Volume 7 Number 8 1979 Nucleic Acids Research

The effects of mercury-substitution on the hybridisation characteristics of nucleic acids

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Received 23 October 1979

ABSTRACT

The effect of different levels of mercury substitution on the rate and extent of hybridisation of globin mRNA with a complementary DNA (cDNA) copy has been investigated. It was found that mercuration significantly reduces both the rate of hybridisation and the extent of the reaction, but that these effects are abolished when at least stoichiometric amounts of 2-mercaptoethanol are included in the hybridisation medium. As a preliminary to using this technique to isolate specific groups of sequences after long-term hybridisations, we have investigated both the rate of demercuration of RNA and its retention on thiol-sepharose columns after extended incubation under commonly employed hybridisation conditions at 43° or 60°. Retention was essentially quantitative even after incubation times of 300 hours at 43°, but decreased significantly after 48 hours at 60°. It is concluded that thiol-sepharose chromatography offers considerable advantages over hydroxyapatite chromatography for the recovery of hybridised sequences, particularly with regard to the lower levels of non-specific binding obtained and its ability to distinguish directly between DNA-DNA and DNA-Hg RNA hybrids.

INTRODUCTION

Mercurated polynucleotides have great potential for dealing with some of the problems involved in the isolation of specific nucleic acid sequences (1). The technique is based on the affinity of mercury-substituted molecules for thiol groups bound to a sepharose matrix (2). The use of mercurated 3V40 cRNA to purify 3V40 DNA has been reported (1), and the method has also been applied to the isolation of specific sequences from recombinant plasmids (3). The potential of the method has, however, not been fully exploited. It should be possible, for example, to remove specific groups of DNA sequences from a highly complex population by hybridisation with the appropriate mercury-substituted RNA and subsequent chromatographic separation of the hybrids. In this way, the difficulties encountered in the separation of hybrid and non-hybrid molecules by the usual method of hydroxyapatite chromatography, which include the poor discrimination between DNA-DNA and DNA-RNA hybrids, could be circumvented. Recently, however,

data have been presented which suggest that this method might have only limited utility in the isolation of material of high complexity. First, doubts have been cast on the usefulness of mercurated nucleic acids in hybridisation analyses by the suggestion that the hybridisability of highly mercurated RNA is low (4,5). Secondly, data presented on the thermolability of the mercury-carbon bond in poly d(A-HgU) implied that the method might not be applicable in hybridisations of long duration owing to progressive loss of retention of mercurated nucleic acid on thiol-agarose (1). Obviously, a necessary prerequisite for the successful use of this technique for the isolation of complex sequences after long term hybridisations is the demonstration that

(a) The rate of hybridisation and total hybridisability of RNA is not adversely affected by Hg substitution.

(b) Demercuration during incubation is not sufficient to preclude the recovery of hybridised material.

We describe here an evaluation of these problems.

MATERIALS AND METHODS

Reagents. Mercuric acetate and E.coli rRNA were obtained from B.D.H., 2-mercaptoethanol (2-ME) was obtained from Sigma Corporation. Formamide was obtained from Fluka. S1 nuclease was obtained from Boehringer Corporation. Thiol-agarose was prepared from Sepharose CL4B using the procedure of Cuatrecasas (6). [^{203}Hg] mercuric acetate (0.5Ci/mmole) and [^{3}H] uridine (45Ci/mmole) were obtained from the Radiochemical Centre, Amersham.

Preparation of RNA. Globin mRNA was prepared from mouse reticulocytes as described previously (7). [^{3}H] labelled total cellular RNA was prepared from clone M2 of Friend cells (8) after labelling for 18 hours with [^{3}H] uridine (1 μCi/ml) during the log phase of growth. RNA was extracted using CsCl (9), desalted by filtration through Sephadex G50 equilibrated with water and stored at -20°.

Synthesis of globin cDNA. Globin mRNA was transcribed using reverse transcriptase from avian myeloblastosis virus as in Birnie *et al.* (10). The cDNA was fractionated in 4-11% (w/w) alkaline sucrose gradients, and cDNA of molecular weight greater than 0.5×10^5 was recovered by neutralisation and precipitation with ethanol. The cDNA was desalted by filtration through Sephadex G50 equilibrated with water.

Mercuration of RNA. RNA samples were mercurated essentially as described by Dale *et al.* (2). Briefly, 0.4mM nucleotide as nucleic acid was

incubated with 4mM mercuric acetate in 5mM sodium acetate, pH 6.0, at 50° for various time periods. Substitution was followed using [^{203}Hg] mercuric acetate at a specific activity of 0.5 mCi/mmole. The mixture of RNA and mercuric acetate was passed over a 20 x 1 cm column of Sephadex G50 medium equilibrated in water. 50 µg of RNA was used for each determination. 1 ml fractions were collected and the A_{260} measured. β emission by ^{203}Hg was detected by liquid scintillation counting in a Triton /toluene-based scintillant. The ratio of [^{203}Hg] to RNA was calculated for the excluded volume fractions and the degree of substitution obtained using the known specific activity of the [^{203}Hg] mercuric acetate. The counting efficiency of ^{203}Hg in mercuric acetate and in polynucleotide was assumed to be the same. The data indicated that it was possible to achieve levels of mercuration approaching 25% of total bases using this method.

RNA - cDNA hybridisations. Hybridisations in solution were performed either in formamide hybridisation buffer (0.5 M NaCl, 25mM Hepes, 5mM EDTA, pH 6.8, 50% v/v formamide) at 43° or in phosphate buffer (0.24M sodium phosphate, 5mM EDTA, 0.1% sodium dodecyl sulphate (SDS), pH6.8) at 60°. 2-ME (1mM) was added to the hybridisation buffers as appropriate (see text). The concentration of nucleic acid was maintained in hybridisation mixtures at 500 µg/ml by the addition of E.Coli ribosomal RNA. Hybridisation reactions were performed in sealed, repelcoted glass capillaries and analysed by S1 nuclease treatment as previously described (10), unless otherwise stated.

Measurement of rate of RNA demercuration. E.coli rRNA was mercurated using [^{203}Hg] mercuric acetate to a level of 12% substitution of total bases. It was dissolved in either formamide hybridisation buffer or phosphate buffer containing 2-ME (1mM) dispensed in 10 µl portions into glass capillaries and incubated at either 43° (formamide) or 60° (phosphate) for varying periods of time. Capillaries were then flushed out with 0.5 ml of NETS (0.1M NaCl, 10mm Tris/HCl, 1mm EDTA, 0.5% SDS pH 7.5) and stored at -20° until completion of the time series. All samples were then passed over 20 x 1 cm columns of Sephadex G50 equilibrated in water and the substitution with mercury determined as described above.

Retention of mercurated RNA on thiol-sepharose. The retention of mercurated RNA on thiol-sepharose was measured using two procedures. One procedure involved chromatography at ambient temperature and the other included a washing step at 60°. 1.5ml columns of thiol-sepharose prepared in 2ml plastic syringes were activated by treatment with 50mM dithio-

threitol (DTT), 0.5M Tris, pH 8.0, for 30 min at ambient temperature. The DTT/Tris was removed by washing with 25ml of NETS. In the ambient temperature procedure the sample of mercurated RNA was applied to the column in 0.5ml of NETS and left for 10 min. The column was then washed with 30ml of NETS and the retained material eluted with 5 x 1 ml of NETS containing 0.1M 2-ME. In the procedure employing elevated temperatures the sample was applied to the column equilibrated at 60° in a water jacket and left for 10 min. It was then washed with 10ml of NETS at 60° and 20ml of NETS at 25°. The elution was carried out as described above. This procedure has been found to give a considerable improvement in background compared with the ambient temperature procedure.

RESULTS AND DISCUSSION. The levels of mercury substitution attained after incubation of E.coli rRNA with [^{203}Hg] mercuric acetate for various time periods are shown in Figure 1. The results are similar to data previously reported by Dale and Ward (1).

Effect of mercuration on rate and extent of hybridisation of RNA. It has been suggested that mercuration adversely affects and can indeed abolish the hybridisability of RNA (4,5). In order to maintain reasonable retention on thiol-sepharose even after prolonged incubation it is important to be able to use high levels of mercuration. If the rates of hybridisation are affected by mercury substitution then certain types of experiment would

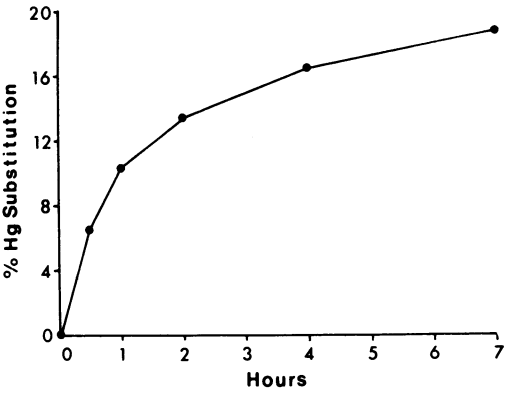

Figure 1. Percentage of mercury-substituted bases in E.coli rRNA after incubation with [^{203}Hg] mercuric acetate for various periods of time. Degree of substitution was estimated as described in Materials and Methods.

be difficult or impossible. The problem was therefore investigated by hybridising globin mRNA, mercurated chemically to different defined degrees of substitution, with [^{3}H] globin cDNA. The hybridisation reactions took place in 0.24M phosphate buffer at 60°. A parallel series of incubations was carried out in which 1mM 2-ME was added to the buffer as ligand for the mercury-substituted bases. Representative $R_o t$ curves obtained are shown in Figure 2, and the full data are summarised in the accompanying Table 1. The results demonstrate that, in the absence of 2-ME in the hybridisation buffer, the degree of mercury substitution in RNA has a dramatic effect on its rate of hybridisation with cDNA. Substitution of 12% of the bases with mercury slows the reaction by a factor of about 3.5, while 22% mercuration results in a 5.3 fold decrease in the rate of hybridisation. At the same time, the overall extent of hybridisation observed at a $R_o t$ value of 3 x 10^{-1} moles sec/l decreased from 83% (0% mercuration) to 59% (22% mercuration). These effects of substitution were essentially abolished when the hybridisation buffer was supplemented with 1mM 2-ME. The $R_o t$ $\frac{1}{2}$ values

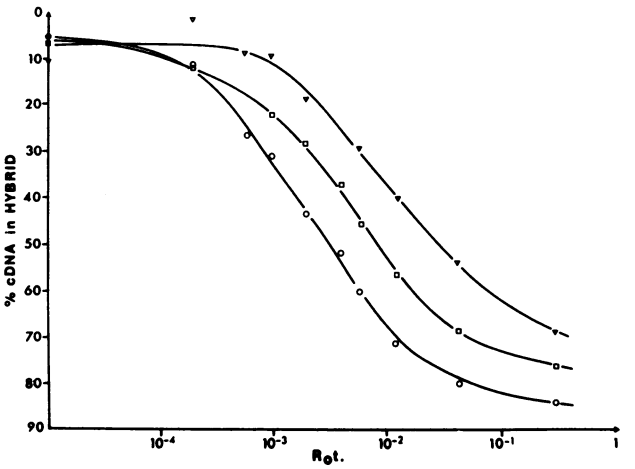

Figure 2. Hybridisation of globin cDNA in phosphate buffer at 60° with globin mRNA mercurated to different levels.

—o— 0% substitution

—□— 12% substitution

—▽— 22% substitution

When the hybridisations were carried out in the presence of 1mM 2-ME, the $R_o t$ curves obtained were similar to that shown for the 0% substituted mRNA.

TABLE 1. Hybridisation of globin cDNA with mRNA mercurated to different levels in phosphate buffer at 60° either in the presence or absence of 2-ME.

% Hg Substitution	$R_0t_{\frac{1}{2}}$ (moles sec./1)	concentration of 2-ME
0	1.6×10^{-3}	
6	2×10^{-3}	
12	1.8×10^{-3}	1mM
17	1.6×10^{-3}	
22	1.7×10^{-3}	
0	1.7×10^{-3}	
12	6×10^{-3}	None
22	9×10^{-3}	
22	3.5×10^{-3}	Stoichiometric*

* Excess 2-ME was removed by passage through a sephadex G-50 column before carrying out the hybridisation in the absence of 2-ME.

observed using globin mRNA at all levels of mercuration were essentially the same, and the extent of reaction was above 80% in each case. Very similar results were obtained when the hybridisations were carried out in formamide buffer at 43° rather than phosphate buffer at 60° (data not shown).

A possible alternative explanation of the above results is that the presence of 1mM 2-ME in the hybridisation buffer catalysed the rapid demercuration of the globin mRNA. If this were the case, the increase in reaction rate in the presence of 2-ME would be due to the demercuration rather than to the stabilising effect of the thiol groups. A control experiment was therefore carried out in which 12% mercury-substituted globin mRNA was hybridised with its cDNA in the presence of 1mM 2-ME in phosphate buffer at 60°. After various time intervals, incubation mixtures were flushed out either with S1 nuclease buffer or with NETS buffer, and analysed by S1 nuclease digestion or SH-Sepharose chromatography as appropriate. If demercuration is appreciable under these hybridisation conditions, it would be expected that the amount of cDNA in hybrid as measured by the SH-sepharose technique would be less than that determined by S1. It can be seen from Figure 3 that the rate of hybridisation is essentially the same when measured by both methods. We conclude therefore that the more rapid reaction rates observed in the presence of 2-ME are

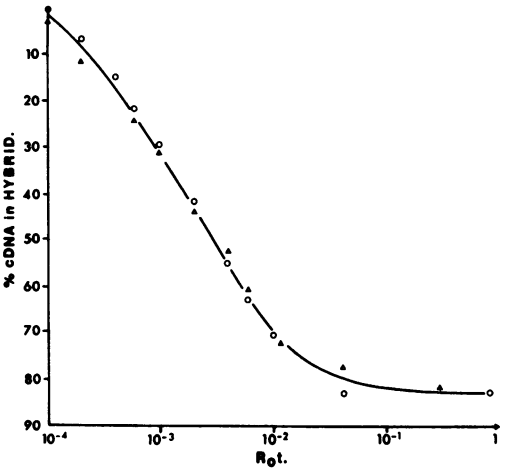

Figure 3. Hybridisation of globin cDNA with 12% mercury-substituted globin mRNA in phosphate buffer at 60° containing 1mM 2-ME. Points were analysed by S1 (—Δ—) or by thiol-sepharose chromatography (—O—).

not due to demercuration of the RNA.

Hybridisation of mercurated RNA in the presence of stoichiometric amounts of 2-ME

In a separate experiment, globin mRNA which had been mercurated to the 22% level was treated with excess 2-ME to saturate all of the substituted bases and then passed through a column of sephadex G50 equilibrated with water to remove the unbound 2-ME. When this RNA was hybridised with globin cDNA in 0.24M phosphate buffer without additional 2-ME, i.e. under conditions where only stoichiometric amounts of ligand should have been present, the rate of the reaction was decreased by a factor of about 2 (Table 1) with respect to the same reaction when carried out in the presence of excess ligand. This indicated either that a slight excess of 2-ME is necessary to allow maximum rate of hybridisation of mercurated RNA with cDNA, or that the mercurated globin mRNA no longer contained a stoichiometric amount of bound 2-ME after passage through the Sephadex G50 column.

These observations may provide an explanation for the discrepancy between our results and previously reported data (4,5) on the hybridisability of mercurated RNA. Although the mercurated RNA transcripts investigated by Beebee and Butterworth(4) and by Zasloff and Felsenfeld (5) were synthesized

in the presence of 2-ME, no additional thiol-containing compounds were included in the hybridisation medium. In view of the results presented above suggesting that at least stoichiometric amounts of thiol groups must be present, it is not surprising that these authors observed slower rates of hybridisation of mercurated probes.

Demercuration of RNA. Hybridisation reactions involving RNA and DNA populations of low complexity should not be appreciably affected by demercuration of the RNA since the incubation times involved are generally short (see Figure 3). However, in the case of complex populations requiring hybridisation to high R_0t values, demercuration of RNA will have implications both for the possibility of obtaining meaningful analytical data and for preparation of specific populations of probe sequences. The extent of demercuration under commonly employed hybridisation conditions was therefore studied using E. coli rRNA as a model. The degree of demercuration of an RNA sample containing initially 12% mercurated cases was determined as described in Materials and Methods. The data shown in Figure 4 demonstrate that even after prolonged incubation of mercurated RNA in 0.24M sodium phosphate at 60° to an equivalent R_0t value of 80,000 moles sec/l there is still a considerable percentage of residual mercury substitution in the RNA. These data are somewhat surprising in view of the findings of Dale and Ward (1), who observed relatively rapid demercuration of mercurated polynucleotides. Possibly, results obtained using synthetic mercurated polynucleotides can not be directly extrapolated to RNA, since it is known that individual mercurated bases show different rates of demercuration (1). Incubation in formamide hybridisation buffer at 43° caused a considerably lower rate of demercuration than that observed in phosphate buffer (Figure 4), but it is worth noting that the equivalent R_0t values which can be attained in a reasonable time are considerably lower than those attainable in the phosphate buffer. Control experiments involving the hybridisation of globin mRNA with its cDNA in both buffer systems indicated that the rate of the hybridisation was approximately 2.3 fold faster in the phosphate buffer at 60°.

Retention of partially demercurated RNA on thiol-sepharose. The demercuration data presented above suggest that it should be possible to retain mercurated RNA on thiol-sepharose even after prolonged incubation under appropriate hybridisation conditions. There may however, be other processes besides demercuration occurring during long-term incubations which might adversely influence retention of the RNA on thiol-sepharose. The most obvious of these is reduction in size of the RNA. It was there-

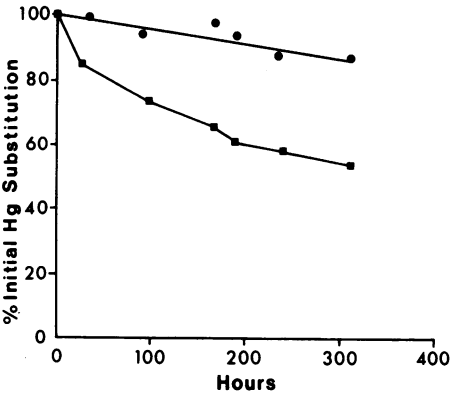

Figure 4. Demercuration of mercurated E.coli rRNA by incubation in formamide-containing hybridisation buffer at 43° or sodium phosphate hybridisation buffer at 60°.

Incubations and analysis of residual mercuration were carried out as described in Material & Methods.

formamide/43° —●—

phosphate/60° —■—

fore decided to make an operational test of the effect of long-term incubations on retention of mercurated RNA by thiol-sepharose.

Retention was measured under two sets of conditions: one in which the samples were applied and the columns washed at 60° prior to the elution and the other in which all stages in the thiol-sepharose chromatography were carried out at ambient temperature (20-25°). The retention of [^{3}H] uridine-labelled Friend cell total RNA chemically mercurated to 12% of total bases was studied. Samples incubated in formamide-containing hybridisation buffer for 300 h showed greater than 95% retention under both the high temperature and ambient temperature chromatography conditions (Figure 5). However, mercurated RNA incubated in phosphate buffer at 60° was retained more efficiently when all stages of the thiol-sepharose chromatography were carried out at ambient temperature (Figure 5). While the overall percentage of mercurated RNA retained remained reasonably constant over the first 48 hours of incubation at 60°, after 360 hours it declined to 62% and 45% when analysed by the hot and ambient temperature column procedures respectively.

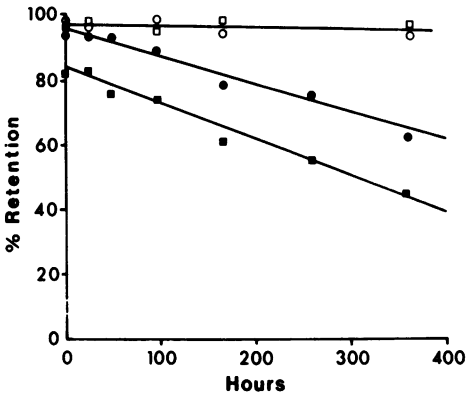

Figure 5. Retention of mercurated Friend cell RNA on thiol-sepharose after incubation in sodium phosphate hybridisation buffer at 60° (—●—, —■—) or formamide buffer at 43° (—○— , —□—).

—○— , —●— : ambient temperature chromatography.

—□— , —■— : 60° chromatography.

Non-specific binding of labelled nucleic acids to thiol-sepharose. It was consistently observed that the hot column procedure described above gave significantly lower levels of non-specific binding than the ambient temperature method. When mercurated *E. coli* rRNA was incubated with labelled cDNA or unique DNA probes from Friend erythroleukemic cells in either the formamide or the phosphate buffer system, the percentage of non-specific binding to thiol-sepharose was about 0.1% when the column was run at 60°, but 2-4% for the 25° procedure. Background levels of this order of magnitude are not particularly important for hybridisation reactions which go essentially to completion (e.g., the globin cDNA mRNA reaction shown in Figure 3 gave the same result when analysed at 25°) but are critical for reactions which terminate at low hybridisation values. For the hybridisation of mercurated Friend cell poly $(A)^+$ polysomal RNA with unique DNA, which attains a final plateau value around 1%, it was essential to run the thiol-sepharose columns at 60° (A. Balmain, D. Brown and G. D. Birnie, (unpublished results).

CONCLUSIONS

Our results indicate that mercury substitution does not affect the hybridisation characteristics of RNA provided that at least stoichiometric

amounts of 2-ME are present during the reaction. Moreover, long term hybridisations involving mercurated RNA should be possible either in formamide buffer at 43°, under which conditions retention on thiol-sepharose in essentially quantitative even after 300 hours, or in phosphate buffer at 60° provided that reaction times do not exceed 48 hours. These techniques therefore offer considerable scope for the isolation of labelled probes of high complexity by RNA-DNA hybridisation. For example, the method has been applied to the isolation of specific unique DNA probes complementary to polyadenylated polysomal and nuclear RNA from Friend erythroleukemic cells (A. Balmain, D. Brown, and G. D. Birnie, manuscript in preparation). In this context, we have found that thiol-sepharose chromatography offers considerable improvements over the more conventional chromatography on hydroxyapatite in terms of low backgrounds, lack of probe degradation, high recoveries and ability to distinguish directly between DNA-RNA and DNA-DNA hybrids. The latter property is particularly useful in experiments involving labelled unique DNA, since the necessity to take steps to remove DNA-DNA hybrids from isolated probes is circumvented.

ACKNOWLEDGEMENTS

The Beatson Institute is supported by grants from MRC and CRC. We thank Miss Anne Sproul and Mr Robert McFarlane for technical assistance, and Drs. G. D. Birnie and P. R. Wilkes for critical reading of the manuscript.

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REFERENCES

1. Dale, R.M.K. & Ward D.C. (1975) Biochemistry 14, 2458-2469.
2. Dale, R.M.K., Martin, F., Livingston, D.C. & Ward, D.C. (1975). Biochemistry 14, 2447-2457.
3. Longacre, S.S. and Mach, B. (1978) J. Biol. Chem. 253. 7500-7507.
4. Beebee, T.J.C. & Butterworth, P.H.W. (1976) Eur. J. Biochem, 66, 543-550.
5. Zasloff, M. & Felsenfeld, G. (1978) Biochemistry 16, 5135-5145.
6. Cuatrecasas, P. & Anfinsen, C.B. (1972) in Methods in Enzymology, W.B. Jakoby ed. Vol. XX11 pp 345-385. Academic Press, New York.
7. Kleiman, L., Birnie, G.D., Young, B.D. and Paul, J. (1977). Biochemistry, 16, 1218-1223.
8. Harrison, P.R., Gilmour, R.S., Affara, N.A., Conkie, D., and Paul, J. (1974). Cell Diff. 3, 23-30.

9. Young, B.D., Birnie, G.D. and Paul, J. (1976) Biochemistry 15, 2823-2829.
10. Birnie, G.D., MacPhail, E., Young, B.D., Getz, M.J., and Paul, J. (1974) Cell Diff. 3, 221-232.