IMMUNOCHEMICAL ANALYSES OF CELLULAR DNA[1]

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Summary:

A goat antiserum was prepared against the base thymine covalently linked to albumin. The antiserum was specific for thymine, complexing only with DNA and not RNA unless the base thymine was present as in E coli transfer RNA. [^{125}I]-DNA was prepared with high specific activity. When reacted against goat antiserum in an Ouchterlony assay the labeled DNA gave several bands which concentrated near the antigenic wells. Similarly, denatured DNA gave several precipitin lines, but with varying diffusion rates. Both iodinated and denatured DNA competed out [^{125}I]-DNA for the goat antiserum in a radioimmunoassay. Absorption of the antiserum with ribosomes did not decrease the binding of DNA. Using this assay, nanogram quantities of DNA were estimated in small samples of tissue.

Materials and Methods:

Preparation of antigen (1) and antiserum (2). The following conditions were used to cleave ribofuranosyl thymine and conjugate the ribose to albumin for antibody production. Ten milligrams of 1-β-D-ribofuranosyl thymine (prepared by Sloan-Kettering Institute for Cancer Research) was reacted with 0.5 ml of 0.1 M $NaIO_4$ and kept at room temperature for 20 min. To destroy excess periodate 0.5 ml of ethylene glycol was added. After 5 min 1 ml of Bovine serum albumin (28 mg/ml in 5% K_2CO_3, pH 9.5) was added and stirred for 45 min at 37°C. $NaBH_4$ was added to a concentration of 15 mg/ml to reduce the resulting Schiff base and the mixture was allowed to stand for 18 hrs. To stop the reaction 0.5 ml of 1.0 M formic acid was added. After 1 hr the pH was adjusted to 8.5 with NH_4OH and the mixture dialyzed 12 hrs against running water. The solution was lyophilized and the antigen injected into goats, as described earlier (2), for production of antibodies.

Purification of DNA. DNA was purified as described previously (5). Isolated nuclei were shaken with 20 vol of SSC buffer (0.15 M NaCl and 0.015 M Na citrate) containing 0.1 M EDTA and 1% sodium dodecylsulfate, at room temperature for 20 min. The DNA was extracted from the solution with an

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equal volume of phenol saturated with SSC buffer. The extractions were continued until no protein interphase was visible. The DNA was then precipitated with 2 vol of cold ethanol, spooled with a pasteur pipette and dissolved in SSC buffer containing 0.1 M EDTA. Pancreatic ribonculease (100 ug/ml) was added and incubated with DNA for 10 min (37°C). The mixture was shaken for 10 min, centrifuged (10,000 x g, 10 min) and the aqueous phase extracted with 50% chloroform. The aqueous phase was extracted twice with ether and the DNA was precipitated with cold ethanol.

Labeling of DNA. After purification the ethanol precipitate was dissolved in 0.2 M acetate buffer, pH 5.0 (1 mg/ml). After mixing, the treated DNA was added to a vial containing 2 mCi [^{125}I] and heated to 70° for 1 hr. The mixture was passed through a Sephadex G-25 column (6) and eluted with 0.2 M sodium acetate buffer, pH 5. The material appearing under the first peak of eluant was pooled and dialyzed overnight against 0.2 M acetate buffer, pH 5. The [^{125}I]-labeled DNA containing 10^8 cpm/OD at 260 mμ was aliquoted to tubes so that each tube contained approximately 8 x 10^5 cpm. The tubes were kept at -70°C until used for analyses.

Preparation of tissue for DNA determination. Tissue was homogenized in 0.2 M sodium acetate buffer, pH 5 and made 1% in sodium dodeculsulfate, (5) homogenized lightly and stirred at room temperature for 30 min. The homogenate was centrifuged (600 x g, 10 min) and the pellet discarded. The supernatant was cooled to 4°C and 2 vol of ethanol (4°C) was added. After 10 min the mixture was centrifuged (600 x g, 10 min). The pellet was dried and dissolved in acetate buffer; further dilutions were made for DNA determinations.

Radioimmunoassay for DNA. Unlabeled DNA obtained from the ethanol precipitate was dissolved in 0.2 M sodium acetate buffer, pH 5. The DNA was denatured by heating to 90°C for 30 min followed by quick cooling (4°C). A stock solution of standard DNA was made 1 OD/ml (260 mμ) and stored frozen. (Twenty OD at 260 mμ was taken as 1 mg of DNA). For the assay 2 ml of acetate buffer containing 0.1% albumin was added to a tube of labeled DNA. One hundred microliters of the labeled DNA mixture was used for each determination, so that each tube contained approximately 4 x 10^4 CPM. One hundred microliters of labeled DNA and 100 μl of unlabeled DNA were mixed together and incubated for 10 min at 37°C. For controls where unlabeled DNA or antibody were omitted a similar vol of acetate buffer was added. Antiserum was diluted 1:100 in acetate buffer, and 100 μl was added to the DNA and incubated for 2 hrs at 37°C. At the end of the incubation period the tubes were centrifuged (600 x g, 15 min, 37°C) and the supernatant decanted. The dried pellets (too small to be visible) were counted in an automatic gamma

counter (Packard). Under these conditions the antigen-antibody complex was pelleted, whereas free antigen remained in solution. Determination of the percentage of bound and unbound antigen was made as described earlier for the radioimmunoassay for ribosomes (7).

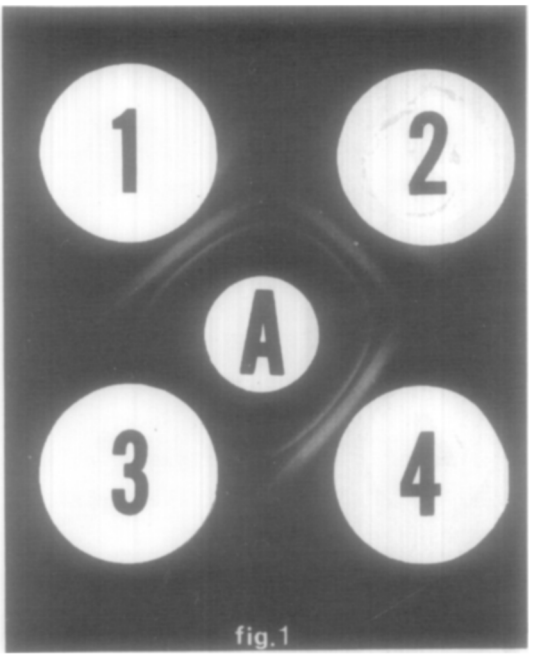
Immunodiffusion assays. Ouchterlony plates were prepared as described earlier (7) except that the agarose was mixed in 0.2 M sodium acetate buffer, pH 5.

Results and Discussion: A simple, precise quantification of DNA is needed as an index for numerous experimental measures. The inefficiency of present techniques in quantitating DNA in a mixture of cellular components (8) and the need for estimating nanogram quantities of DNA (4) lead to the development of an assay system using an antibody specific to the base, thymine, as reported here. Also, an antiserum to thymine will help determine the presence of this base in various RNAs.

An antiserum specific to thymine does not react with mammalian RNA since thymine does not occur naturally as a riboside in mammalian RNA. In order to conjugate thymine to albumin the riboside was used since deoxyribose cannot be cleaved by $NaIO_4$. The synthesized ribonucleoside, 1-β-D-ribofuranosyl thymine, after being oxidized and reduced was complexed to albumin according to a modified procedure of Erlanger et al (1). A high titre of antiserum was developed in goats against the base thymine complexed to albumin.

Denatured DNA gave several bands when reacted with the antiserum in an Ouchterlony assay as shown in Figure 1. In contrast the immunodiffusion analyses of ribosomes with goat antiserum to ribosomes gave a single precipitin line (7). Purified rat liver DNA and crude liver DNA preparations had common antigenic determinants with purified rat myocardial DNA, whereas rat liver ribosomes did not form precipitin lines with the antiserum to DNA (Figure 1). If the DNA was not denatured the diffusion rate was slow, and as shown in Figure 2, the precipitin lines formed near the antigenic wells. [^{125}I]-DNA gave several precipitin lines, which had a diffusion rate similar to that of native DNA. A precipitin line was obtained with E coli transfer RNA (which contains thymine) (9), but not rat liver transfer RNA (Figure 3).

A dilution of antibody was chosen for the immuno-analyses which gave competition of DNA in tissue homogenates to be analyzed. The tissue was treated with sodium dodecylsulfate to release the DNA from proteins, followed by precipitation of the DNA with ethanol. DNA could be quantitated in the ethanol precipitate after denaturing it by heating to 90°C for 30 min followed by quick cooling (4°C). Figure 4 shows the competition of unlabeled purified rat liver DNA against [^{125}I]-DNA of rat liver for the goat anti-DNA



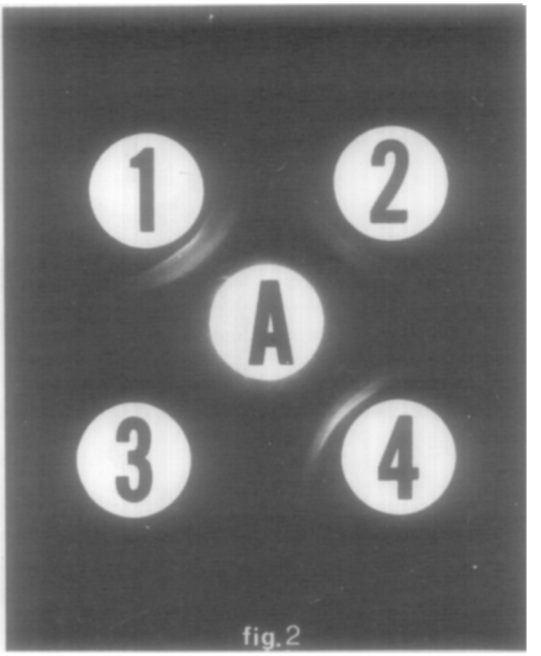


Figure 1
Ouchterlony pattern of denatured DNA (1) purified rat liver DNA, (2) purified rat myocardial DNA, (3) rat liver ribosomes, and (4) crude liver DNA preparations incubated with (A) antiserum to thymine

Figure 2
Immunodiffusion pattern of native DNA (1) purified rat liver DNA, (2) purified rat myocardial DNA, (3) rat liver ribosomes, and (4) crude liver DNA preparations incubated with (A) antiserum to tymine

serum. When plotted on log-log coordinates a straight line was obtained. Results were similar, giving total competition when tissue homogenates were used in place of purified DNA. There was no binding of [^{125}I]-DNA when normal goat serum was used in place of antiserum; results were similar to those reported earlier for the radioimmunoassay for ribosomes (7). Absorption of the antibody with ribosomes did not decrease the binding of DNA to the antiserum. In quantification of DNA in tissue homogenates, based on the standard curve, analyses were similar to those obtained with diphenylamine for determination of deoxypentose in nucleic acids (10). However, DNA data on tissue homogenates was approximately 5% lower with the radioimmunoassay (11). It is believed that analyses of DNA based on estimation of pentose may not give correct analyses when other cellular components are present; whereas, an assay based on an antiserum to thymine gives more specific results.

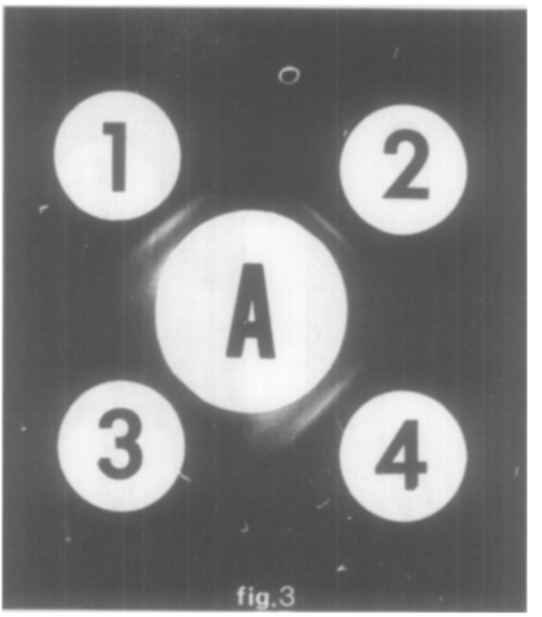


Figure 3
Ouchterlony pattern of E coli transfer RNA (1,2 &4) and rat liver transfer RNA (3) incubated with (A) antiserum to thymine

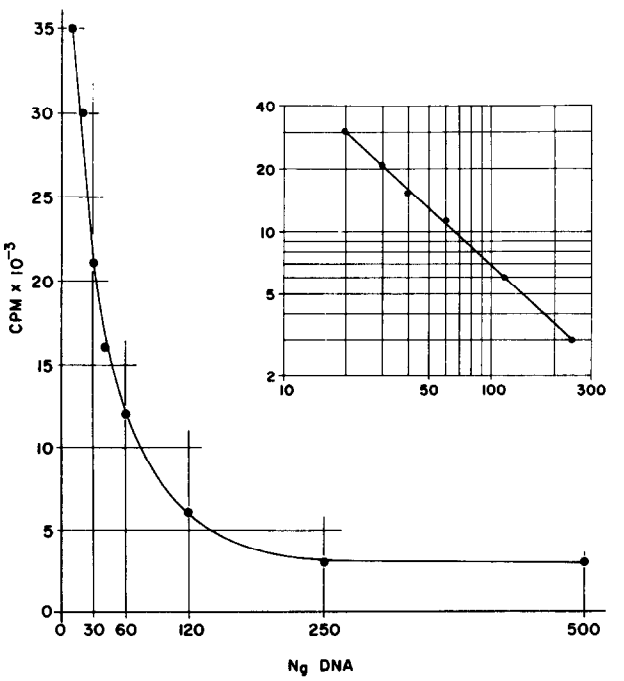


Figure 4
Competition of rat liver DNA against ^{125}I-DNA for antiserum to thymine

References

1. Erlanger, B.S., and Beiser, S.M., Proc. Natl. Acad. Sci., 52, 68 (1964)
2. Busch, H., Busch, R., Spohn, W., Wikman, J., and Daskal, Y., J. Exptl. Biol. and Med., 137, 1470 (1971)
3. Wikman, J., Howard, E., and Busch, H., J. Biol. Chem., 244, 5471 (1969)
4. Yazdi, E., Ro-Choi, T.S., Wikman, J., Choi, Y., and Busch, H., Cancer Res. 29, 1755 (1969)
5. Quagliarotti, G., Hidvegi, E., Wikman, J., and Busch, H., J. Biol. Chem., 245, 1962 (1970)
6. Commerford, S.L., Biochem., 10,993 (1971)
7. Wikman-Coffelt, J., Analyt. Biochem. (May Issue) 1972
8. Enwonwu, C., Strambaugh, R., and Sreenby, L., J. Nutrition, 101,337 (1971)
9. Goodman, H.M., Abelson, J.N., Landy, A., Zadrazil, S., and Smith, J.D., Europ. J. Biochem., 13, 461 (1970)
10. Burton, K., J. Biochem., 62,315 (1956)
11. Wikman-Coffelt, J., Biochim. Biophys. Acta (Submitted)