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Quantitative Determination of Deoxyribonucleic Acid in Rat Brain

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1. A procedure is given for spectrophotometric analysis of rat brain DNA after its resolution into component bases. Amounts of tissue in the range 50–100mg. can be used. 2. The amount of DNA obtained by the present method is 80% greater than that reported for rat brain by a previous procedure specific for DNA thymine. Identity of the material is established by the base ratios of purines and pyrimidines. The features responsible for the higher yield are the presence of dioxan during alkaline hydrolysis of tissue, the determination of the optimum concentration of potassium hydroxide in this step and omission of organic washes of the initial acid-precipitated residues. 3. The requirement for dioxan during alkaline hydrolysis suggests a possible association of brain DNA with lipid. The concentration of potassium hydroxide that gives maximum yield is 0·1M, indicating that there may be internucleotide linkages in this DNA that are more sensitive to alkali than those of liver or thymus DNA. 4. This procedure gives low yields of DNA from liver. It is not suitable for analysis of the DNA from this tissue.

As a basis for the study of brain nucleic acid metabolism in this laboratory, quantitative methods for determination of DNA and RNA were required. In view of the projected experiments the necessary features of such procedures were maximal recovery of nucleic acid from tissue, separation of RNA from DNA, spectrophotometric establishment of the purity of the bases after their isolation and convenience in processing a number of relatively small tissue samples.

Analysis of the nucleic acids of brain has presented difficulties that have led to the development of a number of special procedures (Zamenhof, Bursztyn, Rich & Zamenhof, 1964; Hess & Thalheimer, 1963; Kissane & Robins, 1958). Examination of these indicated that some modifications were needed for our purposes. The singular metabolic characteristics of brain, as shown by virtual absence of cell division in the adult, are not reflected in gross structural differences of its DNA (Emanuel & Chaikoff, 1960). It therefore seemed advisable to be able to examine the base ratios of brain DNA under various experimental conditions. The present paper deals with a method for DNA determination in rat brain that satisfies the above requirements and that has revealed a concentration of DNA in rat brain at least 80% higher than the estimate of 0·7mg./g. wet wt. made by Zamenhof *et al.* (1964). The procedure is based on modifications of the method of Schmidt & Thannhauser (1945) in this and other laboratories (Shibko, Koivistoinen, Tratnyek, Newhall & Friedman, 1967). The original Schmidt–Thannhauser procedure could be employed to give DNA concentrations in fair agreement with those previously reported in rat brain provided that a somewhat different pattern of organic washes was adopted.

MATERIALS AND METHODS

Modified procedures for determination of both brain and liver DNA are given, since the method for latter may be useful as a basis for the determination of DNA in other tissues.

Rats bred in this laboratory from the Sprague–Dawley strain were used. Operations before chromatography, unless otherwise noted, were carried out in the cold. Animals of either sex weighing about 150g. were decapitated by guillotine. The brain above the pons, or the liver, was rapidly removed and placed in tared containers that held an amount of chilled 5% (w/v) trichloroacetic acid sufficient to cover the sample. The maximum time required for removal and immersion of the brain tissue was 1·5min. Weights were noted and the tissues homogenized in 10ml. of 5% trichloroacetic acid/g. of tissue. After being centrifuged for $1{\cdot}8\times10^5g$-min., the supernatants were discarded, the pellets were washed twice with 5% trichloroacetic acid (10ml./g. initial wet wt. of tissue) and either of the following procedures was adopted.

(*A*) Modified procedure. A small amount of water (about 1ml./g. original wet wt.) was added to the brain samples to make a slurry. They were brought to neutrality (Hydrion paper) with KOH, then adjusted to pH $10\pm0{\cdot}2$ and hydrolysed at 37° for 22hr. in 0·1M-KOH in 25% (v/v) dioxan

(Spectroquality; Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.), 10ml./g. initial wet wt. of tissue.

(*B*) Procedure with solvent extraction. The liver precipitates were washed with organic solvents, 10vol./g. initial wet wt. It was necessary to adhere to the following order of solvents for maximum yield: acetone, ethyl acetate, hexane, chloroform–methanol (1:1, v/v), ether, ethanol. Liver samples were then dried under vacuum and hydrolysed at 37° for 18hr. in 1M-KOH, 10ml./g. initial wet wt. of tissue. Brain tissue treated in this way gave considerably lower yields compared with method *A*.

Samples treated as described in procedures *A* or *B* were then neutralized, made 5% with respect to trichloroacetic acid, left for 20min., centrifuged and washed twice with 5% trichloroacetic acid. The combined supernatants and washes were sometimes reserved for RNA analysis. The precipitates, containing the denatured DNA, protein and other tissue residues were neutralized with aq. NH_3 (sp.gr. 0·88) and 0·1M-NH_3 was added (7·5ml./g. initial wet wt. of tissue). The suspensions were left for 10min. with occasional stirring and then centrifuged at 1000*g* for 15min. The turbid supernatant fractions were reserved and the residues were washed twice with 0·1M-NH_3 (5ml./g.). The washes were combined with the respective supernatant suspensions and freeze-dried. The dry samples were hydrolysed with 12M-$HClO_4$ (0·7–1·0ml./g. initial wet wt.) in a boiling-water bath for 1hr. (Marshak, 1951). The samples were diluted with water, neutralized with KOH and centrifuged. The dark supernatants were reserved and the $KClO_4$ residues were washed twice with chilled 0·1M-HCl and then discarded. The combined supernatants and washes were freeze-dried, then dissolved in cold 0·1M-HCl and chromatographed in duplicate. The wet-weight equivalent of 100mg. of tissue was the standard amount taken from the sample, assuming a subsequent elution volume of 4ml. The dry samples could be stored at room temperature in a desiccator, but it was found advisable to proceed with chromatography directly on solution. During storage in solution the degraded materials tend to adsorb the bases, as noted (L. Levenbook, unpublished work, cited by Wyatt, 1955).

Measurement of radioactivity. [6-^{3}H]Thymidine (500μc) was injected intraperitoneally into each of three rats, which were killed 1hr. later. The brains were removed and homogenized in 5% trichloroacetic acid. One half of each homogenate was processed by the modified procedure *A* described in this paper, the other by procedure *B*. When procedure *B* was used the homogenates were centrifuged and the residues were washed twice with 5% trichloroacetic acid, then with acetone, ethyl acetate, ethanol–ether (3:1, v/v) at 50° for 15min., chloroform–methanol (1:1, v/v), ether and ethanol. In view of the high lipid concentration in brain, an ethanol–ether extraction is substituted for the hexane in procedure *B* as applied to liver. The residues were dried and then hydrolysed for 18hr. in 1·0M-KOH at 37°. The hydrolysates were then neutralized, acidified and processed to yield DNA bases as described above.

After chromatography, thymine and its blanks were eluted and thymine was determined spectrophotometrically. Eluates were freeze-dried in counting vials. Cytosine, adenine and guanine spots and their corresponding blanks were also eluted and freeze-dried. Water (0·5ml.) and 15ml. of phosphor solution [1000ml. of toluene, 600ml. of ethylene glycol, 7·5g. of 2,5-diphenyloxazole and 500mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene] were added to each vial and the radioactivity of the samples was then measured in a Packard Tri-Carb scintillation counter.

In a separate trial of recovery efficiency, portions of stock [6-^{3}H]thymidine were added to unlabelled samples obtained by method *A* before hydrolysis with $HClO_4$. These were carried through hydrolysis and chromatography. The eluates of thymine were then freeze-dried and the radioactivity was measured as described above. The radioactivity of [6-^{3}H]thymidine samples from stock was measured at the same time for comparison.

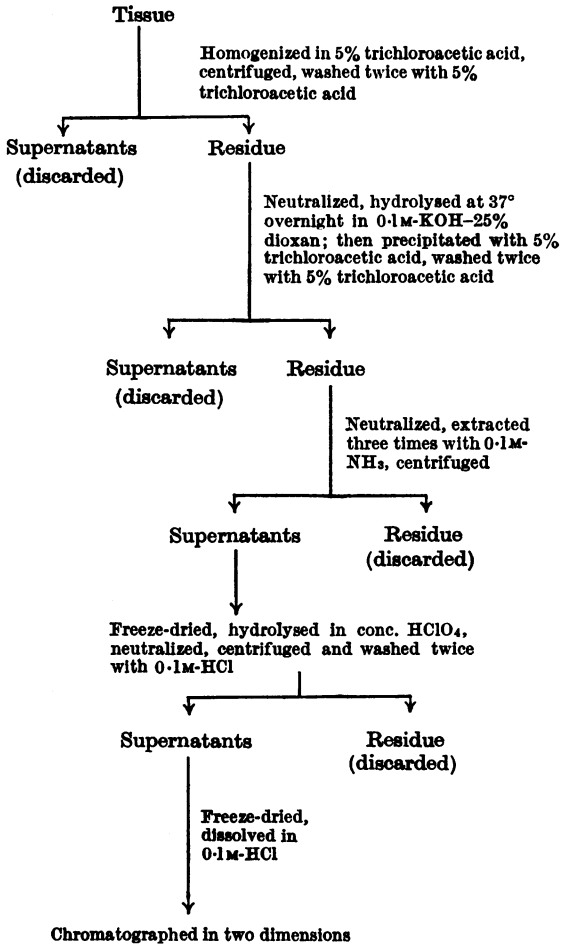
The radioactivity of samples was measured to within 5% accuracy and corrected for quenching by using internal standards.

Chromatography. Two-dimensional ascending chromatography on Whatman no. 1 paper was employed, with the following systems used in order. (1) Chloroform–methanol–conc. HCl–water (200:100:3:3, by vol.). Satisfactory results were obtained with U.S.P. grade chloroform (Matheson, Coleman and Bell); higher-quality reagent does not give good separation of the bases, presumably because of the absence of the alcohol stabilizer. The water content of this solvent system is critical and tanks must be dry before use. At temperatures below 18° serious streaking occurred. R_F values (ascending) were: guanine, 0·10: adenine, 0·26: cytosine, 0·27; thymine, 0·64; uracil, 0·59. Only the movement of thymine was affected by the degraded material, increasing when the amount of the latter was greater. Otherwise samples and standards showed concordant values. Solvent was allowed to run a minimum of 15cm. before removal of sheets. A fluorescent channel of degraded material from the point of application to the solvent front usually masked nucleic acid components. (2) Butan-1-ol–0·1M-NH_3 (6:1, v/v) (Chargaff, Lipshitz, Green & Hodes, 1951). The individual bases were well separated from each other in the second dimension, although an occasional repeated development in the second system was needed for movement of guanine out of the fluorescent region. The bases were eluted in 4ml. of 0·1M-HCl and read in the Beckman model DU spectrophotometer at their respective peaks and at 250, 260, 280 and 300nm. (Bendich, 1957). Two wavelength corrections were sometimes required for thymine and guanine, as indicated by variations in the extinction ratios from the literature values. The quality of DNA/g. wet wt. of brain was calculated from the amount of thymine, taken as 11% (Zamenhof *et al.* 1964). The overall procedure for brain is shown in Scheme 1.

Diphenylamine determinations (Dische, 1955) were carried out on samples freeze-dried from the 0·1M-NH_3 extracts. Commercial DNA (Mann Research Laboratories, New York, N.Y., U.S.A.) with a P content of 7·3% was used as a standard.

RESULTS

Absorption peaks obtained from sample components corresponded to the maxima indicated by comparisons of the R_F values with standards. Extinction ratios usually did not vary by more than 4% from literature values (Bendich, 1957). Uracil standards were also well separated in the two-dimensional ascending systems employed, but this component was absent from tissue samples even when portions corresponding to 1g. wet wt.



Scheme 1. Flow chart for determination of DNA in rat brain.

were chromatographed. When 0·03M- or 0·06M-potassium hydroxide was used in hydrolysis, uracil was detectable. The base ratios obtained by the method given (Table 1) showed reasonable correspondence to those reported for rat DNA (Comb, Sarkar, DeVallett & Pinzino, 1965).

The omission of dioxan resulted in considerable variation in both yield and base ratios of brain DNA, and no significant average values can be presented. About 30–50% less DNA was isolated in its absence. In the presence of dioxan a reproducible high yield was obtained. The concentration of potassium hydroxide had to be carefully controlled, since 0·1M was found to be a fairly sharp optimum concentration (Fig. 1).

Some experimental observations on the procedure may be useful. It was found that trichloroacetic acid and perchloric acid are not equivalent in effect. Much lower concentrations of DNA were found when dilute perchloric acid was employed at any point and were accompanied by erratic base ratios. Concentration in a shaking or rotating evaporator rather than by freeze-drying usually led to an increase in amount of degraded material that was unmanageable by the chromatography systems described. The low-speed centrifugation specified for ammonia extraction of DNA was necessary, since centrifugation at high speeds decreased the yield markedly but produced only a moderate clarification of the turbid supernatant. No method has been found that completely removes the interfering degraded material. It was not significantly affected by the organic washes of the Schmidt & Thannhauser (1945) procedure, or by organic washes subsequent to hydrolysis with concentrated perchloric acid. If Norit activated

Table 1. *Yield and base content of rat DNA*

Tissues were processed by methods *A* or *B* as described in the Materials and Methods section. Results are means of determinations from five animals, the samples being chromatographed in duplicate. Individual values deviated from the means by no more than 5%. Literature values are given in parentheses.

		Base composition (moles/100 moles)				
Procedure	Tissue	A	G	C	T	Yield (mg./g. wet wt. of tissues)
A	Brain	29·0	20·7	21·0	29·5	1·25 (0·7†)
B	Brain	29·5	20·7	20·3	29·7	0·73
B	Liver	28·8	21·6	20·9	28·4	1·7 (2·0‡)
Rat DNA		(28·6*)	(21·4*)	(21·5*)	(28·4*)	

* Comb *et al.* (1965).
† Zamenhof *et al.* (1964).
‡ Kirby (1961).

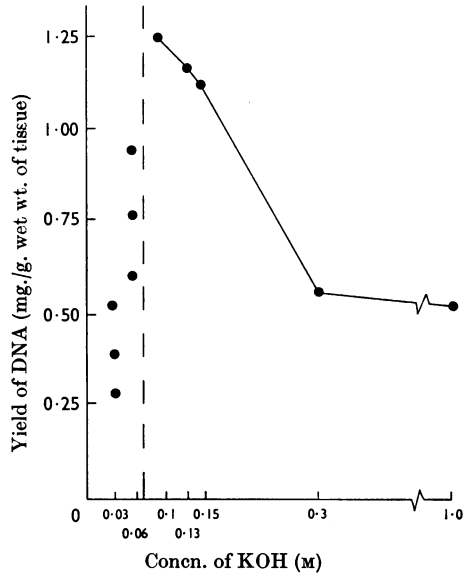


Fig. 1. Effect of alkali concentration on the yield of DNA from rat brain. From 0·1M- to 1·0M-KOH each point represents an average of results from three animals determined in duplicate; variation from means was less than 5% in the range 0·1–0·15M-KOH and less than 8% at 0·3M and 1·0M. Below 0·1M-KOH each point shows the results from one animal. Each KOH solution contains 25% (v/v) of dioxan. At alkali concentrations below 0·1M-KOH, indicated by the broken line, there is RNA contamination of the DNA fraction, indicated by the appearance of uracil on the chromatograms.

charcoal was used to remove this material (Tsuboi & Price, 1959) there were major losses, particularly of guanine. This difficulty was not limited to samples from brain; liver DNA fractions gave the same dark final product. Differential centrifugation of the nuclear fraction through sucrose (Khan & Wilson, 1965) decreased the amount of interfering material, but gave lower yields of DNA, with A/G or T/C ratios varying in the range 1·5–1·7. Solubilization with trichloroacetic acid or perchloric acid gave variable diminished yields with erratic base ratios. The lower yield obtained with procedure *B* applied to brain apparently was not due to losses in the organic-solvent washes; these were uniformly diphenylamine-negative. If the extraction with ammonia was omitted, the base ratios for guanine, cytosine and thymine were unaffected but the amount of adenine was about 100% too high. Since the same result was obtained with liver DNA fractions, this adenine component is not peculiar to brain.

The appreciable variation in the diphenylamine values for brain DNA commented on by Zamenhof *et al.* (1964) is considerably diminished by using procedure *A*, including the extraction with ammonia. Occasional difficulty is encountered in the appearance of cloudy samples after the reaction; these can usually be clarified by the addition of ethanol. Comparison of this base-analysis method with determinations carried out by using the diphenylamine reaction (Dische, 1955) shows fair agreement (Table 2).

Procedure *B* was investigated first and appeared to give satisfactory results for brain DNA content. During this work a more rapid analysis for nucleic acids from liver was reported (Shibko *et al.* 1967). Attempts to apply this method to brain gave exceedingly poor yields of DNA. Dioxan was found to facilitate reaction in the aqueous suspension of brain lipid and protein and markedly improved the recovery of DNA. Examination of various potassium hydroxide concentrations showed 0·1M to be optimum.

The absence of significant RNA contamination was indicated by the DNA base ratios and the absence of uracil. In the range 0·03–0·06M-potassium hydroxide in alkaline hydrolysis, uracil was detectable, accompanied by erratic base ratios.

Although uracil is poorly separated from thymine in system 1 (R_F 0·59 and 0·64 respectively) it is readily distinguished from it in system 2 (R_F 0·39 and 0·53 respectively).

In view of the higher concentration of DNA obtained by this procedure, the amounts in liver and brain were compared. The dry defatted weights of liver and brain were 250 and 100mg. dry wt./g. wet wt. respectively (averages of six animals). The value of 2mg. of DNA/g. wet wt. in liver, uncorrected for possible impurities (Kirby, 1961), is a higher and possibly more reliable value than our procedure *B* gives for liver tissue. By using Kirby's (1961) value for liver DNA and 1·25mg. of DNA/g. in brain, there is thus 8mg. of DNA/g. dry wt. in liver and 12·5mg. of DNA/g. dry wt. in brain.

Efficiency of recovery of label after hydrolysis with perchloric acid and chromatography was examined by adding [6-^{3}H]thymidine to unlabelled samples from three rat brains before hydrolysis. Recoveries averaged 94·1, 94·7 and 101%. Analysis of labelled DNA revealed that thymine isolated by either procedures *A* or *B* had about the same specific radioactivity (Table 3). The yield of DNA, however, was almost twice as great with procedure *A*. In this short-term incorporation period, adenine, guanine and cytosine were not detectably labelled. The radioactivity of their eluates and of all blanks was not above background.

DISCUSSION

It has been pointed out (Emanuel & Chaikoff, 1960) that the unique metabolic characteristics of the brain may be reflected in a 'structural peculiarity' of its DNA. The sharp increase in amount of DNA isolated after alkaline hydrolysis with 0·1M-potassium hydroxide supports this possibility. There is poor recovery of brain DNA at the higher potassium hydroxide concentrations at which liver or thymus DNA may be recovered with little loss (Tamm, Shapiro, Lipshitz & Chargaff, 1953) or none (N. W. Penn & R. Suwalski, unpublished work). An interpretation of this finding is suggested in a previous proposal of the existence of internucleotide alkali-sensitive bridges (Chargaff, 1963). Evidence for peptides that link deoxynucleotide tracts has since appeared (Bendich & Rosenkranz, 1963). Possibly additional non-nucleotide bridges with a greater sensitivity to alkali may be present in brain DNA. It therefore seems that assessment of the nature of brain DNA should be based on study of the maximum amount obtainable from tissue. There is no assurance that a substantial fraction lost in processing may not be the site of a characteristic chemical feature.

Although use of 0·1M-potassium hydroxide is novel in the separation of DNA and RNA fractions, other workers have pointed out that the higher alkali concentrations generally employed in the Schmidt & Thannhauser (1945) method may cause significant losses of DNA, depending on the tissue and the particular animal source (Drasher, 1953; Mauritzen, Roy & Stedman, 1952; McIndoe & Davidson, 1952; Davidson, 1965). It appears that the application of this step in the analysis of tissues whose characteristics are not entirely familiar may require some preliminary testing.

The higher concentration of DNA in brain compared with liver is particularly notable, since protein synthesis and breakdown in the brain proceed at a lower rate than in liver (Lajtha, 1959; Lajtha & Marks, 1965). The high concentration of DNA in brain revealed by this method may thus be characterized not only by a structural difference, but also by some unknown functional

Table 2. *DNA values by diphenylamine and base-analysis methods*

Each diphenylamine value is based on determinations of DNA in three rats; diphenylamine assays were performed in triplicate on tissue sections from one half of each brain, base analyses in duplicate on the other half. Individual values deviate from given averages by 5% or less.

	DNA (mg./g. wet wt. of brain)	
Expt. no.	Diphenylamine	Base analysis
1	1·07	1·25
2	1·16	1·19
3	1·09	1·33
Average	1·11	1·26

Table 3. *Recovery of* [6-^{3}H]*thymine from DNA*

Values from three animals are given. Each sample was run in duplicate. Individual values differ from means by less than 5%.

	Procedure *A*			Procedure *B*		
Animal no.	Thymine (μmoles/g.)	Radioactivity (c.p.m./g.)	Sp. radioactivity (c.p.m./μmole of thymine)	Thymine (μmole/g.)	Radioactivity (c.p.m./g.)	Sp. radioactivity (c.p.m./μmole of thymine)
1	1·06	1595	1505	0·59	940	1603
2	1·08	1833	1697	0·54	794	1470
3	1·18	1164	986	0·63	570	905

difference, compared with other DNA species. In addition, the effectiveness of the dioxan raises the possibility of a specific association between brain DNA and lipid that is absent from the corresponding liver fraction. The reasonable agreement between the diphenylamine determination of DNA and the method given above suggests that the latter may serve as a control on the validity of the more rapid colorimetric or fluorimetric assays for DNA (Burton, 1956; Kissane & Robins, 1958).

RNA cannot be simultaneously determined in these samples. At 0·1 M-potassium hydroxide it is not quantitatively hydrolysed to mononucleotides and further hydrolysis is necessary. The absence of uracil components from the DNA fraction indicates that determination of RNA is practicable with the acid-soluble supernatants of these residues.

Similar specific radioactivities are found for thymine by both methods (Table 3). It appears that the additional DNA obtained in procedure *A* is homogeneous with that in method *B* with respect to rate of incorporation of label and size of precursor pools. There is therefore a reasonable correspondence between the increased DNA content, determined on the basis of thymine, and the higher total radioactivity yielded by the procedure *A*. The incorporation into DNA presumably occurs in the neuroglia of the central nervous system, as observed by other methods of analysis (Messier, Leblond & Smart, 1958; Messier & Leblond, 1960; Altman, 1963; Altman & Das, 1964).

In view of the difficulties that have beset the various methods proposed for this analysis, it cannot at present be decided from the data whether the amount of DNA per brain cell in the rat or the total estimated number of cells in the rat brain should be revised upward by some 80% (Zamenhof, 1964). The former alternative appears more reasonable.

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