Thiol Proteins in Nuclei from Rat Liver and Thymus

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Kedrova, Antokol'skaya & Rodionov (1961, 1963) and Deakin, Ord & Stocken (1963) have described thiols in nuclei from rat liver and thymus gland whose reactions were altered after X-irradiation. In thymus nuclei 70 % of the thiol groups appeared to be associated with the arginine-rich histone fraction, although only traces of cysteine or cystine have been reported in histones (Daly & Mirsky, 1954-55; Crampton, Stein & Moore, 1957; Phillips, 1963; Konikova, Pogosova, Rapoport, Guljamov & Kritzman, 1963). To investigate any metabolic significance of these thiol compounds an attempt has been made to characterize them more precisely. Besides glutathione, acid-extractable peptides containing thiol groups have been found in sea-urchin eggs by Sakai & Dan (1959) and variations in the amount of these compounds occur during the mitotic cycle.

The recent introduction of a sensitive method for the determination of thiol groups (Ellman, 1959) has presented the opportunity for a further characterization of the thiol components of acid-extractable proteins from nuclei.

METHODS

Animals. This laboratory strain of Wistar rats was used throughout. No difference was found in thiol concentrations between rats of either sex. In most of the experiments with nuclei prepared from liver, animals of 200-300 g. body wt. were used; with nuclei prepared from thymus, the rats were 100-140 g.

Preparation of cell fractions. Liver nuclei were isolated as described by Chaveau, Moulé & Rouiller (1956) using 2.2M-sucrose with the addition of CaCl_2 (5 mM) and tris-HCl (5 mM, pH 7.2). The nuclei were suspended in 0.25M-sucrose-5 mM- CaCl_2 -5 mM-tris-HCl, pH 7.2, before use. Fed rats were normally used but no difference in thiol content was found if the animals had been starved for 24 hr. previously.

Thymus nuclei were isolated in 0.25M-sucrose-5 mM- CaCl_2 -5 mM-tris-HCl, pH 7.2 (Creasey & Stocken, 1959).

Mitochondria and microsomes were obtained from rat liver. The tissue was dispersed in 0.25M-sucrose-5 mM-tris-HCl, pH 7.2, and centrifuged twice for 15 min. at 800g to remove nuclei, red cells and debris. The mitochondria were sedimented by centrifuging at 6000g for 15 min. and were

washed once. The microsomes were obtained after further centrifugation at 105 000g for 60 min.

Extraction procedures. (a) Acid extraction. The time from death to the first extraction was kept as short as possible and averaged 2 hr. for liver and 20 min. for thymus. All steps were carried out at 2°. Cell fractions were prepared from two to four livers or from 12 thymus glands and were finally suspended in 20 ml. of 0.25M-sucrose medium. The fractions were washed three times with 1 mM-HCl and extracted three times with 50 mM-HCl, and once with 250 mM-HCl; 5-10 ml. was used each time for the nuclei and 5 ml. for the mitochondria and microsomes. About 5 min. was required for each stage in the extraction process. All samples for amino acid analysis or electrophoresis were dialysed overnight against 20-100 vol. of water.

(b) Salt extraction (Frenster, Allfrey & Mirsky, 1960; Zbarskii & Samarina, 1963). Nuclei were extracted three times with 10 ml. of 0.1M-tris-HCl, pH 7.1, to remove nuclear ribosomes (Frenster *et al.* 1960). The residue was shaken for 1 hr. with 20 or 40 ml. of 2M-NaCl. In some experiments extraction with 20 ml. of M-NaCl preceded that with 2M-NaCl.

The extracts were examined at 360-230 m μ with a Beckman DK 2A spectrophotometer.

Electrophoresis. This was performed on polyacrylamide gel in glycine-acetic acid buffer, pH 4.0. The procedure was essentially that described by McAllister, Wan & Irvin (1963). The samples were examined at 18° immediately after dialysis to reduce the possibility of aggregation (Ui, 1957). Where possible, 0.2 mg. of sample in 0.1 ml. of 1M-sucrose-glycine buffer was applied to the gel columns (0.5 cm. $\times$ 8 cm.). The electrophoretic runs lasted 2.5 hr. at 5-6 mA/tube, 60 v/cm. The de-staining solution differed from that of McAllister *et al.* (1963) because of difficulties in getting constant gels by photopolymerization of acrylamide in the presence of riboflavin. The de-staining medium finally used was 7% (v/v) acetic acid made 1M with sucrose. This medium prevented back-diffusion of dye and appeared to introduce no artifact in gel structure or removal of background colour.

Thiol groups. These were determined by the method of Ellman (1959). When the total thiol content was required the nuclei were allowed to stand for 5 min. at 18° in 4M-urea before the addition of the reagent. A final concentration of 75-100 mM-phosphate buffer, pH 7.4, was used in a volume of 3 or 4 ml. When whole nuclei or insoluble compounds were present the suspensions were filtered through Whatman no. 30 paper 5 min. after the addition of the reagent. Reduced glutathione was used as a standard.

Disulphide. This was measured as SH after reduction with sodium borohydride (Moore, Cole, Grundlach & Stein, 1958) in the presence of 4M-urea. About 10 mg. of borohydride was used/nuclear sample (25-40 μ g. of DNA phosphorus) and the reduction was stopped after 30 min. at 40°

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by the addition of 0.2 ml. of 5N-HCl. After a further 5 min. at 40° the samples were readjusted to pH 7.4, and phosphate buffer and reagent immediately added in the usual way.

Deoxyribonucleic acid. DNA was determined by the diphenylamine method of Burton (1956) and the results were expressed in terms of its phosphorus content.

Ribonucleic acid. RNA was determined by the orcinol method of Dische & Schwartz (1937).

Amino acid analysis. This was by the procedure of Crestfield, Moore & Stein (1963).

The dialysed proteins were freeze-dried, and 2 mg. was hydrolysed with 2 ml. of constant-boiling HCl (twice-distilled) for 17 hr. *in vacuo*, the tubes being sealed as described by Crestfield *et al.* (1963). The hydrolysates were dried *in vacuo* over NaOH pellets and anhydrous CaCl₂.

Glutathione reductase (EC 1.6.4.2). The activity was measured by the decrease in absorption at 340 m μ in the presence of 1 mM-GSSG and 0.33 mM-NADPH. Nuclear suspension (1 ml.) was used in a final volume of 3 ml. and the reaction was allowed to proceed for 15 min. at 40°. The suspensions were centrifuged for 1 min. at 3500g and filtered before spectrophotometric analysis. The reductase activity was completely inhibited by 0.1 mM-ZnSO₄.

N[1-¹⁴C]-Ethylmaleimide. This was obtained from Schwartz Bio-Research Inc., Mount Vernon, N.Y., U.S.A., specific activity 8 μ C/mg. Acid extracts from nuclei were brought to pH 4–5, a three- to four-fold excess of N[¹⁴C]-ethylmaleimide was added, and the extracts were allowed to stand at 18° for 60 min. After dialysis overnight at 2°, the extracts were analysed electrophoretically. No SH groups were detectable after labelling and no difference in pattern was found between control and labelled samples. The bands were cut out and the ¹⁴C activity was determined either by extracting the band with 80% formic acid and assaying samples of the supernatant by scintillation counting (Tritomat Isotope Developments Ltd.), or by grinding the bands with water, plating the suspension and counting (Nuclear-Chicago gas flow counter).

RESULTS

Total thiol content of nuclei. Earlier experiments with thymus (Deakin *et al.* 1963) had shown that the apparent thiol content of nuclei was increased both by X-irradiation *in vivo* or by allowing the nuclei to stand in a medium of greater ionic strength. This indication that masked thiol groups were present has been confirmed by measuring the thiol content of nuclei reacting directly with the Ellman reagent (apparent SH) and the amount which is detectable after standing nuclei from either thymus or liver in 4M-urea for 5 min. (total SH) (Table 1). Longer incubations with urea did not lead to any further increase in SH. In freshly prepared liver nuclei the disulphide content was fairly low, but wide variations in the ratio of SS to total SH + SS were occasionally found (0–70%). Standing the nuclear suspension at 2° overnight increased the relative content of disulphide. Glutathione reductase was very much more active in nuclei from liver than in those from

thymus (160 μ M-moles of NADPH utilized/min./mg. of DNA phosphorus at 40° compared with 25–30 μ M-moles/min./mg.) and the presence of this enzyme and the high concentration of glutathione in the liver could affect the oxidation–reduction state of the thiol groups in the nuclei during the course of the isolation.

In an attempt to investigate the association of the thiol compounds with nuclear constituents, liver nuclei were incubated with either deoxyribonuclease (EC 3.1.4.5) or ribonuclease (EC 2.7.7.16), and the apparent thiol content was compared with untreated incubated controls (Table 2). Total removal of the DNA did not affect the reactivity of the thiol compounds but a slight

Table 1. *Thiol and disulphide concentrations in nuclei from rat thymus and liver*

Tissue	Apparent SH*	Total SH†	Total SH + SS
Thymus	0.36	1.03	1.14
	0.37	0.67	1.30
	0.38 (7)	—	—
Liver	0.86 $\pm$ 0.246 (14)	1.91 $\pm$ 0.471 (11)	2.31 (4)
Young	0.67	—	2.24
liver	1.50	1.50	2.50
	1.13	2.43	—

* Direct reacting.

† Reacting after treatment with 4M-urea (cf. text).

Table 2. *Effect of nuclease treatment on the apparent thiol content of liver nuclei*

Nuclei were isolated in 2.2M-sucrose–5 mM-CaCl₂–5 mM-tris, pH 7.2, and suspended in 0.25M-sucrose–5 mM-CaCl₂–5 mM-tris, pH 7.2. Nuclei (1 ml.) were incubated with 0.2 ml. of 0.1M-MgCl₂ and deoxyribonuclease (Sigma Chemical Co., crystalline) for 60 min. at 25° before estimating the apparent SH content, or with ribonuclease (L. Light and Co. Ltd.) for 30 min. at 37°. Thiol concentration is given as μ moles of SH groups/mg. of DNA phosphorus.

Deoxyribo-nuclease (μ g./incubation)	Apparent thiol content		Percentage reduction in DNA or RNA
	Control	With nuclease	
100	1.41	1.41	34
150	1.00	1.00	100
150	0.95	0.95	86
Ribonuclease (μ g./incubation)			
200	1.16	1.28	—
200	1.46	1.76	47
500	1.46	1.76	47

though consistent increase was produced by removal of some of the RNA, suggesting that a small part of the thiol content might be associated with ribonucleoprotein.

Acid extraction of nuclei. The spectra of sequential acid extracts of liver nuclei are shown in Fig. 1. The first wash with 1 mM-hydrochloric acid was usually turbid; this turbidity was not present in the second wash and very little material with u.v. absorption was removed by the third wash. The first extraction with 50 mM-hydrochloric acid removed protein with an absorption peak at 275 m μ . The second extraction at this same pH gave a much smaller amount of protein accompanied by material absorbing at 260 m μ . This flattening and broadening of the spectral curve of the second extraction with 50 mM-hydrochloric acid has been quite characteristic of the extraction procedure, with both liver and thymus nuclei. Subsequent analyses were performed on the loosely bound histone represented by the dialysed material from the first extraction with 50 mM-hydrochloric acid. The first extraction with 250 mM-hydrochloric acid produced a further fraction of histone which was usually much less than that shown in Fig. 1. Additional washing with 250 mM-hydrochloric acid again showed reduced amounts of protein and increased amounts of substances absorbing at 260 m μ . The reason for the fluctuations in the relative amounts of the loosely and tightly bound histones of rat-liver nuclei is not known. Experiments with thymus nuclei treated identically showed a much greater proportion of tightly bound histone.

Fig. 1. Ultraviolet-absorption spectra of sequential acid extracts from liver nuclei. (1) First extraction with 1 mM-HCl; (2) second extraction with 1 mM-HCl; (3) third extraction with 1 mM-HCl; (4) first extraction with 50 mM-HCl; (5) second extraction with 50 mM-HCl; (6) first extraction with 250 mM-HCl.

The thiol + disulphide content of the liver and thymus nuclear extracts (Table 3) showed that about 25 % of the SH + SS content was found in the fraction extracted with 50 mM-hydrochloric acid. In thymus approximately equal amounts were distributed between the two acid extracts.

Electrophoretic analysis of the dialysed material from the first extraction of liver nuclei with 50 and 250 mM-hydrochloric acid showed that both extracts contained a mixture of components (Fig. 2). Eight bands have so far been distinguished; the first is material remaining at the origin which was present in all runs using acid extracts of whole liver nuclei. This component may be the acidic component described by Laurence, Simson & Butler (1963). The 50 mM-hydrochloric acid extract showed seven bands, of which 1, 4 and 5 (Fig. 2) would be considered main constituents, and the

Table 3. *Acid-extractable thiol groups from liver and thymus nuclei*

Nuclei were suspended in 0.25M-sucrose-5 mM-CaCl₂-5 mM-tris, pH 7.2. A, Supernatant obtained after centrifuging the suspension for 1 min. at 3500g. B, Supernatant obtained after washing the residue from A with water. C, supernatant obtained after extracting the residue from B three times with 50 mM-HCl. D, Supernatant obtained after extracting the residue from C three times with 250 mM-HCl. Between stages B and C the nuclei were washed with 1 mM-HCl. Values are given as % of total thiol + disulphide in the original nuclear suspension. Figures in parentheses are the numbers of experiments.

Suspension	A	B	C	D
Liver	22.6 (3)	8.8 (3)	24.7 (7)	Trace (1)
[3.03 (3) μ moles of SH + SS/mg. of DNA P]				
Thymus	—	2.2 (1)	10.3 (3)	10.6 (3)
[1.37 μ moles of SH + SS/mg. of DNA P]

Fig. 2. Electrophoretic separation of acid extracts from liver and thymus nuclei and from liver mitochondria and microsomes. (1) Liver nuclei, 50 mM-HCl extract; (2) liver nuclei, 250 mM-HCl extract; (3) thymus nuclei, 50 mM-HCl extract; (4) thymus nuclei, 250 mM-HCl extract; (5) liver mitochondria, 50 mM-HCl extract; (6) liver microsomes, 50 mM-HCl extract. Bands are numbered from origin 1 to 8. Minor components are shown by hatching.

250 mM-hydrochloric acid extract had six components, of which 1, 4, 5 and 6 were prominent.

Thymus nuclei showed components similar to those from acid extracts of liver, but in the 50 mM-hydrochloric acid extract the four bands 4, 5, 6 and 7 showed equal intensity of staining and the first band was absent. In the 250 mM-hydrochloric acid extract, band 5 was very prominent.

Amino acid analyses of the unfractionated protein from the first 50 mM-hydrochloric acid extract from liver nuclei (Table 4) indicated that it had an amino acid composition and lysine:arginine ratio resembling the slightly lysine-rich fraction of Hnilica, Johns & Butler (1962). Analyses of the separated components of the 50 mM-hydrochloric acid extract have not been performed.

Salt extraction of nuclei. The acid-extraction procedure did not allow unequivocal demonstration that the material extracted with either 50 or 250 mM-hydrochloric acid had been associated with DNA. Deoxyribonucleoprotein was therefore isolated from the washed nuclei (Frenster *et al.* 1960). Of the SH+SS content 10 % was associated with the nuclear ribosomes (Table 5) and about 30 % of the total SH+SS was extracted by 2M-sodium chloride. Acidification of the 2M-sodium chloride extract to 250 mM-hydrochloric acid gave a protein fraction with identical spectral characteristics to that obtained by extracting the whole nuclei with 50 mM-hydrochloric acid, although only about 20 % of the SH+SS content of the nucleoprotein could be recovered in the acid extract. In one experiment, extraction with 1M-sodium chloride gave nucleoprotein which, after acidification, yielded a histone containing SH+SS which migrated as a single component in the electrophoretic analysis, corresponding in position to component 4 or 5 (Fig. 2).

Identification of the thiol-containing histone fractions. The presence of the single component in the electrophoresis migrating in position 4 or 5, obtained after extraction of liver nuclei with 1M-sodium chloride, suggested that in the acid extractions of whole nuclei the material migrating in one of these positions might be the SH-containing constituent. This was confirmed by the use of $N[^{14}C]$ -ethylmaleimide, which reacted to an amount equivalent to that found by the Ellman method. Electrophoresis of labelled extracts from liver and thymus nuclei and determination of the ^{14}C activity in the different bands showed that bands 4 and 5 were radioactive (Table 6). In some runs evidence was obtained that band 5 could be resolved into at least two components. When this occurred the more slowly moving component contained the radioactivity. Counts were also found at the origin in all the extracts examined, and in the 250 mM-hydrochloric acid extracts, when band 3 was present, it was slightly radioactive.

Table 4. *Amino acid composition of 50 mM-hydrochloric acid extracts of liver cell fractions*

Values are given as moles/100 moles.

Amino acid	Nuclei	Mitochondria	Microsomes
Lysine	12.58	7.09	8.38
Histidine	2.38	2.58	2.94
Arginine	7.87	5.13	4.77
Aspartic acid	6.72	10.18	9.95
Threonine	5.67	5.66	5.26
Serine	6.41	6.48	6.46
Glutamic acid	10.19	11.09	11.70
Proline	5.22	5.21	5.57
Glycine	8.84	8.80	7.97
Alanine	10.80	7.98	7.74
Valine	4.63	6.80	5.74
Methionine	1.52	2.58	2.42
Isoleucine	3.76	4.57	3.97
Leucine	7.32	8.60	8.44
Tyrosine	2.55	3.12	2.67
Phenylalanine	2.40	4.00	4.34
$\frac{1}{2}$ Cystine	Trace	0.89	1.09
Basic/acidic	1.35	0.66	0.74

Table 5. *Salt extraction of liver nuclei*

Suspensions of nuclei in 0.25M-sucrose medium were centrifuged for 1 min. at 3500g and the supernatant was discarded. The nuclei were then suspended in 0.1M-tris-HCl, pH 7.1, and a sample was reserved for SH analysis. The remaining nuclei were sedimented and washed again with tris-HCl buffer, before being extracted for 60–120 min. at 2° with either 40 ml. of 2M-NaCl or with 20 ml. of 1M-NaCl followed by 20 ml. of 2M-NaCl. Values are given as % of the total SH+SS present in the first suspension in tris buffer.

Expt. no.	...	...	1	2	3
Extractant					
0.1M-Tris			10	8	7
1M-NaCl			17	—	—
2M-NaCl			4	49	35
Initial SH+SS (μ m-moles/ml.)			125	252	74

Table 6. *Radioactivity of bands obtained by electrophoretic separation of $N[1-^{14}C]$ -ethylmaleimide-treated histone fractions from liver and thymus nuclei*

Acid extracts were treated with excess of $N[1-^{14}C]$ -ethylmaleimide and appropriate amounts applied to each column as described in the text.

Bands	Counts/sec.			
	Liver		Thymus	
	50 mM-HCl extract	250 mM-HCl extract	50 mM-HCl extract	250 mM-HCl extract
1	332	81	112	30
3	—	13	—	9
Blank	1	—	—	—
4	35	40	22	32
5 (slow)	0	19	18	61
5 (fast)	—	0	3	0
6	0	—	0	—

Mitochondrial and microsomal extracts. Possible contamination of the nuclear extracts by proteins from mitochondria and microsomes necessitated study of the effect of the acid-extraction procedure on these cell fractions. Some turbidity was present in the first extract in each case owing to a small amount of lipid which layered on top of the centrifuge tube and which re-dispersed on decantation. The solutions were otherwise clear and faintly yellow after centrifugation. With mitochondria the material extractable with 1 mM-hydrochloric acid showed an absorption maximum at about 265 m μ whereas that from microsomes showed a peak at 260 m μ . Two to three times as much absorbing substance was removed from mitochondria and microsomes as from nuclei from the same wet weight of liver and this was also true in the extractions with 50 mM-hydrochloric acid. As with the nuclei, the first extraction with 50 mM-hydrochloric acid removed a considerable amount of basic protein with a maximum at 275 m μ , but continued extraction of mitochondria and microsomes with 50 or 250 mM-hydrochloric acid did not appear to alter the character of the protein being extracted.

Electrophoresis of the dialysed material from the first extraction of mitochondria and microsomes with 50 mM-hydrochloric acid showed a single component migrating to a similar extent in both cell fractions. Runs for different times and with different dilutions have so far failed to produce evidence of heterogeneity with material from either source. The protein migrated in a position similar to components 4 or 5 from the nuclear extracts (Fig. 2). Amino acid analyses (Table 4) confirmed the similarity in composition of the acid-extractable material from mitochondria and microsomes and showed also the presence of cysteine, as had been found by the Ellman method.

Exhaustive washing with 0.25 M-sucrose medium and sedimentation at 800g failed to reduce the amounts of components 4 or 5 extracted from liver nuclei with 50 mM-hydrochloric acid, so that though cytoplasmic contamination cannot be excluded as the source of these bands it seems more likely that basic proteins in the microsomes and mitochondria have similar electrophoretic properties to the histone fractions represented in bands 4 or 5.

DISCUSSION

The distribution of SH + SS groups was similar in thymus and liver nuclei; about 10 % was associated with nuclear ribosomes, 20–30 % was removed in dilute hydrochloric acid and the remainder was not readily extractable. The higher content of SH + SS in nuclei from liver is consistent with their greater protein content; radioautographic analysis of liver nuclei labelled with N[¹⁴C]-ethylmaleimide (M. G.

Ord & L. A. Stocken, unpublished work) showed that the amount of fixed thiol was roughly proportional to the nuclear size. As the acid-extractable material was electrophoretically inhomogeneous it was necessary to study the extraction procedures in detail and also to determine the possibility of contamination from other cell fractions. The heterogeneity of histones is well established and numerous procedures have been evolved for their isolation. Since it was not known whether high salt, prolonged extraction and the use of surface-active agents affected the amount of SH groups, it was initially decided to employ simple acid extraction for short periods.

Butler, Davison, James & Shooter (1954) demonstrated the degradative action of intranuclear cathepsins at neutral pH; 1 mM-hydrochloric acid was therefore chosen for the preliminary washing, as Daly & Mirsky (1954–55) had found that histones were not extracted at pH 3. Ui (1957) showed that successive extraction of thymus nuclei with acid of increasing strength resulted in virtually total extraction of histones. He also noted that extraction with 0.1 M-sulphuric acid gave histones with properties differing from histones extracted with 0.2 M-acid. This is confirmed by our results and it is suggested from the spectrophotometric evidence that short time-intervals of extraction give rise to high protein concentrations, whereas prolonged or multiple extraction allows greater contamination with nucleotide-containing material and extraction of protein which would otherwise have been removed at lower pH.

The pattern of components from liver nuclei was very similar to that previously reported (Driedger, Johnson & Marko, 1963). Experiments in which extraction with 50 mM-hydrochloric acid was complete within 5 min. suggested that brief treatment reduced the number of components extracted, although SH + SS was always detected. Labelling the SH-containing proteins with N[¹⁴C]-ethylmaleimide confirmed that thiol groups were associated with specific histone fractions, which were found in both liver and thymus nuclei. Amino acid analysis of the unfractionated material extracted from liver nuclei with 50 mM-hydrochloric acid showed the presence of traces of cysteine, but it has not yet been possible to determine the amino acid composition of the separated thiol-containing histones.

The large amount of basic sulphur-containing protein which could be extracted from mitochondria, and which was electrophoretically homogeneous, was somewhat surprising, although basic protein (Geyer, 1960) and thiol groups (Lehninger & Neubert, 1961; Christian, Knoblock Purdy, 1963) have been reported earlier. Acid-extractable protein from microsomes has been examined by Cohn

& Simson (1963) and the amino acid composition found in our experiments is in most respects similar to that reported by Cohn & Simson (1963) for ribosomal protein, but the values for the basic amino acids in our extracts are lower and resemble those given by Butler, Cohn & Simson (1960) for total microsomal protein. The apparent homogeneity of the material extracted from liver microsomes with 50 mM-hydrochloric acid may reflect the use of more dilute acid and shorter extraction times than in previous work.

SUMMARY

1. The total thiol + disulphide content of nuclei obtained from rat livers was approximately twice that found in nuclei from rat thymus gland. Amounts of disulphide were usually small, and the amount of thiol was increased about twofold in the presence of 4M-urea.

2. Of the total thiol + disulphide in liver and thymus nuclei 10 % was associated with nuclear ribosomes and 30 % was extractable in dilute hydrochloric acid.

3. The acid-extractable thiol groups were associated with two of the histone fractions, which could be separated electrophoretically.

4. Mitochondria and microsomes from rat liver contained basic thiol-containing proteins which were extracted with 50 mM-hydrochloric acid. These proteins had similar amino acid compositions and were each apparently homogeneous on electrophoresis.

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