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The Presence of 5-Hydroxymethylcytosine in Animal Deoxyribonucleic Acid

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A method is given for small-scale preparation of DNA from 1.0–1.5g of adult rat tissues. The product from brain or liver is characterized by base ratios and phosphorus content which accord with reported values for rat tissue. It is reasonably free of RNA, protein and glycogen. It contains 5-hydroxymethylcytosine at a content of about 15% of the total cytosine bases present. 5-Hydroxymethylcytosine is also demonstrable in mouse and frog brain DNA and in the crude cytidylic acid fractions obtained from RNA hydrolysates of rat brain and liver. 5-Hydroxymethylcytosine is identified by paper chromatography, u.v. spectra in acid and alkaline solutions and by its conversion into 5-hydroxymethyluracil.

It has been proposed by several investigators that the DNA of brain might differ from that of other tissues in view of the virtual absence of regeneration and mitosis in the adult neuron (Emanuel & Chaikoff, 1960; Robinson, 1966). However, no specific differences in composition were demonstrated in these studies. As part of this laboratory's programme it was necessary to prepare total tissue DNA, which conformed to accepted analytical criteria, from 1.5–3g of rat brain. Under these conditions it was found that standard methods (DuBuy *et al.*, 1966; Kirby, 1968) gave low yields or degraded products or both. Attempts to develop a satisfactory procedure based on extraction with concentrated sodium bromide solution (Emanuel & Chaikoff, 1960) appeared to give conventional DNA preparations, but perchloric acid hydrolysis followed by determination of base ratios revealed low cytosine values. This observation and the possibility that the method had preserved a labile DNA fraction usually lost in some analytical methods (Penn & Suwalski, 1969), suggested a more intensive analysis of the product. Formic acid hydrolysis and two-dimensional chromatography of the DNA components revealed the presence of 5-hydroxymethylcytosine. It constituted 15% of the total cytosine bases and brought the mole percentage of these bases into conformity with conventional base ratios. Application of the same preparative method to 1–2g of rat liver gave a similar DNA fraction, although in low yield. The 5-hydroxymethylcytosine was 17% of the cytosine bases. Similar DNA preparations have been isolated from rat spleen and mouse and frog brain.

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Materials

Lysozyme (3 × crystallized), 5-hydroxymethylcytosine, 5-hydroxymethyluracil, glutathione (crystalline), cytosine, cytidine, uracil, deoxycholic acid, phenylalanine, glycine, glutamic acid, glycogen, glucose, galactose, fructose, fucose, mannose, ribose, deoxyribose, streptomycin, protamine, calf thymus DNA (type 1), yeast RNA (type XI) and electrophoretically purified deoxyribonuclease I were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Sodium bromide, potassium citrate, sodium arsenate and sodium arsenite were Baker Chemical Co. products, American Chemical Society grade. Ethanol was glass-bottled, Gold Shield, 200 proof, supplied by Commercial Solvents Corp., Terre Haute, Ind., U.S.A. The authors wish to thank Dr. S. S. Cohen, Department of Therapeutic Research, University of Pennsylvania, for the gift of primary standards of 5-hydroxymethylcytosine and 5-hydroxymethyluracil.

The five working solutions A–E required are described in Table 1. At high concentrations of NaBr, as in solution A, the deoxycholate tended to precipitate. The mixture was then evenly suspended by gentle inversion of the vessel before each use. The deoxycholate was nonetheless effective since at lower concentrations of this component in solution A the DNA yield was decreased. The sodium arsenite and GSH solutions were freshly prepared for each experiment. Their pH adjustment was carried out with 1.0M-HCl and 0.5M-NaOH respectively. Iodoacetamide was employed in solution E when the sample was prepared for analytical determinations,

Table 1. *Solutions for preparation of DNA*

Either GSH or iodoacetamide is added to solution E. These reagents are not used together.

Component	Concn. (M)				
	A	B	C	D	E
Sodium bromide	4.5	—	—	2.0	—
Potassium citrate, pH8.6	0.01	0.01	0.01	0.01	0.001
Sodium arsenate	0.01	0.01	0.01	0.01	—
Sodium arsenite, pH8.6	0.01	0.01	0.01	0.01	—
Sodium deoxycholate, pH8.6	0.04%	0.02%	—	0.04%	—
GSH, pH8.6	0.03	0.03	0.003	0.03	0.001
Sodium chloride	—	—	—	—	0.14
Iodoacetamide	—	—	—	—	0.02

GSH was added when it was to be used for biological studies. The two compounds were not used simultaneously in solution E.

Rats of either sex were of the Sprague–Dawley strain. Mice used in these trials were of the ICR strain. Bullfrogs (*Rana catesbiana*) were purchased from Sargent Welsh Scientific Co., New York, N.Y., U.S.A. A small homogenizing pestle that loosely fitted the high-speed centrifuge tubes to be used was a desirable tool. Diameter of the pestle was 0.6 in for the 0.625in diameter tubes employed. Deviations in diameter greater than ±0.005 in decrease the yield of DNA and lower the percentage of 5-hydroxymethylcytosine markedly. This homogenizer was power-driven and should rotate along a straight axis without eccentric motion when in use. It was used in all extractions after the first homogenization. The initial tissue homogenization was performed in a 50ml size vessel with a Teflon pestle (machined to give 0.01 in clearance), purchased from A. H. Thomas, Philadelphia 5, Pa., U.S.A.

Methods

Preparation of lysozyme solution

Lysozyme was dissolved at 50mg/ml, pH5.0, with 0.1M-HCl to adjust the pH. The solution was placed in a boiling-water bath for 20min and then chilled in ice. Bentonite (10mg/ml) was added, the suspension was stirred at room temperature for 10min and then centrifuged for 5min at about 20000*g* at 4°C. The clear supernatant solution was decanted, shaken with an equal volume of butan-1-ol and centrifuged for 20min at 1000*g*. The organic phase and interfacial material were discarded. Traces of butan-1-ol in the lysozyme solution was removed by dialysis in the cold against 1.0mM-potassium citrate, pH5, and the dialysis residue was stored at −15°C.

Analytical methods

The following chromatography systems were used: solvent 1, 0.1M-sodium acetate buffer (pH3.5)–ethanol (1:4, v/v) (Loeb & Cohen, 1959); solvent 2, butan-1-ol–aq. 0.1M-NH_3 (6:1, v/v) (Chargaff *et al.*, 1951); solvent 3, propan-2-ol–conc. HCl–water (170:41:39, by vol.) (Wyatt, 1951); solvent 4, butan-1-ol–water (43:7, v/v) (Markham & Smith, 1949); solvent 5, propan-2-ol–water–conc. aq. NH_3 soln. (sp.gr. 0.88) (85:15:1.3, by vol.) (Hershey *et al.*, 1953); solvent 6, 5M-ammonium acetate (pH9.5)–saturated sodium tetraborate–ethanol (1:4:11, by vol.) (Reichard, 1958); solvent 7, chloroform–methanol–conc. HCl–water (200:100:3:3, by vol.) (Penn & Suwalski, 1969); solvent 8, propan-2-ol–pyridine–water–acetic acid (8:8:4:1, by vol.) (Gordon *et al.*, 1956); solvent 9, ethyl acetate–pyridine–water (5:2:7, by vol.; upper phase) (McFarren *et al.*, 1951); solvent 10, ethyl acetate–acetic acid–water (3:1:3, by vol.) (Jermyn & Isherwood, 1949); solvent 11, phenol–water (43:7, v/v).

(*a*) *Bases.* Formic acid (88%) hydrolysis at 175°C of DNA samples was required to obtain 5-hydroxymethylcytosine (Wyatt & Cohen, 1953). Perchloric acid hydrolysis for these quantities of DNA (0.4–1.0mg) has been described (Penn & Suwalski, 1969). Samples from formic acid or perchloric acid hydrolysates of DNA from one rat brain or 1.0–1.5g of liver were dissolved in 0.1M-HCl and were subjected to chromatography. One-third to one-half of the total products was applied as a 2cm streak when 5-hydroxymethylcytosine was to be determined. Two-dimensional chromatography on Whatman 3MM paper was required for separation of the 5-hydroxymethylcytosine. Solvents 1 and 2 were employed sequentially for this step. The amounts of guanine in such samples were too high for resolution as a discrete spot. To determine the major bases more precisely, 5–10% of the total sample was subjected

to chromatography in duplicate on Whatman no. 1 paper with solvent 3. After chromatography the components were eluted in 0.1M-HCl, extinction peaks were determined in the Beckman model DU and the samples were also read at 250, 260, 280 and 290nm. Values for 5-hydroxymethylcytosine were also obtained for these points in 0.1M-NaOH. The mol percentages of the major bases, including the combined values for cytosine and 5-hydroxymethylcytosine, were derived from the samples analysed in solvent 3, which does not resolve the latter two components. The percentages of 5-hydroxymethylcytosine and, on occasion, 5-methylcytosine, in the total cytosine bases were the results of analyses of the larger samples subjected to chromatography in solvents 1 and 2.

A 6–9% portion of the total sample was taken for determination of phosphorus in triplicate (Norton & Autilio, 1966).

The 5-hydroxymethylcytosine samples from brain and liver DNA were compared with cytosine, cytidine, 5-methylcytosine and 5-hydroxymethylcytosine by rechromatography on Whatman no. 1 paper in solvents 1 to 7 inclusive.

As a further confirmation of the identity of 5-hydroxymethylcytosine, it was converted into 5-hydroxymethyluracil (Wyatt & Cohen, 1953) and separated in two-dimensional chromatography against standards of cytosine, uracil, 5-hydroxymethylcytosine and 5-hydroxymethyluracil in solvents 1 and 2. U.v. spectra of the sample eluates were determined as for the DNA bases.

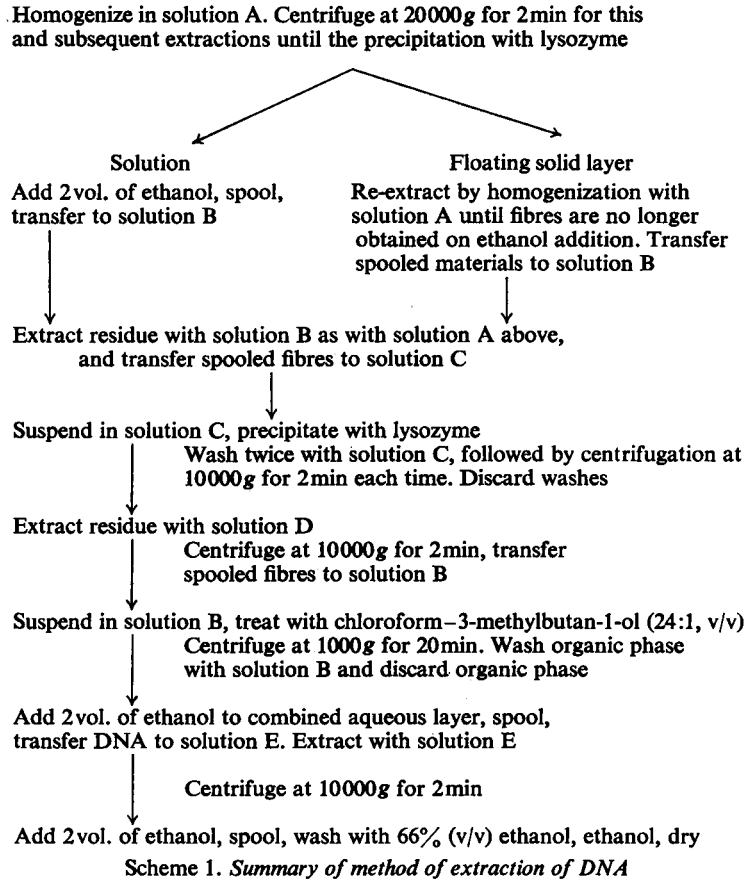
(*b*) *Sugars.* The total yields of DNA from brain (1.5g) or liver (1.0–1.5g) were treated with 1.0ml of 1M-HCl in a boiling-water bath for 1h to liberate ribose from possible RNA contaminants. The solution was diluted with 9ml of water and freeze-dried before chromatography in solvents 8, 9 and 10. Chromatograms were stained with silver nitrate (Trevelyan *et al.*, 1950). Standards of glucose, galactose, fructose, fucose, mannose, ribose and deoxyribose were employed.

(*c*) *Amino acids.* The total DNA samples obtained from brain and liver as in (*b*) were hydrolysed overnight in sealed glass tubes with 1.5ml of 6M-HCl at 125°C. The solutions were dried *in vacuo* over KOH before chromatography with standards of glycine, glutamic acid and phenylalanine in solvent 11. Chromatograms were sprayed with ninhydrin and dried at room temperature.

Preparation of DNA from brain

All operations, unless otherwise specified, were carried out at 0–4°C. Centrifugation was performed at 4°C. Rats of either sex weighing about 150g were killed in a cold-room by guillotine. The brain above the pons, assumed to weigh about 1.5g, was rapidly removed in one piece and homogenized in 10vol. of solution A with a motor-driven pestle. About six to seven up-and-down strokes were employed after the pestle was gently forced through the brain. The suspension was centrifuged at 28000*g* for 2min in the Spinco no. 65 rotor. A fine stirring rod was inserted through the solid surface layer and the underlying solution was decanted. Ethanol (2vol.) was added and the mixture was left without mixing for 4–5min. While the precipitate was forming, the solid layer was re-extracted in the centrifuge tubes by homogenization with 10ml of solution A and centrifuged as before. The DNA precipitate in the original somewhat milky suspension was then spooled with gentle mixing of the phases. At all times in this procedure, the DNA fibres were collected and compacted below the liquid surface before prompt transfer to the next solution. This markedly decreased GSH oxidation and DNA losses. The fibres were then placed in 10ml of solution B in a plastic tube of about 20ml capacity with internal diameter of 0.625in. The floating layer obtained from the second centrifugation was punctured and the solution and solid were treated as before. The process of extraction was continued with collection of DNA in the same 10ml of solution B until no spooled material was obtained after addition of ethanol to solution A supernatant fractions. The crude DNA was extracted into solution B by homogenization and the suspension was treated as for the solution A stage except that a sedimented pellet was extracted after the centrifugations. Fibres were collected in 3ml of solution C and were extracted twice to give a final volume of 5–6ml of solution C.

Lysozyme solution (approx. 0.1–0.15ml/ml of DNA solution) was added dropwise with vigorous stirring. A white precipitate formed before addition was complete. The mixture was allowed to stand for 30–60min with occasional stirring to break up large fibrous or viscous masses. It was then centrifuged at 10000*g* for 2min and the supernatant solution was discarded. The precipitate was washed twice by homogenization in solution C followed by centrifugation. The original supernatant solution and washes gave no detectable reaction with diphenylamine (Dische, 1955). The lysozyme–DNA complex was then extracted into 3ml of solution D by homogenization, centrifuged at 10000*g* for 2min and then was treated as in solution A. The spooled DNA was collected in 3ml of solution B, extracted to give a final volume of about 5ml, and centrifuged at 10000*g* for 2min. The solution was shaken with an equal volume of chloroform–3-methylbutan-1-ol (24:1, v/v) in 12ml glass-stoppered tubes. This size was chosen to minimize air space above the mixture. Shaking should be just vigorous enough to give a well-dispersed emulsion, to minimize oxidation of GSH. The mixture was centrifuged at 1000*g* for



Scheme 1. *Summary of method of extraction of DNA*

20min and the aqueous phase was removed. The organic phase was washed with 2ml of fresh solution B and the aqueous phases were combined. Ethanol was added to the aqueous solution, the spooled DNA was transferred to 3ml of solution E and extracted, with centrifuging at 1000*g* for 2min. DNA was precipitated by ethanol addition, washed in 66% (v/v) ethanol and then in ethanol. It was dried *in vacuo* over a mixture of NaOH and $CaCl_2$, stored overnight at −15°C and analysed. The method is summarized in Scheme 1.

Mice were killed by cervical dislocation and four to five brains were combined as a single sample in a chilled pre-weighed container of solution A. Total weight was determined promptly and the procedure was then the same as for rat brain. Frogs were decapitated by guillotine and three to four brains were combined as one sample. The same method was employed.

Preparation of DNA from liver

Rats were decapitated by guillotine and 1.0–1.5g of liver was placed in 3–5ml of pre-chilled, measured solution A. The tissue was rapidly minced and transferred to the homogenizer. Solution A was then added to give a ratio of 10:1 (v/w) and the tissue was homogenized. The procedure described for brain was then employed.

Isolation of 5-hydroxymethylcytosine from brain RNA: 2′,3′-cytidylic acids

After hydrolysis in 0.1M-KOH–25% dioxan at 37°C for 22h, DNA and protein were removed by precipitation with trichloroacetic acid (Penn & Suwalski, 1969). The samples were then extracted with ether until they were neutral. Ether was removed by warming under a stream of N_2 and the aqueous solutions were freeze-dried. The samples were then hydrolysed again overnight in 0.3M-KOH at 37°C to complete conversion of polynucleotides into mononucleotides. The solutions were neutralized in ice with perchloric acid and $KClO_4$ was removed by centrifugation. The sediment was washed twice with ice-cold water; the original supernatant solution and washes were combined and freeze-dried. The crude

cytidylic acids were isolated by chromatography in solvent 3, eluted and freeze-dried.

Isolation of 5-hydroxymethylcytosine from liver RNA: 2',3'-cytidylic acids

Rats weighing about 150g were decapitated and the tissue was removed, weighed in a pre-chilled container with a small amount of 5% (w/v) perchloric acid and rapidly minced with scissors. The mixture was transferred to a homogenizer, 5% perchloric acid was added to give a ratio of 10:1 (v/w) and the tissue was homogenized. The suspension was centrifuged for 10min at 12000*g* and washed twice with fresh 5% perchloric acid equal in volume each time to that employed for homogenization. Water (3–4ml) was added to the residue, which was adjusted to approx. pH8 with KOH and hydrolysed overnight in 0.3M-KOH at 37°C. The samples were neutralized with perchloric acid, brought to a final concentration of 5% perchloric acid and the DNA fraction and salts were removed by centrifugation. The residue was washed twice with 5% perchloric acid and the washes were combined with the original supernatant solution. The solution was neutralized with KOH. The $KClO_4$ was removed by centrifugation and washed twice with cold water. The combined supernatant solution and washes were then treated according to the procedure for the corresponding brain sample.

5-Hydroxymethylcytosine from cytidylic acids

The freeze-dried eluates of cytidylic acids from either brain or liver RNA were washed out of the vessels with small amounts of formic acid to give a final volume of about 1.0ml, hydrolysed by the method for DNA cited above and subjected to chromatography in solvents 1 and 2.

Preparations of DNA from brain and liver have also been carried out by standard methods for the latter tissue (Kirby, 1968) and by a modified phenol procedure for the former.

Results and Discussion

The DNA preparations isolated from brain and liver gave a positive diphenylamine reaction, their solutions were highly viscous, viscosity was markedly decreased by electrophoretically purified deoxyribonuclease, the major bases were adenine, guanine, cytosine and thymine, the ratios agreed with values for rat DNA (Wyatt, 1951) and the phosphorus/base ratios were approximately unity (Table 2). All calculations of DNA yield were based on a thymine content of 11% (Zamenhof *et al.*, 1964). The DNA obtained ranged from 0.4 to 1.0mg in brain and about 0.7mg in liver/g wet wt.

Table 2. *Composition of rat DNA*

Formic acid hydrolysates were subjected to chromatography in duplicate, phosphorus determinations were performed in triplicate. Determinations were made on three preparations. Deviations from means, ±3%.

	Content (mol/100mol of DNA)		
	Brain	Liver	Wyatt (1951)
Adenine	28.9	28.8	28.6
Guanine	21.1	21.5	21.4
Cytosine	18.5	17.7	20.4
5-Hydroxymethylcytosine	3.3	3.6	—
5-Methylcytosine*	—	—	1.1
Thymine	28.1	28.2	28.4
Ratio P/base	1.01	1.03	1.01

* This component is observed only in brain DNA samples at a yield of 1.0mg of DNA/g wet wt. At the lower yields usually obtained it is not detectable.

Identification of 5-hydroxymethylcytosine was based on its identity with standards in chromatography solvents 1–7 (Table 3), spectrophotometric constants in 0.1M acid and alkaline solutions (Table 4) and preparation of 5-hydroxymethyluracil (Wyatt & Cohen, 1953) from pooled samples of 5-hydroxymethylcytosine after rechromatography. The derivative was compared with cytosine, uracil, 5-hydroxymethylcytosine and a reference standard of 5-hydroxymethyluracil by chromatography systems 1 and 2. It was not detectable in pooled starting materials. The standard separated poorly from cytosine but the extinction peaks at pH1, 261nm and 276nm respectively, sharply differentiated the two compounds (Table 5).

Table 2 shows that the sum of the molar percentages of cytosine and 5-hydroxymethylcytosine is required for reasonable correspondence to the values for guanine, indicating that the 5-hydroxymethylcytosine is in fact a DNA component. Although the percentages of 5-hydroxymethylcytosine are similar in the DNA fractions isolated from brain and liver, the relative yields, 0.8mg and 0.7mg of DNA/g respectively, indicate a substantially higher content of 5-hydroxymethylcytosine in brain DNA. There was reported to be about 1.25mg of DNA/g wet wt. in brain (Penn & Suwalski, 1969) and at least 2.0mg/g in liver (Kirby, 1961).

The presence of 5-hydroxymethylcytosine in DNA suggested an examination of RNA for this base. The percentage of 5-hydroxymethylcytosine in the cytidylic acid fractions obtained after alkaline hydrolysis

Table 3. *Chromatography of 5-hydroxymethylcytosine from brain and liver DNA*

See the text for details of chromatography solvent systems.

	R_F			R_F of unknown	
Solvent	Cytosine	5-Hydroxymethylcytosine	5-Methylcytosine	Brain	Liver
1	0.47	0.41	0.54	0.40	0.39
2	0.28	0.12	0.36	0.11	0.09
3	0.43	0.43	0.53	0.43	0.40
4	0.19	0.11	0.24	0.09	0.10
5	0.38	0.27	0.42	0.26	0.25
6	0.56	0.47	0.59	0.44	0.49
7	0.17	0.10	0.31	0.09	0.09

Table 4. *Spectrophotometric results for 5-hydroxymethylcytosine from brain and liver DNA*

Tissue	Solvent		Peak (nm)	E_{250}/E_{260}	E_{280}/E_{260}	E_{290}/E_{260}
Brain	0.1 M-HCl	Standard	279.5	0.45	2.05	1.51
		Experimental	279.5	0.46	2.04	1.54
	0.1 M-KOH	Standard	283	0.81	2.9	2.58
		Experimental	283	0.87	3.1	2.81
Liver	0.1 M-HCl	Standard	279.5	0.45	2.05	1.51
		Experimental	280	0.47	2.08	1.58
	0.1 M-KOH	Standard	283	0.81	2.9	2.58
		Experimental	282	0.86	3.2	2.72

of RNA from brain and liver is given in Table 6. A very high percentage of 5-hydroxymethylcytosine appeared to be present in the crude RNA fraction of brain. Such a conclusion would, however, assume that 5-hydroxymethylcytidylic acid and cytidylic acid were equally susceptible to hydrolysis with formic acid. Since it is not definitely established that the percentage yield of free base from each of these compounds is the same, the value of 42% is viewed as a provisional maximum for the 5-hydroxymethylcytosine content in cytosines from crude RNA of the central nervous system. The relative amount of 5-hydroxymethylcytosine in brain RNA was nonetheless significantly greater than in liver RNA. It is apparent that a substantial contamination of brain DNA by RNA would raise doubts as to which nucleic acid was the source of 5-hydroxymethylcytosine.

Examination of the analytical methods of choice for RNA indicates that 5-hydroxymethylcytosine would not be detected by these procedures. The 2′,3′,5-hydroxymethylcytidylic acid does not separate from the corresponding cytidylic acid in the standard solvent 3, propan-2-ol–HCl. The pK values of the amino groups of cytosine and 5-hydroxymethylcytosine, 4.45 (Shugar & Fox, 1952) and 4.3 (Fissekis *et al.*, 1964) respectively, are too close to permit clear electrophoretic separation at the pH values (2–5) customarily employed for separation of the products of the alkaline hydrolysis of RNA (Smith, 1955).

Previous failure to observe 5-hydroxymethylcytosine in DNA preparations from animal tissue may have been due to the coincidence of this component with cytosine in several chromatographic systems. However, it appears that enzymic or chemical degradation, or both, of the DNA fraction containing 5-hydroxymethylcytosine during the isolation procedures is primarily responsible, as shown by experiments with a standard phenol procedure (Kirby, 1968). If a portion of the crude nucleic acids is precipitated and spooled from the aqueous phase after the first centrifugation, 5-hydroxymethylcytosine is present in the mixture of DNA and RNA fibres. Formic acid hydrolysis of this crude spooled material followed by chromatography together with 5-hydroxymethylcytosine and cytosine standards in solvents 1 and 2 gave a considerable amount of degraded material that partially obscured the products. Elution and rechromatography in the same systems showed 5-hydroxymethylcytosine to be present to the extent of about 20% of the total cytosine bases in brain and 6% in liver. Analysis of the nucleic acid mixture at the next precipitation from ethanol–cresol revealed no detectable

Table 5. *Production of 5-hydroxymethyluracil from brain and liver 5-hydroxymethylcytosine*

	R_F values					
	Sample					
Solvent	Brain	Liver	5-Hydroxymethyluracil	Cytosine	5-Hydroxymethylcytosine	Uracil
1	0.50	0.52	0.53	0.54	0.40	0.61
2	0.19	0.18	0.20	0.24	0.12	0.31
Extinction peaks (nm) at pH1	261	262	261	276	279.5	260

Table 6. *5-Hydroxymethylcytosine content of crude rat cytidylic acids from RNA*

The cytidylic acids were isolated as described in the text. Chromatography of formic acid hydrolysates was performed in duplicate. Values are means of samples from three animals. Overall variation is ±6%.

	Concn. (μmol/g)	
	Brain	Liver
Cytosine	0.11	0.8
5-Hydroxymethylcytosine	0.082	0.027

5-hydroxymethylcytosine. It appears possible that denaturation of all degradative enzymes may not be immediate on homogenization of tissue in phenol–cresol, but becomes complete with removal of tissue components during continued purification. These results further support the possibility that 5-hydroxymethylcytosine is present in the DNA isolated by the alternative preparative method of this report.

In some tissues the DNA species containing 5-hydroxymethylcytosine may also be lost owing to an additional lability towards the standard preparative reagents. Brain and liver DNA preparations obtained by the NaBr method were processed through the first step of the cited phenol procedure. Analysis of treated and untreated samples indicated negligible effect on liver DNA, but there was marked degradation of the brain DNA (Table 7).

It was possible to stabilize brain DNA to a limited extent in the presence of phenol. The addition of GSH, arsenate and arsenite in the stated concentrations to all solutions employed in the phenol procedure was found to permit occasional isolation of DNA with 5-hydroxymethylcytosine as 1–3% of the total cytosine bases. Further experimentation indicated that a separate group of inhibitors was required before this small percentage was consistently

Table 7. *Effect of phenol treatment on DNA preparations obtained by the sodium bromide method*

Samples were isolated from NaBr homogenates as described in the text, and then treated by the phenol procedure up to stage of first alcohol precipitation.

	Content (mol/100 mol of DNA)	
	Brain	Liver
Adenine	33.9	28.1
Guanine	23.7	21.3
Cytosine	20.7	18.1
5-Hydroxymethylcytosine	—	3.4
Thymine	21.4	28.8
Percentage loss after treatment	41	6.3

found. This procedure was cumbersome and appeared to offer no advantage over the method described here.

The DNA isolated by the method described was reasonably free of the usual major contaminants. There was, however, an unknown u.v.-absorbing component found in all DNA preparations from rat, mouse and frog. The material was absent in perchloric acid hydrolysates and it may have been an adventitious contaminant. Its R_F values in solvents 1 and 2 were 0.60 and 0.18 respectively. The extinction peaks were 264nm at pH1 and 263nm at pH13.

RNA was essentially absent from these preparations. On occasion, ribose was barely detectable after treatment with silver nitrate of chromatograms of 1M-HCl hydrolysates of brain or liver DNA, indicating a maximum contamination by RNA of about 0.5% depending on the individual preparation. It was frequently not detectable. The observed concentration of 5-hydroxymethylcytosine in the brain DNA samples thus could not be ascribed to RNA,

Table 8. *Comparison of perchloric acid and formic acid hydrolysis of brain and liver DNA preparations*

Samples were divided in half before hydrolysis and chromatographed in duplicate.

	Content (mol/100 mol of DNA)			
	Brain		Liver	
	Formic acid	Perchloric acid	Formic acid	Perchloric acid
Adenine	28.5	28.8	28.9	29.1
Guanine	21.1	22.5	21.4	21.7
Cytosine	18.7	19.8	17.3	19.1
5-Hydroxymethylcytosine	3.2	—	3.5	—
Thymine	28.8	29.1	28.3	29.7

Table 9. *Content of 5-hydroxymethylcytosine in the brain of mouse and bullfrog*

Values are averages of two determinations, each performed in duplicate. Variation from means ±4%.

	Content (mol/100 mol of DNA)	
	Mouse	Bullfrog
Adenine	28.4	27.8
Guanine	22.5	22.4
Cytosine	18.8	20.0
5-Hydroxymethylcytosine	2.2	1.28
5-Methylcytosine	—	1.25
Thymine	27.9	27.1

which may have a high percentage of this base. In chromatograms of hydrolysates of 300–500 μg of DNA from brain or liver, no uracil was detectable under u.v. light.

There was no significant protein contamination of DNA obtained from either liver or brain. When chromatograms of the total product from about 1.0 mg of DNA after 6 M-HCl hydrolysis were sprayed with ninhydrin, they showed only two definite spots with R_F values of glycine and phenylalanine in solvent 11.

Glycogen was removed in the supernatant solutions of the lysozyme–DNA complex after centrifugation. Control experiments with commercial glycogen showed that it was not precipitated by lysozyme and it did not sediment at the centrifugal forces employed to remove and wash the lysozyme–DNA complex. It was soluble in the solution C employed for the washes of the insoluble lysozyme–DNA complex. The first supernatant solution of the liver DNA–lysozyme precipitate was opalescent, whereas that from brain DNA was relatively clear.

Concentrated perchloric acid hydrolysis of liver and brain DNA samples resulted in the disappearance of 5-hydroxymethylcytosine (Table 8), a result similar to that reported with T-even bacteriophage DNA (Wyatt & Cohen, 1953). The initial observation suggested, by analogy to the findings on viral DNA, not only that 5-hydroxymethylcytosine might be present in brain DNA but that glucose (Loeb & Cohen, 1959) or another sugar component might be combined with this base. This possibility was supported by the appearance of a component on chromatograms of the 1 M-HCl hydrolysates, which corresponded to the glucose standards in solvents 8, 9 and 10. Proof of these inferred linkages will require isolation of the presumptive glucose compound of the deoxynucleotide of 5-hydroxymethylcytosine from these animal tissues.

In addition to rat brain and liver, two-dimensional chromatograms of hydrolysates of spleen DNA, prepared as described, showed a component with the R_F values and spectrophotometric characteristics of 5-hydroxymethylcytosine. It constituted 5–7% of the total cytosine bases. Spleen did not give any solid residue or floating layer on homogenization in solution A, but this step must still be followed in application of the method.

The occurrence of 5-hydroxymethylcytosine was not a peculiarity of the rat strain of this laboratory colony. It was also present in brain obtained from mice of the ICR strain and in the bullfrog brain (Table 9).

Control experiments showed that the appearance of 5-hydroxymethylcytosine in these DNA preparations was not an artifact, nor was it detectable in other DNA samples. Repeated trials with commercial calf thymus DNA revealed no trace of this component when up to 2.0 mg of hydrolysate was subjected to chromatography. Analysis of rat liver DNA prepared by standard methods (Kirby, 1968)

also failed to give 5-hydroxymethylcytosine. The presence of 5-hydroxymethylcytosine was not due to some interaction between unusual trace impurities remaining in the DNA and cytosine residues. The total amount of 5-hydroxymethylcytosine was not changed when commercial calf thymus DNA and rat brain DNA were mixed before formic acid hydrolysis. As a further test, 1.0mg of thymus DNA (P content 8.3%) was added to one-half the NaBr homogenate of two rat brains and the portions were processed independently. The fraction containing the added DNA was found to have 7.1% more 5-hydroxymethylcytosine and 61% more cytosine. The difference in 5-hydroxymethylcytosine content is well within experimental variation, indicating it did not arise during the isolation procedure.

The introduction of lysozyme afforded a more highly purified product. Its use is not obligatory and it may be omitted for the sake of speed if there is no objection to a somewhat contaminated material. Its omission resulted in a yield of about 1mg/g wet wt. of brain. In such samples 5-hydroxymethylcytosine constituted about 18% of the total cytosine bases. These small increases were apparently due to the shorter period of treatment. It had been found that an effective separation of commercial DNA from RNA through their lysozyme complexes could be achieved by adjustment of salt concentration. The RNA complex is soluble in 0.15M-NaCl, the DNA complex is insoluble. It was, however, found that the conditions for precipitation or separation established in these control trials were not applicable to crude products from tissue. The use of lysozyme affords several advantages in the treatment of tissue nucleic acid extracts: it offers the possibility of a rapid separation of a major part of the RNA from DNA when the specific requirements of the given sample are established; an insoluble complex with DNA that can be extensively washed with dilute salt solutions is formed rapidly and quantitatively; the DNA content of the precipitate can be directly assayed by the diphenylamine reaction since the lysozyme does not interfere; the complex with commercial DNA is readily soluble in moderately concentrated salt solutions such as 0.2M-NaCl or 0.1M-$MgSO_4$; the treated lysozyme does not precipitate on addition of 2vol. of ethanol; glycogen does not precipitate with lysozyme, thus facilitating removal of this carbohydrate by simple centrifugation; the DNA–lysozyme complex can be sedimented quantitatively at 1000*g* for 10–15min, offering a variety of centrifugation schedules for purification from unusual contaminants. Investigation of the reaction between protamine or streptomycin (Cohen & Lichtenstein, 1960) and commercially available nucleic acids indicates that these reagents are not as serviceable as lysozyme in this procedure. The lysozyme must be purified as specified in the Methods section to remove interfering materials, which frequently are present.

Initial attempts to apply the NaBr method to the preparation of DNA from small amounts of total brain tissue gave a degraded product in poor yield. Addition of arsenite improved the yield but adenine/thymine molar ratios in such preparations ranged from 1.6 to 5. The presence of the thiol group in certain deoxyribonucleases (Price *et al.*, 1969) led to the addition of iodoacetamide as a possible inhibitor. Correct base ratios can be obtained with this reagent, but P/base ratios (about 1.3) showed a phosphorus-containing impurity to be present. The possibility that a phosphopeptide might account for these values suggested the use of GSH or cysteine at a concentration great enough to inhibit a thiol-containing enzyme and to displace the hypothetical peptide. GSH gave the greater yields of DNA, higher 5-hydroxymethylcytosine percentages and acceptable P/base ratios.

There appear to be several species of brain as well as liver DNA whose isolation is dependent on the experimental route employed. 5-Methylcytosine was not detectable in preparations originally obtained by the large-scale NaBr method (Emanuel & Chaikoff, 1960). The present results confirm that, at a DNA yield of about 0.8mg/g in rat brain, 5-methylcytosine is not visible on chromatograms of hydrolysates. It is possible to identify and measure this base at the infrequent yield of about 1.0mg/g and it appears that at this yield another minor species of brain DNA containing 5-methylcytosine with, or in addition to, 5-hydroxymethylcytosine has been isolated.

The occurrence of 5-hydroxymethylcytosine can no longer be considered a biochemical oddity limited to the T-even bacteriophages (Cohen, 1968), since it is found in the DNA of rat, mouse and frog brain and in rat liver and spleen. Its role is not established, but there is the possibility of a glucosylated form in these DNA preparations suggesting a similarity to the glucosylated 5-hydroxymethylcytosine of bacteriophage DNA (Loeb & Cohen, 1959). The high concentration of 5-hydroxymethylcytosine in brain nucleic acids may possibly bear a relation to the central nervous system's primary metabolic dependence on glucose.

The results presented therefore indicate that 5-methylcytosine as well as 5-hydroxymethylcytosine or the molecular species that contain these bases are lost by current methods for preparing DNA from brain. The possibility therefore exists that other components may also be lost from DNA during isolation. The native structure thus could be more complex than current concept suggests.

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