A Procedure for the Isolation and Determination of Deoxyribose Derivatives

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A method of determining deoxyribose derivatives in biological material is described. It has high sensitivity, and is particularly useful in that it can be applied to a large range of tissues for which the other available assays are unsuitable. This is because the method is applicable to complex mixtures of nucleotides in which such substances as ribonucleotides are present in very large excess over deoxyribonucleotides, and it is not necessary to equilibrate the nucleotide-precursor pool with radioactive phosphate. The method has mainly been developed with the object of determining deoxyribonucleoside triphosphates, but it can be used to assay ribonucleoside triphosphates, as well as the mono- and diphosphates of both types of nucleoside. The procedure used involves three basic techniques: (1) periodate oxidation and methylamine-induced cleavage of the sugar ring to destroy 2'- and 3'-unsubstituted ribonucleosides; (2) column chromatography to separate the deoxyribonucleotides from each other and from other substances, such as the products of the periodate oxidation; (3) fluorimetric determination of deoxyribose after labilization of the pyrimidine-glycosidic bond by bromination of the heterocyclic ring. Each of these three procedures can be used independently, in conjunction with other analytical procedures.

The main problem in the determination of deoxyribonucleotides is that they invariably occur as minority components in the complex cellular pool of low-molecular-weight substances. Several types of method for determining deoxyribonucleotides have been reported, but they are of somewhat limited application and are unsuitable for investigating pool sizes in many potentially interesting tissues. The first type relies on the great separation of ribo- and deoxyribo-nucleotides possible with t.l.c. systems (Randerath & Randerath, 1967). The problem that derivatives of ribose are present in great excess over those of deoxyribose is overcome by equilibrating the cells under investigation with $^{32}\text{P}_i$ and working with only very small amounts of nucleotide. This method has obvious application in the study of micro-organisms and of cultured eukaryotic cells (see, e.g., Neuhaard & Munch-Petersen, 1966; Nordenskjöld *et al.*, 1970), but it is more difficult to apply to the organs of multicellular animals, because of the problem of proving that the organs studied are truly equilibrated with the isotopically labelled substance. The second type of method involves utilizing the dependence of bacterial DNA polymerase on the presence of all four deoxyribonucleoside triphosphates (Solter & Handschumacher, 1969). This method has the disadvantage that only deoxyribonucleoside triphosphates can be determined and that pool extracts may contain substances

that inhibit DNA polymerase. The more sensitive modification of this method by Lindberg & Skoog (1970) suffers from the additional disadvantage that it measures only dATP and dTTP.

Of the two existing chemical assays for deoxyribose derivatives, the diphenylamine method (Dische, 1955) and the fluorimetric method (Kissane & Robins, 1958), the latter is the more sensitive and was used as the method for determining the amount of material in each of the deoxyribonucleoside triphosphate pools. Both methods involve the measurement of free deoxyribose but are not equally applicable to the four deoxyribonucleoside triphosphates. Under the usual conditions of DNA assay, free deoxyribose is liberated only from purine nucleotides. To make the pyrimidine deoxyribose available for assay it is necessary to labilize the pyrimidine-glycosidic bond by modifying the pyrimidine ring (Michelson, 1963). Of the methods tried, bromine addition across the C-4–C-5 double bond of the heterocyclic ring has been found to be the most suitable. Therefore a bromination step is included in the assay.

The reaction leading to the formation of the fluorescent compound in the Kissane & Robins (1958) assay is specific for α -unsaturated aldehydes, therefore it should be possible to assay the deoxyribose derivatives in the presence of the ribose derivatives. However, in practice, because of the high ribonucleotide compared with deoxyribonucleotide content of the tissue, this proved to be

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impossible. As the separation of the ribo- from the deoxyribo-nucleotides is not practical in the circumstances of this procedure, periodate oxidation was introduced to destroy the ribose derivatives selectively.

Therefore the assay involves three distinct processes: (1) the selective destruction of the ribo-nucleotides by periodate oxidation and methylene-induced cleavage of the dialdehyde thus formed; (2) the separation of the deoxyribonucleotides by Dowex-1 (HCO_3^- form) chromatography; (3) the determination of the deoxyribose of the deoxyribonucleotides, with prior bromination to labilize the pyrimidine-glycosidic bond.

Our main interest is in the characteristics and control of DNA synthesis during embryonic development and for this reason we have characterized our method mainly with respect to the deoxyribonucleoside triphosphates. Evidence that this method is applicable to embryonic material is provided in the following paper (Woodland & Pestell, 1972).

Materials and Methods

Materials

Nucleotides and 3,5-diaminobenzoic acid were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Radioactive substances were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Nucleotide solutions

All of the work described in this paper utilizes solutions of nucleosides and nucleotides obtained commercially and standardized by the measurement of u.v. absorption. It was found to be necessary to purify the deoxyribonucleoside triphosphates to produce satisfactory standard curves. The method used was an abbreviated version of the full procedure described below. Samples (10ml) of the nucleotide solutions were treated with a twofold excess of NaIO_4 and a 20-fold excess of methylammonium formate as described. Dowex-1 (0.5g), previously washed by the procedure of Hurlbert *et al.* (1954), was added and the suspension agitated on a vortex mixer. The resin was collected by centrifugation and washed three times with 10ml of water. The deoxyribonucleotide was eluted with 1M- NH_4HCO_3 and the resin filtered off. The solution was standardized by its u.v. absorption, by using the maximum extinction coefficients shown in Figs. 3 and 4.

A procedure that is suitable for the preparation of deoxyribonucleotide solutions from animal tissues is described in the following paper (Woodland & Pestell, 1972).

Periodate oxidation

The purpose of this step is to destroy the ribose derivatives with unsubstituted 2'- and 3'-hydroxyl groups. An upper limit for the amount of these substances is obtained by measuring the E_{260} and assuming $\epsilon_{\text{mm}}=10$. If the u.v.-absorbing substances present are believed to consist almost wholly of adenosine derivatives, the higher value of 14 may be used. At this stage a portion may be taken for the analysis of the ribonucleotides. The pH of the rest of the solution is adjusted to between 5 and 6 with formic acid. A twofold molar excess of 0.1M- NaIO_4 over the calculated ribonucleotide content of the solution is added, and the solution is stirred for 15min at room temperature. If necessary the pH is readjusted to between 5 and 6 during this period. A quantity of 1M-glycerol equimolar to the NaIO_4 is then added to destroy excess of NaIO_4 . After 20min the pH is adjusted to 8.0 with NaOH, a 20-fold excess of methylammonium formate (pH 8.0) over the NaIO_4 is added and the solution stirred for 40min at room temperature. The Schiff's base thus formed is destroyed by adjusting the pH to 6.5 with formic acid. After 20min the solution is ready for column chromatography, or it may be stored at -70°C .

Chromatography

Dowex-1 (formate form) columns were prepared by the method of Hurlbert *et al.* (1954); 50cm $\times$ 0.4cm columns were used for test solutions and 50cm $\times$ 1.0cm columns for tissue extracts. If the formate concentration of the sample exceeds 0.1M it must be diluted, otherwise the binding of certain nucleotides to the column is prevented. It is run on to the column, which is then washed with water until the eluate is of constant E_{260} . The elution system is based on that of Cohn & Bollum (1961) and is a linear gradient from 0 to 1.0M- or 1.5M- NH_4HCO_3 . The E_{260} and E_{280} of the eluate are measured. Spectra may also be measured at this stage to identify the individual peaks, but it is better done after the removal of the NH_4HCO_3 .

If it is desired to measure ribonucleotides, the sample previously removed may be chromatographed by the same method as the deoxynucleotides.

Labilization of the pyrimidine-glycosidic bond

The fractions from the column chromatography are freeze-dried and NH_4HCO_3 is removed *in vacuo* at 60°C . The remaining material is dissolved in 0.5ml of water (at which stage spectra may be measured) and 0.5ml of freshly saturated bromine water is added. The samples are heated for 1h at 60°C , and if any are decolourized, further bromine water is added to them. The samples are then dried *in vacuo*. The brominated pyrimidine is unstable and the double bond would be regenerated in the conditions of the fluorimetric

assay. This can be avoided by treating the dried sample with a solution of alkali in the form of Na_2CO_3 , which serves to degrade the brominated pyrimidine ring (Soodak *et al.*, 1949). A portion (0.4ml) of 0.2M- Na_2CO_3 is added. The samples are then dried again.

Fluorimetric assay

This is a modification of the method of Kissane & Robins (1958). 3,5-Diaminobenzoic acid is dissolved in 4M-HCl at a concentration of 0.3g/ml, and treated six or seven times with acid-washed activated charcoal at a proportion of 1g/100ml. When the solution reaches a constant pale-yellow colour, it is filtered through Whatman no. 1 paper. It may then be stored at -70°C until required. If the solution begins to show a blue coloration, it should be repurified. The dried samples from the bromination procedure are dissolved in 0.2ml of 0.8M-HCl to neutralize the Na_2CO_3 . A portion (0.2ml) of the above diaminobenzoic acid solution is added and the samples are heated at 60°C for 1h. The samples are cooled and diluted with 1.0ml of 0.6M- HClO_4 . The fluorescence is then measured. In all the work described here

an Aminco-Bowman spectrophotofluorimeter was used. The excitation and emission wavelengths used were 410 and 510nm respectively, both being maxima. Standards for the fluorimetric assay are prepared in 1.0M- NH_4HCO_3 and are treated as described for the fractions from the chromatography.

Results

The aim of this section is to give the rationale for the procedure described in the Materials and Methods section and to demonstrate that it enables deoxyribonucleoside triphosphates to be measured accurately in the presence of large excesses of ribonucleotides. It must first be shown that periodate oxidation destroys ribonucleotides without degrading deoxyribonucleotides. Secondly, the four deoxyribonucleoside triphosphates (or any other deoxyribonucleotides whose assay is desired) must be separated in such a way that their identification and assay are possible, and the recovery must be high. Finally, it must be demonstrated that the fluorimetric assay measures the deoxyribose content of nucleotides accurately. The results which follow show that all these conditions are fulfilled.

Fig. 1. Column chromatography of ribo- and deoxyribo-nucleoside triphosphates after periodate-methylamine treatment

Approx. 15mmol of each ribonucleoside triphosphate and 300nmol of deoxyribonucleoside triphosphate were dissolved in 11.0ml of water and treated with periodate and methylamine as described in the Materials and Methods section. The resulting solution was run onto a column (50cm $\times$ 0.4cm) of Dowex-1, which was washed with water until the E_{260} was constant. Nucleotides were then eluted with a 1.5 litre linear gradient of 0–1.5M- NH_4HCO_3 . Fractions (approx. 10.0 ml) were collected. Results for the wash fractions are not shown. $\circ$, E_{260} ; —, E_{280} . dCTP, dTTP, dATP and dGTP (represented by C, T, A and G respectively) were identified by their u.v.-absorption spectra.

Table 1. *Spectrophotometric identification of peak fractions from the column chromatography*

These results were obtained from the column chromatogram shown in Fig. 1. The absorption measured was corrected for the background absorption in the relevant region. The u.v.-absorption spectra of these fractions were also determined after the removal of the NH_4HCO_3 solution. These spectra supported the identification made on the basis of the extinction ratio.

Fraction no.	Putative identification	E_{260}	E_{280}	Calculated E_{280}/E_{260}	Predicted E_{280}/E_{260}
83	dCTP	0.042	0.044	1.05	0.98
91	dTTP	0.045	0.034	0.76	0.73
108	dATP	0.075	0.017	0.23	0.15
119	dGTP	0.038	0.022	0.58	0.66

Periodate oxidation and column chromatography

The purpose of the periodate step is to destroy the ribonucleotides, thus enabling the deoxyribonucleotides to be purified by a relatively simple chromatography system. Ribose, but not deoxyribose, is a *vic*-glycol, since it contains both 2'- and 3'-hydroxyl groups. In most acid-soluble ribonucleotides found in the cellular pool, only the 5'-hydroxyl group is substituted, and they are therefore susceptible to oxidation by periodate, a reagent specific for $-\text{CHOH}-\text{CHOH}-$, $-\text{CHNH}_2-\text{CHOH}-$ and similar groups (Whitfield, 1954; Brown *et al.*, 1955; Lehman *et al.*, 1958). The periodate oxidation breaks the sugar ring by forming a dialdehyde, the base and the phosphate remaining attached to the ruptured sugar ring. If the u.v.-absorbing base were allowed to remain associated with the phosphate groups, it would be eluted in the same region as deoxyribonucleotides and would confuse the analysis of these compounds. Therefore the phosphate is separated from the base by treating the dialdehyde with methylamine (Khym & Cohn, 1961). At alkaline pH values the methylamine and the dialdehyde form a Schiff's base, which is destroyed by adjusting the pH to 6.5. This treatment splits the sugar backbone, thus separating the phosphate from the base. The u.v.-absorbing material derived from the degraded ribonucleotides is then washed straight through the anion-exchange column.

It is important to remember that 2'- and 3'-substituted ribonucleotides (e.g. 3':5'-cyclic AMP) will be unaffected by this periodate-methylamine treatment and will be eluted from the column in their usual positions. This aspect of the procedure could have useful applications.

The effectiveness of the periodate-methylamine treatment and of the subsequent column-chromatography step have been judged in a single type of experiment. Known quantities of the four common deoxyribonucleoside triphosphates, in the presence of a 50-fold excess of the four corresponding ribonucleoside triphosphates, were treated with periodate

and methylamine and then chromatographed as described above. Fig. 1. shows the extinction profile obtained. The two large peaks that are eluted at low salt concentrations result from the periodate-methylamine reaction. The peaks of interest were identified by their absorption spectra. Table 1 shows the E_{280}/E_{260} ratio for the peak fractions, compared with the values expected for pure deoxyribonucleotides. Table 2 shows the quantity of each deoxyribonucleoside triphosphate added to the column compared with the quantity recovered, calculated from the sum of the extinctions under the appropriate peaks.

Two conclusions may be drawn from this experiment: (1) the chromatographic system adequately separates the four deoxyribonucleoside triphosphates in a manner that enables them to be identified and determined by u.v.-absorption measurements; (2) the periodate oxidation completely removes at least a 50-fold excess of ribonucleotides, without degrading the deoxyribonucleotides or interfering with their chromatography.

Fluorimetric assay

The original method of Kissane & Robins (1958), for assay of the purine nucleotides in DNA, has been considerably altered in our assay. It is therefore necessary to demonstrate that deoxyribose can be assayed with adequate sensitivity under the conditions finally adopted. Fig. 2. shows that the assay enables about 1 nmol of deoxyribose to be measured.

The greatest difference from the method of Kissane & Robins (1958) is that a step has been introduced to labilize the pyrimidine-glycosidic bond. Of the several different methods that we have tried (e.g. hydrogenation) bromination has proved to be the most suitable. Cohn (1956) has considered bromination as a general method of addition across the C-4-C-5 double bond of pyrimidines, and various methods of bromination suitable for limited numbers of samples of relatively pure pyrimidines

Table 2. *Determination of deoxyribonucleoside triphosphates after periodate-methylamine treatment, column chromatography and bromination*

A portion (1 mg) of each of the ribonucleoside triphosphates was added to the solution containing the amounts of deoxyribonucleoside triphosphate indicated. The solution was then treated with periodate and methylamine as described in the text. It was chromatographed on a column (50cm×0.4cm) of Dowex-1 by using a 1.5 litre gradient from 0 to 1.5M-NH₄HCO₃.

	Quantity added to column (nmol)	Quantity recovered from column (nmol)	
		Measured by E_{260}	Measured by fluorescence
dATP	292	266	262
dCTP	356	370	391
dGTP	348	321	372
dTTP	—	289	261
dTDP	—	9	52
dTTP+dTDP	313	298	313

Fig. 2. *Fluorimetric assay of deoxyribose*

A standard solution of deoxyribose was prepared by weight and individual samples were measured by volume. Each was then made up to 0.2 ml with water. Diaminobenzoic acid solution (0.2 ml) was added and the assay performed as described in the text. In this and subsequent Figures the fluorescence is plotted as arbitrary units in terms of the 0.1 scale of the Aminco-Bowman spectrophotofluorimeter used.

strated in Fig. 3, which presents results from the assay of deoxycytidine, deoxythymidine, deoxyadenosine and deoxyguanosine. A similar experiment in which the four corresponding deoxyribonucleoside triphosphates were determined is shown in Fig. 4. It can be seen that in this experiment the four curves are not coincident. dCTP always gives a convex curve and dATP gives less fluorescence than the other deoxyribonucleotides. The divergences are much greater when standard solutions are prepared from the unpurified commercial triphosphates. Since only a very simple purification procedure has been used, the differences may result from impurities still present in the standard nucleotide solutions. The standard curves indicate that the deoxyribose content of as little as 10–20 nmol of a nucleoside or nucleotide can be measured. The lower sensitivity obtained when deoxyribonucleosides or deoxyribonucleotides are measured, as compared with deoxyribose, may result from the presence in the former of products from the bromination reaction.

Validation of complete assay procedure

This was carried out by the fluorimetric assay of brominated fractions obtained from the column chromatography of known quantities of deoxyribose and ribonucleoside triphosphates after periodate oxidation. Sufficient deoxyribonucleotide was used to enable the recovery to be determined by u.v. absorption. These results are compared with those obtained from the fluorimetric assay in Table 2. It seems that in this experiment the dTTP was contaminated with dTDP, which was eluted a little before dCTP. It was identified by its elution position and spectrum.

Table 2 shows that the fluorescence assay is

have been described (Massart & Hoste, 1947; Soodak *et al.*, 1949; Yanagisawa & Isono, 1966; Trim & Parker, 1970). The method that we have used is not dissimilar to these, but it is necessary to show that it enables pyrimidine deoxyribose derivatives to be determined accurately, and that it does not interfere with the determination of the purine derivatives. That both these requirements are fulfilled is demon-

Fig. 3. Determination of deoxyribose in nucleosides

The pyrimidine-glycosidic bond was labilized by bromination and the fluorescence assay performed as described for fractions from chromatography columns in the Materials and Methods section. The concentrations of the standard solutions were calculated on the basis of extinction maxima in an aqueous solution. The millimolar extinction coefficients used were: dA, 15.0; dC, 9.0; dT, 9.6; dG, 13.7. ○, dA; ●, dC; ▲, dT; △, dG.

capable of determining the deoxyribonucleoside triphosphates, since the measurements by u.v. absorption and fluorimetry agree both with each other and with the value for the amount of deoxynucleotide present at the beginning of the experiment. The sensitivity of the assay procedure is sufficient to enable as little as 10–20 nmol of a deoxynucleotide to be measured.

The recovery of deoxyribonucleoside triphosphates may also be measured by adding radioactive triphosphates at the beginning of the extraction procedure. This was done in the experiments just described above, and in those on eggs and oocytes reported in the following paper (Woodland & Pestell, 1972). When either [^3H]dTTP or [^3H]dATP were added, the distribution of radioactivity between the nucleoside and the mono-, di- and tri-phosphates was the same as that of the stock radioactive solution used, indicating that no measurable breakdown had occurred as a result of the assay procedure. Full details for determining recoveries by using radioactivity methods are given by Woodland & Pestell (1972). In the experiment for which results are given in Table 2, a small peak of radioactivity was coincident with the putative dTDP. This is additional evidence that the peak is dTDP.

Discussion

The procedures described above have proved to be the most effective and reliable methods for the deter-

Fig. 4. Determination of deoxyribose in deoxyribonucleoside triphosphates

All experimental details are as described for Fig. 3, except that the millimolar extinction coefficients used were: dATP, 15.4; dCTP, 9.1; dTTP, 9.6; dGTP, 13.7. ○, dATP; ●, dCTP; ▲, dTTP; △, dGTP.

mination of deoxyribonucleotides that we have tested. Alternative procedures have been tried, but found to be ineffective. For example, it proved to be essential to remove ribonucleotides if they were present in large excess, otherwise extensive charring occurred during the formation of the fluorescent product, and the assay of any fluorescent product was prevented. We also tried triethylammonium bicarbonate in place of NH_4HCO_3 in the column chromatography, because this compound is very volatile and could have been removed more easily before the subsequent stages of the assay. Unfortunately it did not give adequate resolution of the nucleotides.

It is clearly important that the method we describe should determine deoxyribonucleotides in biological extracts as well as it does those in standard solutions. The accompanying paper (Woodland & Pestell, 1972) shows that it does so, at least for extracts of eggs and oocytes of *Xenopus laevis*. It therefore seems likely that our procedure can be applied to the determination of deoxyribonucleotides in many tissues, provided that an appropriate extraction procedure is used.

The three techniques used in this method should also prove useful when combined with other procedures. For example, polyethyleneimine t.l.c. is a powerful technique for the separation of nucleotides (see, e.g., Randerath & Randerath, 1967), but it is not directly applicable to the separation of deoxyribonucleotides because they are usually associated with such large amounts of ribonucleotides. A preliminary periodate-methylamine step would destroy most of the ribonucleotides. The deoxyribonucleotides could be freed from the products of the ribonucleotide degradation and concentrated by a simple

chromatography step followed by freeze-drying, as described by Neuhaard & Munch-Petersen (1966).

The periodate reaction does not destroy 2'- and 3'-substituted ribose derivatives. Followed by the chromatography step it therefore enables the isolation of these compounds, some of which (e.g. cyclic nucleotides) are already known to be of great importance in the cell. In the following paper (Woodland & Pestell, 1972) we report the isolation, though not the identification, of some such substances from amphibian eggs.

Although the method described has been presented mainly as an assay for deoxyribonucleoside triphosphates, it is applicable to other deoxyribose derivatives. This is one of its main advantages over enzymic methods, by which only the deoxyribonucleoside triphosphates can be determined.

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