activities (equal to that of pool) will be the same, namely A^0 , and will be approached asymptotically.

(2) Suppose that at time T , at which the specific activity of the product has reached the value a_T , the pool activity is suddenly decreased to $A = 0$. On the assumption that the growth rate is unaffected, the radioactivity in fraction i will diminish with time exponentially according to:

$$a_i = a_T e^{-(v+w_i)(t-T)} \quad (2)$$

(3) In practice the situation is not so clear-cut, and the pool activity will rise sharply to a maximum value and then decrease gradually. The application of eqn. (1) to this case is simplified if $a_i \ll A$, say $a_i < 0.1A$, as in this case variations of a_i on the right-hand side of the equation may be ignored in the first-order approximation. In justification of this simplification in our short-term experiments, we note that even with a tumour with growth rate of

50% per day the value of a_i in the absence of additional turnover would be less than $0.02A$ after 1 hr. incorporation. In other tissues it may be much smaller. The experiments on the decay of radioactivity in whole histone over several days [during which the pool size will have diminished 50–100-fold or more according to R. M. Campbell, D. P. Cuthbertson & A. S. McFarlane (quoted by McFarlane, 1957)] indicate a period of half-decay of about 1 week for rat liver, and a_i would then be about $0.004A$ after 1 hr.

Integration of eqn. (1) with $a_i \ll A$ gives:

$$a_i = (v + w_i) \int_{t=0}^t A dt$$

and so the value $(v + w_i)/(v + w_j)$ for two fractions i and j is given by a_i/a_j , as in the initial period of linear incorporation described in (1). No information about turnover has been lost due to variation in the rate of incorporation under these conditions. In the case where A decreases until it approaches a_i , the rates of incorporation will cease to reflect the relative values of $(v + w_i)$ as the value of a_i in eqn. (1) can no longer be ignored. The rates will, however, be less by a factor at least 10 than the original rate of incorporation, and it would require a period at least 10 times as long for these misleading results to seriously affect the original evidence of turnover. Thus under our conditions, where incorporation is linear for at least half the experimental interval and still increasing in the remainder, it is very likely that the results are a true measure of turnover.

When the specific activities of lysine in various cell protein fractions from the Krebs and lymphoma ascites cells are compared (Table 4) it is seen that

three of the histone fractions, namely F1, F2a and F3, have practically the same values, which are, moreover, the lowest observed in any fraction. This result was independent of whether fraction F1 was extracted first (as in method B) or last (as in method A), and also of variations in the 'cut' that the acetone precipitation made in the F2a + F3 mixture. The activity in fraction F2b is usually appreciably higher than in the other fractions. This might be due to fraction F2b being contaminated with a more active protein. It is notable that when the order of preparation of fractions is reversed the activity of fraction F2b is appreciably reduced (Expt. 8). It must be observed, however, that none of the protein fractions isolated (Table 2) has a much higher activity. This does not exclude the possibility that the high values of fraction F2b are due to a more highly active impurity, but only that such a protein has not been isolated.

The conclusion to be drawn from these experiments is that all cell proteins examined have approximately the same average rate of synthesis except the acid-insoluble nuclear residue, in which a certain amount of 'turnover' is observed. This fraction may include nuclear ribosomes and attached newly formed proteins.

The activities observed in the fractions obtained from ascites cells labelled 2 days previously were higher than the activity observed for cells labelled during the usual 1 hr. period. The activity after 2 days was about $1\frac{1}{2}$ times the highest activity observed for experiments of 1 hr. duration, though the amount of isotope used was $2\frac{1}{2}$ times as great. These results show that the activity incorporated into ascites cells is not lost as a result of turnover but is retained for a considerable time after the usual 1 hr. period of labelling. There is a levelling of activity in all fractions, which suggests that the increased activity in the acid-insoluble residue after 1 hr. incorporation is probably due to rapid incorporation into a small part of this fraction.

A somewhat different picture is found with liver (Table 7). In liver both fractions F1 and F2b tend to have higher activities than the other histone fractions, but the values obtained are somewhat variable, as would be expected if there is some contamination with more active proteins. The lowest values are of the same order as that of the other histone fractions. In liver (Table 7) both the 'cytoplasm' and 'nuclear-sap' fraction and the nuclear residue have much higher activities than the histone fractions. This would be the case if these fractions contain proteins for use elsewhere.

The results of the experiments on the decrease of radioactivity of liver histone fractions over a period of 1 week given in Table 9 supplement previous work by Allfrey, Daly & Mirsky (1954) and by Holbrook, Irvin, Irvin & Rotherham (1960), who showed that

the activity of glycine-labelled liver histones either remained unchanged or decreased to about half the maximum value over a period of 3–4 days, depending on the nutritional state of the animal and the histone fraction studied. In the present experiments the average decrease in activity over 1 week was 47%, but a histone obtained by ethanol fractionation decreased in activity by only 24%. From the results with uptake experiments it would be expected that the ethanol fractionation would isolate those parts of the main histone fractions with minimum turnover, namely fractions F2a and F3 and the less rapidly metabolized part of F2b. In contrast, the activity of the acid-insoluble nuclear residue fell by 70% in 1 week. On the assumption that the decreases are due to experimental decay over 1 week, the turnover time $(v + w)^{-1}$ of the slow histone fraction and the acid-insoluble nuclear residue would be about 4 weeks and 6 days respectively. This is approximately the 1:4 ratio found in the uptake experiments.

None of these rates is high enough to lead to any error by prolonging the uptake somewhat beyond the end of the period of linear incorporation of isotope.

The similarity of the radioactivities in the various liver histone fractions and the low level of those activities relative to the activities of the cytoplasmic and acid-insoluble nuclear proteins are consistent with histone metabolism occurring only (or mainly) as required for cell division. The observation that with the August rats bearing a tumour the ratio of the labelling of histone in tumour and liver is about 3:1 suggests, however, a more vigorous metabolism in the liver than the growth requirement would allow.

The problem of whether histone turnover of liver is in excess of the rate of DNA turnover or of the requirement for growth is difficult to resolve, as the relation between DNA turnover and growth is itself not clear.

The growth rate of the August rats was about 1.5% per day, whereas the rate of growth of the tumour was about 30%. This should lead to a 20-fold difference in the rates of incorporation into liver and tumour. However, Kelly & Jones (1950) and Kelly, Payne, White & Jones (1951) found that the rate of incorporation into liver DNA may be stimulated up to fivefold by the presence of a tumour in the same animal. The nature of this extra incorporation is not clear.

The effect of a tumour on the DNA metabolism of liver is a particular case of the general problem of DNA synthesis not related to growth of the organ. Pelc (1964) has summarized results based on thymidine incorporation that give the turnover time of DNA in resting liver as about 4 weeks, about the same as the turnover time of our slowest-metabol-

ized histone fraction. On the other hand, values based on mitotic index suggest a half-life of liver DNA due to growth alone about 20 times as great as this. The determination of mitotic rates in liver may be considerably in error, especially as in older livers most of the thymidine incorporation is into non-parenchymal cells, for which detection of mitosis is apparently more difficult than for parenchymal cells. However, Pelc (1964) gives evidence that many of the cells incorporating thymidine do not divide even by some cryptic form of amitosis.

In conclusion, it is necessary to observe that our findings do not support the observations of Hnilica & Busch (1963) and Busch, Steele, Hnilica, Taylor & Manoglu (1963), who reported that in Walker tumour (rats) the specific lysine activity of fraction F3 is about twice that of fraction F1, with fractions F2a and F2b intermediate, and a similar finding in rat liver. However, their fraction F3 sample contained a high content of acidic amino acids (21.4%) (Hnilica & Busch, 1963). There are undoubtedly proteins of high specific lysine activity in liver and these can easily contaminate histones unless precautions are taken. We have not observed, in tumours, fractions of markedly higher activity than the histones; i.e. synthesis is occurring in the different fractions at approximately the same rate, so that protein synthesis in these tumours is predominantly that required for the enlargement of the cell mass.

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