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THE ROLE OF SULFHYDRYL GROUPS IN RNA METABOLISM

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THE work of Brachet [1, 2] and Brachet and Rapkine [11] has attracted attention to the role of various sulfhydryl reagents in morphogenesis in embryos and in Acetabularia. Work in Brachet's laboratory [11, 12] has shown, for instance, that β-mercaptoethanol increases the uptake of thymidine into DNA, and of uridine and cytidine into RNA. These observations had suggested to Brachet that β-mercaptoethanol might induce the formation of abnormal RNA, which could lead to the production of abnormal proteins. In Acetabularia β-mercaptoethanol binds to the nucleolus and nuclear sap, as well as to structures of the differentiating apex of the stem, and this localization suggests that this SH compound might affect differentiation by interfering with the transfer of nuclear RNA to the cytoplasm, where it is involved in the synthesis of specific proteins. At Professor Brachet's suggestion, we have attempted in the present paper, to determine whether β-mercaptoethanol, as well as other related agents, interfere with the transfer to the cytoplasm of label incorporated into nuclear RNA.

MATERIAL AND METHODS

The transfer to cytoplasmic RNA of ^{3}H-cytidine taken up in 1 hr into HeLa-cell nuclear RNA is studied, using the culture methods and incubation procedures already described [9]. Cells are cultured in Petri dishes on coverslips in ϕ_{10} medium, and before the experiments the coverslips are transferred to individual cuvettes containing Eagle's solution. After 2 hr incubation at 37°C in this solution, ^{3}H-cytidine (360 mC/m*M*; 0.8 μc/ml, Amersham) is added for 1 hr with or without the SH reagents, to study their effect on RNA synthesis. To study the transfer of label to the cytoplasm, cells are similarly labeled for 1 hr without any SH reagent and further incubated for another 4 hr in Eagle's solution, with or without the reagent to be tested. Immediately after these treatments, the cells are fixed in acetic ethanol

[1] Fellow of the National Science Foundation. *Present address*: Department of Biochemistry, College of Physicians and Surgeons, New York. Abbreviations: N, non-nucleolar part of the nucleus; n, nucleolus; C, cytoplasm; PCMB, parachloromercuribenzoate; NEM, *N* ethylmaleimide.

(25/75 in volume), rinsed in 70 per cent ethanol, dried and extracted first with 1 per cent cold perchloric acid and then with a non-radioactive cytidine solution (5 μg/ml at room temperature). The cells are rinsed afterwards for 2 hr in running tap water and processed for autoradiography with liquid Ilford L4 emulsion, as previously described [10]. Exposure times are of the order of 6–8 days. The grains are scored in the nucleolus (n), in the non-nucleolar part of the nucleus (N) and in the cytoplasm (C). The reagents used are β-mercaptoethanol and dithiodiglycol, its oxidized dimer, which has strikingly opposite effects on differentiation. L. Light & Co. products are used and the solutions containing these reagents are made up immediately before each experiment. A few experiments are made with parachloromercuribenzoate (PCMB) (California Foundation for Biochemical Research) and *N* ethylmaleimide (NEM) (L. Light & Co), which both combine with SH groups.

RESULTS

β-Mercaptoethanol.—After 1 hr in 10^{-3} *M* β-mercaptoethanol, some unspecific silver precipitate over the nucleus and cytoplasm can be seen; at 10^{-4} the cells look normal, but already after this short time the number of

TABLE I. *Incorporation of ^{3}H-cytidine in the presence of β-mercaptoethanol.*

		Control	$2{\cdot}10^{-3}$ *M*	10^{-3} *M*	$5{\cdot}10^{-4}$ *M*	$2{\cdot}10^{-4}$ *M*	10^{-4} *M*
				Total counts[a]			
	I	854	—	956	—	—	938
	II	654	—	240 (36)	162 (25)	—	—
	III	2227	151 (6.5)	264 (12)	—	824 (37)	1280 (56)
	IV	920	111 (12)	96 (10.4)	—	520 (55)	716 (77)
				Distribution of grains[b]			
n	I	36.7	—	33.1	—	—	36.7
	II	34.2	—	28.2	27.0	—	—
	III	27.6	15.9	26.0	—	26	26.3
	IV	37	42	38.0	—	31	37
N	I	54.5	—	58.2	—	—	56.2
	II	54.5	—	58.5	58.0	—	—
	III	60.3	76.3	70.5	—	64.2	59.2
	IV	49	42	62.0	—	59	55
C	I	7.8	—	8.7	—	—	6.9
	II	11.3	—	13.3	15	—	—
	III	12.1	7.8	3.5	—	9.8	14.5
	IV	14	16	0	—	10	8

I, II, III, IV: no. of experiment.
[a] Total grain counts for 30 cells. Figure in parentheses: percentage of control value.
[b] Percentage of total count in nucleolus (n), rest of the nucleus (N) and cytoplasm (C).

abnormal mitotic figures has increased and sometimes an apparently uneven distribution of chromatin at anaphase can be observed.

After 4 hr many cells have lost their cytoplasm and the bare nuclei look condensed. Unspecific silver precipitate can be seen in the nuclei and on the cell membranes. However, only normal-looking cells have been scored. The effects on RNA metabolism are the following:

(1) β-Mercaptoethanol decreases the labeling by ^{3}H-cytidine of nuclear (N) and nucleolar (n) RNA even at a concentration of 10^{-4} *M*. There is an equal effect on both these nuclear compartments (Table I).

(2) If the cells have been labeled for 1 hr in normal conditions, and are then further incubated for 4 hr in non-radioactive medium either containing or not containing various concentrations of β-mercaptoethanol, a lower RNA labeling is found when the SH reagent has been used at the concentration of $2 \cdot 10^{-4}$ *M* and maybe still at 10^{-4} *M* (Table II). During the 4 hr incubation

TABLE II. *Transfer of RNA-incorporated ^{3}H-cytidine in the presence of β-mercaptoethanol.*

		Control	$2 \cdot 10^{-3}$ *M*	10^{-3} *M*	$5 \cdot 10^{-4}$ *M*	$2 \cdot 10^{-4}$ *M*	10^{-4} *M*
				Total counts[a]			
	I	1298	574 (45)	—	—	—	1949 (>100)
	II	621		51 (8)	419 (67)	—	—
	IV	1630	Cytolyzed	740 (46)	—	940 (58)	1110 (68)
				Distribution of grains[b]			
n	I	23	31.4	—	—	—	22
n	II	15.2	—	23.5	12.3	—	—
n	IV	18.5	Cytolyzed	33.5	—	20	16
N	I	42	57.5	—	—	—	39.4
N	II	35.5	—	51	37.7	—	—
N	IV	29.0	Cytolyzed	45	—	34	33
C	I	35	11.1	—	—	—	36.8
C	II	49.3	—	25.5	50	—	—
C	IV	52.5	Cytolyzed	21.5	—	46	51
				Cytoplasmic gain during transfer			
	I	27	3	—	—	—	29
	II	38	—	14	38.7	—	—
	III	38.5	—	7.5	—	32	37

I, II, III, IV: no. of experiment.
[a] Total grain counts for 30 cells. Figures in parentheses: percentage of control value.
[b] Percentage of total count in nucleolus (n), rest of the nucleus (N) and cytoplasm (C).

in non-radioactive medium, the cells continue to take up cytidine from the nucleotide pool of precursors (compare total counts in the controls of corresponding experiments of Tables I and II); however, in the case of mercaptoethanol-treated cells, when the concentration of the agent is higher than $5 \cdot 10^{-4}$ *M*, the number of grains found after 4 hr (Table II) is smaller than that found immediately after the 1 hour of the normal labelling period (Table I, controls). Therefore not only is the uptake of label from the radioactive pool formed in 1 hour into RNA diminished, but there is also a measurable loss of incorporated radioactivity.

(3) If one looks at the last entry in Table II (cytoplasmic gain during transfer), it can be seen that with 10^{-3} *M* mercaptoethanol there is a striking inhibition of the relative rise in cytoplasmic grain count: at this concentration there is not only a considerable loss of incorporated radioactivity but also a relative inhibition of the transfer of the remaining label to the cytoplasm.

Dithiodiglycol.—After 1 hr at $2 \cdot 10^{-3}$ *M* there is often some non-specific silver

TABLE III. *Incorporation of ^{3}H-cytidine in the presence of dithiodiglycol.*

		Control	$2 \cdot 10^{-3}$ *M*	10^{-3} *M*	$5 \cdot 10^{-4}$ *M*	$2 \cdot 10^{-4}$ *M*	10^{-4} *M*
				Total counts[a]			
	I	854	—	702 (81)	—	—	897 (>100)
	II	659	—	240 (36)	162 (24)	—	—
	III	2227	600 (27)	1583 (69)	—	1235 (55)	1806 (80)
	IV	670	132 (20)	210 (31)	—	580 (85)	654 (97)
				Distribution of grains[b]			
n	I	37	—	36	—	—	38
n	II	34	—	28	27	—	—
n	III	27	25	25	—	28	29
n	IV	41	45	40	—	40	40
N	I	55	—	59	—	—	59
N	II	55	—	58	58	—	—
N	III	60	69	65	—	56	56
N	IV	54	55	60	—	55	54
C	I	8	—	5	—	—	3
C	II	11	—	14	15	—	—
C	III	13	6	10	—	16	15
C	IV	5	0	0	—	5	6

I, II, III, IV: no. of experiment.
[a] Total grain counts for 30 cells. Figures in parentheses: percentage of control value.
[b] Percentage of total count in nucleolus (n), rest of the nucleus (N) and cytoplasm (C).

precipitate over the cell nuclei and on the cytoplasmic membrane, chiefly in the case of dividing cells; after 4 hr the cells contain vacuolated nuclei and cytoplasm; in some instances the cytoplasm has been cytolyzed, leaving bare nuclei; metaphases appear pycnotic for the higher concentration, and the chromosomes are somewhat smaller at the lower concentration. Only normal-looking cells were counted.

The effects of dithiodiglycol on RNA metabolism are the following:

(1) As in the case of β-mercaptoethanol, there is a striking inhibition of the uptake of ^{3}H-cytidine into nuclear and nucleolar RNA, for concentrations

TABLE IV. *Transfer of RNA-incorporated ^{3}H-cytidine in the presence of dithiodiglycol.*

		Control	$2\cdot10^{-3}$ *M*	10^{-3} *M*	$5\cdot10^{-4}$ *M*	$2\cdot10^{-4}$ *M*	10^{-4} *M*
				Total counts[b]			
	I	1288	1066 (83)	—	—	1160 (90)	—
	II	621	—	377 (60)	625 (100)	—	—
	III	2573	1801 (59)	1708 (65)	—	2703 (106)	2708 (106)
	IV	1552	210 (15)	390 (25)	—	1385 (89)	1570 (102)
				Distribution of grains[b]			
n	I	23	23	—	—	23	—
	II	15	—	20	15	—	—
	III	19	21	24	—	18	21
	IV	16	42	29	—	18	18
N	I	42	57	—	—	43	—
	II	36	—	48	45	—	—
	III	32	50	48	—	40	39
	IV	31	45	43	—	34	27
C	I	35	20	—	—	34	—
	II	49	—	32	40	—	—
	III	49	29	28	—	42	40
	IV	53	13	28	—	48	55
				Cytoplasmic gain during transfer			
	I	27	12	—	—	26	—
	II	38	—	21	27	—	—
	III	36	16	15	—	39	27
	IV	48	7	23	—	43	50

I, II, III, IV: no. of experiment.
[a] Total grain counts for 30 cells. Figures in parentheses: percentage of control value.
[b] Percentage of total count in nucleolus (n), rest of the nucleus (N) and cytoplasm (C).

of $2 \cdot 10^{-4}$ *M* and higher. There is no change in grain distribution, showing that both N and n are equally affected (Table III).

(2) If the cells have been prelabeled in normal conditions for 1 hr with ^{3}H-cytidine, a smaller amount (20 to 80 per cent) of labeled RNA (N + n) is found after the further incubation with high concentrations (10^{-3} and higher) of dithiodiglycol (Table IV). At these concentrations (except in experiment I), the total grain count is lower than that found immediately after the 1 hr normal incubation in radioactive cytidine (control of Table III). There is therefore probably also a measurable loss of label from the RNA in the presence of dithiodiglycol, in addition to an inhibition of incorporation from the labeled pool.

(3) Cytoplasmic RNA becomes only weakly labeled when cells prela-

TABLE V. *Incorporation of ^{3}H-cytidine in HeLa cells in the presence of PCMB and NEM (1 hr incubation).*

	Control	PCMB		NEM	
		$2 \cdot 10^{-5}$ *M*	$2 \cdot 10^{-6}$ *M*	$2 \cdot 10^{-4}$ *M*	$2 \cdot 10^{-5}$ *M*
		Total counts[a]			
	784	695	828	523	681
	—				
	667				
	762				
	753				
		Distribution of grains[b]			
n	30.4	53.5[c]	29.0	31.4	31.4
n	28.5				
n	26.9				
n	25.5				
N	60.0	40.3[c]	61.0	61.5	59.5
N	65.5				
N	60.7				
N	61.7				
C	9.6	6.2[c]	10.2	7.1	9.1
C	6.0				
C	12.5				
C	12.7				

[a] Total grain counts for 30 cells. Figures in parentheses: percentage of control value.
[b] Percentage of total count in nucleolus (n), rest of the nucleus (N) and cytoplasm (C).
[c] At $2 \cdot 10^{-5}$ *M* PCMB there are heavy grain artefacts, especially over the nucleoli, which makes counting very inaccurate.

beled with ^{3}H-cytidine are transferred to a non-radioactive medium in the presence of dithiodiglycol. The relative nucleolar loss of label in these cells is only slightly inhibited, whereas the loss from N is relatively more inhibited (Table IV).

Parachloromercuribenzoate.—In $2 \cdot 10^{-5}$ *M* PCMB many cells contain heavy non-specific silver grains over the nucleus and nucleoli, due probably to the presence of the heavy metal; dividing cells appear to be blocked in metaphase. After 4 hr these silver precipitates increase and the slides become impossible to read. At the lower concentrations the cells appear normal.

In the presence of PCMB there may be a small inhibition of the uptake of cytidine at the higher concentrations used (Table V), and the higher relative nucleolar count could be an artefact due to non-specific precipitates. The lower concentrations do not inhibit the uptake of cytidine during 1 hr (Table V). When transfer of nuclear label to cytoplasmic RNA is studied, only the lower concentrations can be studied and only a small effect, if any, is observed (Table VI).

TABLE VI. *Transfer of RNA-incorporated ^{3}H-cytidine in the presence of PCMB and NEM.*

1 hr incubation + 4 hr chase in inhibitor

	Control	PCMB $2 \cdot 10^{-6}$ *M*	NEM $2 \cdot 10^{-4}$ *M*	NEM $2 \cdot 10^{-5}$ *M*
		Total counts[a]		
	614 861	899	917	1082
		Distribution of grains[b]		
n	19.5 12.1	17.8	18.0	16.7
N	21.8 32.3	36.7	37.4	32.5
C	58.7 55.6	45.4	44.6	50.8
		Cytoplasmic gain during transfer		
	47 44	35	36	41

[a] Total grain counts for 30 cells. Figures in parentheses: percentage of control value.

[b] Percentage of total count in nucleolus (n), rest of the nucleus (N) and cytoplasm (C).

At $2 \cdot 10^{-5}$ *M* PCMB there are heavy grain artefacts over the cells with less marked localizations than in the 1 hr incubation (Table V). After 4 hr the nucleoli do not seem to be radioactive, but the rest of the nuclei have some grains.

N Ethylmaleimide.—This agent does not appear to affect the cells very strikingly after 1 hr incubation. After 4 hr the compound seems to cause the metaphase figures to become more condensed and even pycnotic (especially at the higher concentration). The higher concentration of NEM appears to inhibit the uptake of cytidine into the cells, without changing its general distribution between N, n and C (Table V).

After 4 hr further incubation in non-radioactive medium, there is no drastic change in the transfer of label at the dosages of NEM used (Table VI).

DISCUSSION

Validity of the radioautographs.—It could be claimed that the presence of reducing or oxidizing agents might alter the radioautographic efficiency. The presence of reducing agents could have the effect of increasing the grain count. Mercaptoethanol does seem to affect the emulsion by creating non-specific silver precipitates, which, however, can be clearly distinguished from grains due to ^{3}H electrons. On the other hand, dithiodiglycol could act as an oxidizing agent and, if not properly eliminated by the various washing procedures, might inhibit grain formation during radioautography. However, this is not the case: cells which have taken up ^{3}H-cytidine in normal conditions and which are thereafter transferred to a medium containing 10^{-3} *M* dithiodiglycol either before (for 5′ or 20′) or after (for 60 min) fixation, have identical counts to control cells incubated in normal medium and processed in the usual way.

Nature of the RNA labeled during the 1 hr incubation in ^{3}H-cytidine.—Several experiments in this laboratory [13, 14] and others [5–9] point to the concept that, besides informational or messenger RNA, ribosomal RNA and possibly transfer RNA are formed in the nuclei during this relatively short pulse. The mode of formation of the ribosomes, the relationship between nuclear and cytoplasmic ribosomes and the mode of association of messenger RNA to these structures are still far from understood and it is not possible from the present data to say which of these RNA's are most affected in these inhibition studies; only biochemical analysis might answer these questions.

Uptake of ^{3}H-cytidine into RNA.—β-Mercaptoethanol, dithiodiglycol and NEM at concentrations of the order of 10^{-4} *M* inhibit the incorporation of ^{3}H-cytidine with apparently no greater effect on N and n, suggesting that the synthesis of RNA in both the cell compartments may be controlled by similar type systems. The results obtained with mercaptoethanol seem to be at variance with those described by Brachet and his collaborators for amphibian

and chick embryos [3], where an increased uptake was found. Lower concentrations of the reagents should be assayed to check this point. PCMB at $2 \cdot 10^{-5}$ *M* had only a slight inhibitory effect and cannot be used at higher concentration for autoradiography.

Increase in cytoplasmic RNA.—If one studies the *relative* increase of ^{3}H-cytidine in cytoplasmic RNA, irrespective of the fact that some "RNA" has been lost during label transfer from the nucleus to the cytoplasm, there is a definite inhibition of transfer for the higher concentration (10^{-3} *M*) of β-mercaptoethanol and dithiodiglycol, but no effect was observed with PCMB or NEM, which were, however, used at lower concentrations.

SUMMARY AND CONCLUSIONS

The role of SH groups in the synthesis of RNA from nucleosides is not yet clearly understood, in spite of the finding by Chamberlin and Berg [4] that intact SH groups are necessary for the DNA-primed synthesis of RNA in bacterial extracts. The effects of dithiodiglycol, PCMB and NEM on the uptake of cytidine into nuclear RNA (n + N) might very well interfere with the RNA polymerase system. Mercaptoethanol might act at some different level still to be discovered, unless it is the SS⇌SH equilibrium which is involved.

The loss of label, once it has been introduced into RNA, and its failure to reappear in the cytoplasm with the higher concentrations of mercaptoethanol and dithiodiglycol may indicate that this step also involves SS⇌SH equilibrium. The transfer of RNA label from nucleus to cytoplasm may involve all types of RNA and might be accompanied, at least for ribosomal and transfer RNA's by some remodelling of the molecules formed in the nucleus [7]. As most of the cytoplasmic RNA is ribosomal, one might also postulate that the formation of new ribosomes is affected by both these reagents, perhaps at the level of the cystine residues [15] of the ribosomal proteins. These are merely hypotheses, which may be useful to orient further work on the subject.

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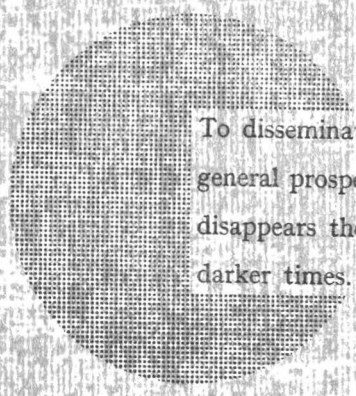

To disseminate knowledge is to disseminate prosperity — I mean general prosperity and not individual riches — and with prosperity disappears the greater part of the evil which is our heritage from darker times.

Alfred Nobel