CIRCADIAN RHYTHMS IN THE PHOSPHORYLATION OF RAT LIVER HISTONES AND SIMILAR BASIC PROTEINS

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SUMMARY: In the rat liver, the phosphorylation of histones is subject to a circadian rhythm. Most classes of histones, which had been obtained by polyacrylamide gel electrophoresis of nuclear HCl-extracts, exhibited maximum phosphorylation at 21.00 h and at 08.00 h. This is in correlation to maxima of RNA synthesis (23.00 h) and protein synthesis (24.00 h and 12.00 h), reported in the literature. A series of basic proteins, not as yet described, could be separated from the histones. These proteins exhibit a high extent of phosphorylation and a rhythmicity analogous to that of the histones. It is suggested that these proteins are of importance in the expression of genetic information.

Diurnal rhythmicity of a great variety of metabolic reactions has been demonstrated in virtually all plant and animal species. In recent years, interest was focused primarily on reactions involved in the expression of genetic information. Circadian rhythms have been demonstrated concerning the genetic activity of mouse liver (1, 2) and the rate of protein synthesis in the rat liver, including the synthesis of individual enzymes (3-5). Rhythmicity has also been observed in the incorporation of ^{3}H-thymidine into the DNA of mouse organs, such as kidney, parotid gland and duodenum (6).

Although the regulation of transcription remains partially obscured, there is growing evidence that chemical modification of histones is involved in this process. It has repeatedly been suggested that this modification might be due to the phosphorylation of individual amino acids of the histone fractions (7-9). This would result in conformational alterations of these proteins, followed by an activation of previously repressed gene sequences (10, 11).

Assuming the accuracy of this concept, the diurnal rhythmicity of genetic activities would presuppose a dependence of histone phosphorylation with daytime. In the present paper the phosphorylation kinetics of histones and other basic pro-

teins, extracted from rat liver nuclei, are described.

METHODS: Male Sprague-Dawley rats, 2.5 months old, were used throughout these investigations. Each animal was injected intraperitoneally with 1 ml neutralized ^{32}P-orthophosphate (1.7 mCi/ml) in saline, containing 16.1 µmole of inactive orthophosphate, one hour prior to decapitation. Four animals each were killed at 10.00 h, 13.00 h, 16.00 h, 21.00 h, 24.00 h, 04.00 h, 08.00 h, and 10.00 h and their livers homogenized in a Potter-Elvehjem homogenizer. Homogenization procedures and purification of nuclei was as described by Tidwell et al. (12). Nuclei were extracted twice with ice cold 0.2 N HCl and histones were precipitated in the combined extracts with 10 volumes of acetone.

Fractionation of the histones was carried out by electrophoresis in 15% polyacrylamide gels, containing 2.5 M urea, at pH 2.8 (13, 14). For densitometry of the amido black stained histone bands, one hundred micrograms of histones (dissolved in 20 µl water) were applied to the gels. Determinations of the radioactivity of individual fractions were made with gels of the same run, loaded with 200 µg histone. These gels were frozen immediately after the run in dry ice, and were cut into 35 slices with a Joyce and Loebl gel-slicer. Slices were incubated overnight at 54°C with 200 µl 35% H_2O_2 for solubilization and the radioactivity was determined in a Packard liquid scintillation spectrometer under 10 ml of Bray's solution. The relation counts per minute/mm scale deflection was denominated "specific activity".

RESULTS: The separation of histones into the classes F1, F3, F2b, F2a1 and F2a2 by polyacrylamide gel electrophoresis is demonstrated in Fig.1. In some cases further separation into subfractions may be achieved, as for instance with histones F1' and F1'' or with the two subfractions of histone F2a1. In addition, a series of small bands with an electrophoretic mobility appreciably lower than that of the 5 major bands, is demonstrated. Under certain conditions the material of these fractions is phosphorylated to a very high degree (Fig.2). In accord with most classes of histones, the specific activity of

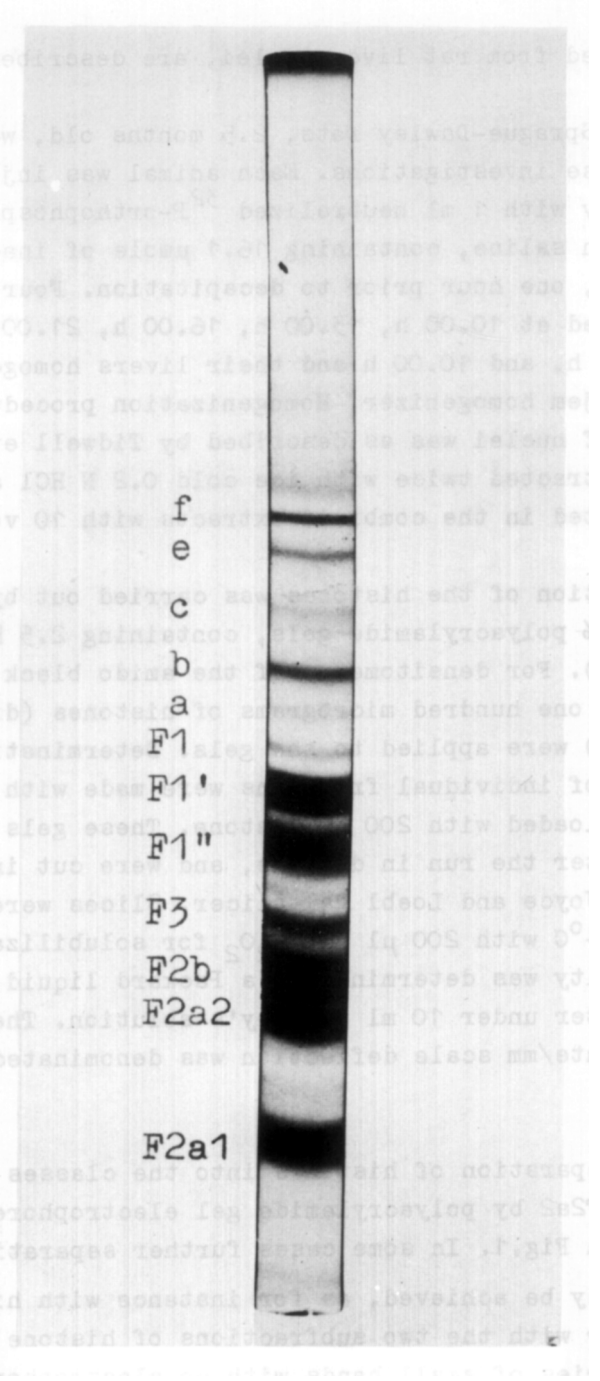

Fig. 1. Separation of histones by electrophoresis on 15% polyacrylamide gel. Electrode buffer: 0.9 N acetic acid, pH 2.8. Electrophoresis was carried out for 2.5 hours at 2 mA/gel. The gels were stained for 3 hours with 0.1% amido black in 7% acetic acid-20% ethanol.

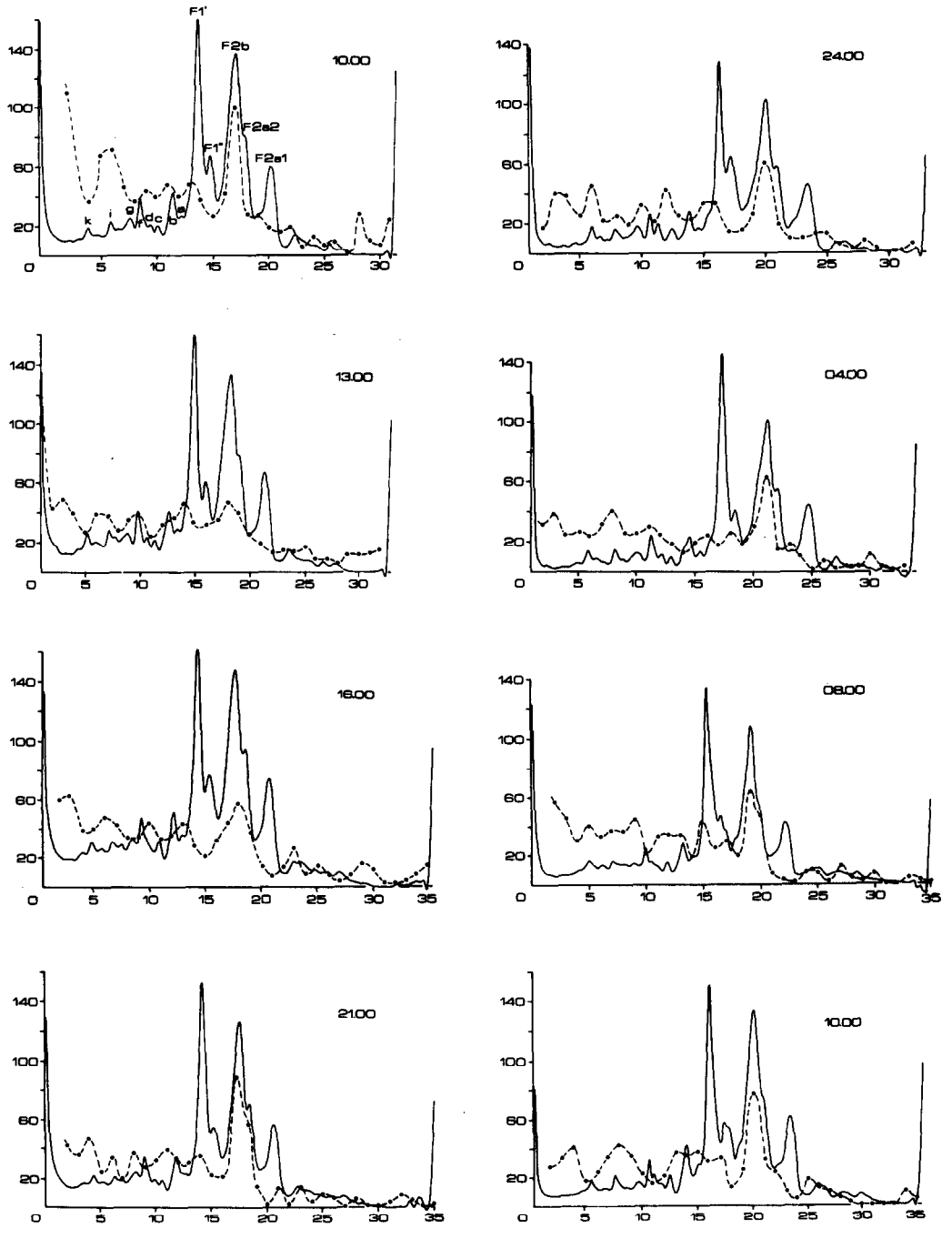

Fig. 2. Densitometry of 8 different gels with histones, having been phosphorylated at 8 different times of the day (fully drawn lines). The broken lines result from the radioactivities of gel slices, obtained in a parallel run. Abscissa: Fraction number, Ordinate: Optical density in mm scale deflection, or radioactivity in c.p.m.

Table I

Arithmetic means ($\bar{x}$) and standard deviation (s) of the specific radioactivity of histone fractions at different times of the day. Number of animals at any given time: 4.

histone fraction		time 10.00	13.00	16.00	21.00	24.00	04.00	08.00	10.00	Σ
f	$\bar{x}$	0.973	1.033	0.995	0.745	0.848	0.923	0.745	0.718	0.873
	s	0.038	0.062	0.041	0.076	0.061	0.092	0.071	0.070	0.133
c	$\bar{x}$	1.930	1.660	1.353	2.200	1.705	1.913	2.020	1.743	1.816
	s	0.087	0.465	0.221	0.346	0.282	0.324	0.295	0.124	0.355
b	$\bar{x}$	1.040	0.953	0.980	1.123	0.900	0.830	1.015	0.893	0.967
	s	0.074	0.109	0.148	0.045	0.141	0.140	0.076	0.075	0.130
F1	$\bar{x}$	1.040	1.090	1.168	1.000	0.990	1.090	1.048	0.938	1.046
	s	0.057	0.069	0.156	0.001	0.156	0.105	0.145	0.075	0.117
F1'	$\bar{x}$	0.343	0.298	0.200	0.258	0.203	0.145	0.208	0.200	0.232
	s	0.041	0.060	0.035	0.045	0.041	0.025	0.015	0.008	0.069
F3	$\bar{x}$	0.745	0.718	0.668	0.730	0.578	0.765	0.668	0.623	0.687
	s	0.048	0.046	0.126	0.070	0.059	0.138	0.013	0.021	0.292
F2b	$\bar{x}$	0.735	0.550	0.478	0.700	0.570	0.628	0.593	0.465	0.590
	s	0.049	0.157	0.091	0.022	0.055	0.046	0.053	0.077	0.115
F2a2	$\bar{x}$	0.323	0.525	0.400	0.868	0.488	0.275	0.755	0.453	0.511
	s	0.019	0.026	0.130	0.037	0.131	0.017	0.105	0.097	0.208
F2a1	$\bar{x}$	0.288	0.268	0.238	0.288	0.215	0.173	0.173	0.208	0.231
	s	0.065	0.017	0.108	0.081	0.039	0.083	0.075	0.077	0.078
Σ	$\bar{x}$	0.824	0.788	0.720	0.879	0.722	0.749	0.803	0.693	0.772
	s	0.495	0.451	0.417	0.559	0.457	0.542	0.536	0.458	0.489

these proteins varies appreciably with the time of the day.

To test the significance of the dependence of histone phosphorylation on daytime, a statistical evaluation of the experimental material was carried out. Arithmetic means and standard deviations of the specific radioactivity of individual histone fractions, which had incorporated ^{32}P at various times of the day, are shown in Table I. Further calculations by two-way analysis of variance (Table II) demonstrate that a.) individual histone classes are phosphorylated to a different degree at various times of the day (level of signifi-

Table II

Two-way analysis of variance of the data from Table I.

	sums of squares	degrees of freedom	mean square	F
between columns (different time)	0.984	7	0.141	5.647
between rows (diff. hist. classes)	60.952	8	7.619	306.010
error (within subclass)	6.772	272	0.025	
total	68.709	287		

cance 99.9%) and b.) the degree of phosphorylation at a definite time is different for various histone classes (level of significance 99.9%). A two-way analysis of variance computed from the logarithms of the original data yields exactly the same results.

In Fig.3 the specific radioactivity of individual histone classes is shown in dependence of the daytime. It is demonstrated clearly that there is a prominent maximum of phosphorylation at 21.00 h in histones F2a2, F1', F2b, F3, F2a1 and in the fractions c and b. A second, minor maximum is observed at 04.00 or 08.00 h. Phosphorylation kinetics of fractions F1 and f are different insofar as the more prominent maximum is at 13.00 - 16.00 h. Interestingly, fraction F2a2 is characterized by an additional, third phosphorylation maximum at the same time. The differences between the arithmetic means of maximum and minimum phosphate incorporation are significant in most cases, according to the difference-of-means test (Table III).

DISCUSSION: Separation of histones into subfractions and determination of the diurnal phosphorylation rhythmicity of individual bands revealed a significant dependence of phosphate incorporation on daytime in most cases. Generally, highest incorporation rates were observed at 21.00 h and between 04.00 and 08.00 h. Minimum incorporating activity was at midnight and

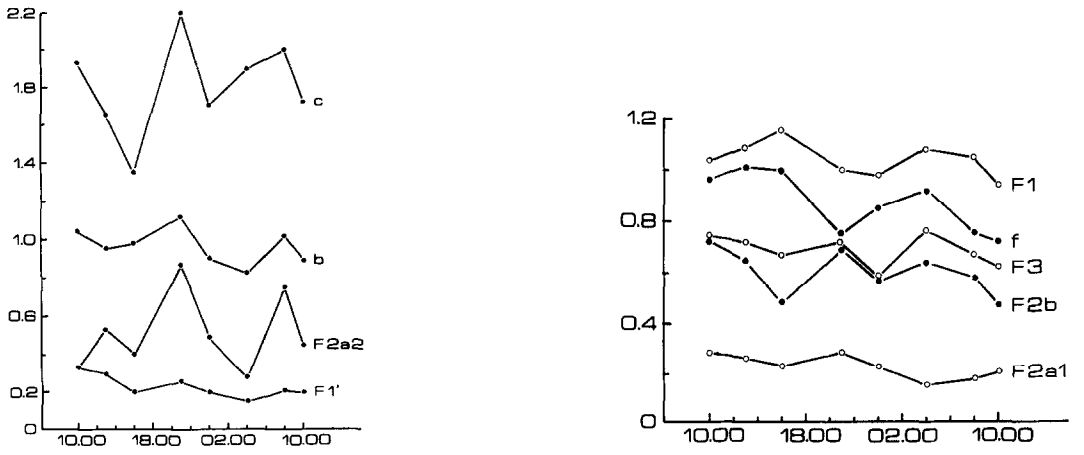

Fig. 3. Specific activity of individual histone bands at various times. Abscissa: Time of the day, Ordinate: arithmetic means of the specific radioactivities (c.f. Table I).

Table III

Time of the day, when maximum or minimum phosphorylation of individual histone fractions occur.

histone	max.	min.	max.	min.	max.	min.
F2a2	21.00	04.00	08.00	10.00	13.00*	16.00*
F2a1	21.00	04.00	10.00	16.00		
F1'	21.00*	04.00	08.00*	16.00*		
F2b	21.00	24.00*	04.00*	16.00		
F3	21.00*	24.00	04.00*	16.00		
F1	16.00*	24.00	04.00	10.00*		
f	13.00*	21.00	04.00	10.00*		
c	21.00	24.00	08.00	16.00		
b	21.00	04.00*	08.00	13.00*		

Underlined time: arithmetic means of the specific activities differ significantly from the means of both neighbouring minima (maxima) at a level of significance of 95% (t-test). Time marked with an asterisk: significant difference to one of the neighbouring minima (maxima).

at 16.00 h. This is in good correlation with the results of LeBouton and Handler (3), who reported a diurnal rhythmicity of protein synthesis in the rat liver. According to these authors the highest incorporation rate of L-leucine-4,5-^{3}H into the protein fraction, insoluble in trichloroacetic acid, is at about midnight. The maximum of RNA synthesis, as determined by the incorporation of ^{14}C-UTP into mouse liver RNA, was reported to occur at 23.00 h (1). Thus, the phosphorylation of histones would fit very well into the sequence of events leading from gene activation to the synthesis of proteins. This would demonstrate once more the significance of histone phosphorylations in the stimulation of gene activity.

The synthesis of some trichloroacetic acid-soluble proteins (3), including the enzymes glycogen synthetase (4) and adenyl cyclase (5), are characterized by a different rhythmicity, as these proteins are synthesized primarily at about noon. It is quite possible that the histone phosphorylations at 08.00 h or 10.00 h are responsible for the stimulation of gene activity, leading to the synthesis of these components.

The occurence of bands c, b and f deserves further interpretation. These and similar components had been specified by former investigators mainly as contaminating proteins or aggregated histones (15). However, the comparatively high phosphorylation rate as well as the phosphorylation kinetics very similar to that of the main histone classes, indicate the importance of these compounds in gene expression. We cannot discriminate for the moment whether these proteins belong to a group of hitherto undetected histones, or whether they have another function in the course of the cell cycle (16) and liver regeneration (17). We are now engaged in the isolation and characterization of these proteins.

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