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DETERMINATION OF RUTHENIUM ON DNA BY XRF

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ABSTRACT

An x-ray fluorescence (XRF) technique is used to quantitate the binding of $[H_2O(NH_3)_5Ru^{II}]^{2+}$ to DNA. This method is shown to be more sensitive, precise and convenient than conventional optical absorption (OA) spectroscopy, differential pulse voltammetry (DPV), or atomic absorption (AA) techniques. XRF is insensitive to the oxidation state or coordination environment of the Ru, and so can be used to determine total Ru. The minimum detectable amount of Ru is 10 ng in one hour of counting time using a 100 mCi ^{125}I source. The specific advantages of the XRF method over the conventional methods are outlined.

INTRODUCTION

A number of Ru complexes with amonia and organic amines have exhibited both mutagenic and anticancer properties, and a rational approach to their development as both anticancer chemotherapeutic and radiodiagnostic agents have been devised (1-3). Since their biochemical effects and chemotherapeutic action appear to be similar to that of cis-$[Cl_2(NH_3)_2Pt]$, which is now the most widely used anticancer drug in the US, it is reasonable to assume that both the Ru and Pt anticancer drug, have a similar target molecule i.e., DNA (4). Compounds containing ^{97}Ru are also candidates as radioscintigraphic agents, since this isotope has excellent radiophysical properties for organ imaging (5,6). Complexes containing the beta-emitting isotopes ^{103}Ru or ^{106}Ru might also be used as radiotherapeutic pharmaceutical providing they can be sufficiently localized in neoplastic tissue (3,6).

In ascertaining the biological and biochemical effects of ruthenium it is necessary to accurately determine the concentration of this element at low concentrations. While characteristic spectra are observed when $[(NH_3)_5Ru^{III}]$ is coordinated to the purine or pyrimidine bases of DNA, often more than one type of base is coordinated and the overlapping spectral bands make quantitation difficult since absorbance readings cannot be directly translated into concentrations (7). Since reduction potentials are sensitive to the coordination environment of the metal ion, quantitation by differential pulse voltammetry (DPV) (8) can also be less than straightforward. Moreover, interferences from buffer artifacts or biological residues further contribute to errors arising from this technique. Atomic

absorption spectrophotometry (AA) has been the method of choice for this element (9), however, flame techniques require relatively large volumes (5 mL) and are often not suitable for viscous samples, such as occur at high DNA concentrations. Graphite furnace techniques have not been widely used for Ru, perhaps owing to its high atomization temperature, which is at the limit of commonly used temperature sensors.

In an effort to quantitate ruthenium binding to DNA, a superior analytical technique was developed using XRF. While being less discriminatory as to the type of Ru present, XRF is nondestructive, allows for the convenient determination of total ruthenium, is several orders of magnitude more sensitive than conventional optical absorption (OA) methods and requires minimum sample volumes.

METHOD

The XRF apparatus consisted of an external source (100 mCi. ^{125}I emitting Te x-rays at 27.97 and 30.99 keV) to induce the emission of the Ru chaacteristic x-rays (19.28 and 21.65 keV) present in the sample. Emitted x-rays were detected by a solid state, [Si(Li)], detector whose signal was electronically processed and stored in digital form. A more detailed description of the system is given elsewhere (10).

Stock solutions of $[Cl(NH_3)_5Ru^{III}]^{2+}$ were prepared by dissoliving 100 mg (0.33 mmol) of its chloride salt with the addition of two equivalents of AgTFA (where TFA = trifluoroacetate) to remove the ionic chloride. The resulting solutions were adjusted to a pH of 2-3 and a final $[Cl(NH_3)_5Ru^{III}]^{2+}$ concentration of approximately 0.03 or 0.3 M. Under these conditions the complex slowly hydrolyzes to yield $[(H_2O)(NH_3)_5Ru^{III}]$; however, this has no effect on the results reported here. Reduction to $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ was carried out in an argon purged solution over Zn amalgam for 30-60 minutes.

$[(NH_3)_5Ru^{III}]_n$-DNA. Stock solutions of calf thymus DNA (Sigma. Type I) were prepard by dissolving the DNA in 0.1 M phosphate buffer at pH 7.2 or TA buffer (40 mM Tris, 5mM sodium acetate adjusted to pH 7.8 with acetic acid) and diluting to a DNA-phosphate concentration (P_{DNA}) of 1.5 mM (A_{260} = 12). Heat-denatured DNA was prepared by heating the DNA solution in a boiling water bath for 20 min. and then cooling rapidly in an ice bath. Ruthenium-DNA complexes were prepared from aliquots of these solutions, which had been purged with argon for 30 minutes and then injected with varying amounts of the $(H_2O)(NH_3)_5Ru^{II}]^{2+}$ solution. Reactions were allowed to proceed for 1 hr. at 20° with continuous argon bubbling. Oxidation to yield $[(NH_3)_5Ru^{II}]_n$-DNA was accomplished by a 1 hr. purge with O_2 which caused the initially yellow $[(NH_3)_5Ru^{II}]_n$-DNA to turn a pronounced purple color. Unreacted Ru^{III} species were removed by dialysis against the appropriate buffer or by three successive ethanol precipitations of the DNA.

Samples for XRF were prepared by pipeting 0.1 mL of the Ru-DNA solution onto a thin (0.012 mg/cm^2) Formvar foil and air dried. The sample was then positioned in the beam and counted for one hour. When necessary, the counting period was increased for better accuracy.

Hexaammineruthenium chloride $[(NH_3)_6Ru]Cl_3$, and chloropentaammineruthenium chloride, $[Cl(NH_3)_5Ru]Cl_2$, were used as primary standards. Drying of these materials can be effected by heating in a vacuum desicator before use and both are stable as solids for periods of years. Either is much better characterized than $RuCl_3.xH_2O$, which is often used as a ruthenium standard even though it is usually supplied as a mixture of hydrates of Ru(III) and Ru(IV).

However, solutions of the ammine complexes cannot be stored indefinitely and standards were usually adjusted to pH 2-3 in order to supress hydrolysis. Standards made up from $[(NH_3)_6Ru]Cl_3$ deviated appreciably from those made directly from $RuCl_3.xH_2O$ or a commercial standard solution.

RESULTS

Linearity of solution standards was excellent with ($r = 0.999$, see Figure 1). Standards prepared with added calf-thymus DNA (Sigma. Type 1) fell on the same calibration line as those in weak acid solution, verifying non-interference of the biochemical matrix.

Spectra of 100 ppm (989 μM) and 3.125 ppm (30.92 μM) are shown in Figure 2. The Fe, Cr, and Ar peaks seen in this figure result from the source holder and Ar present in air. The minimum detection limit (MDL) for Ru by XRF (defined as three times the square root of the background) was 0.1 ppm for a one hour counting time of a 0.1 mL sample (10 ng Ru). The sensitivity of the system was ~ 10 counts /hr/ppm Ru/mCi.

Coordination of $[NH_3)_5Ru^{III}]$ to guanine and adenine bases results in strong ligand to metal charge transfer bands (LMCT) which occur in the visible region of the spectra. Since guanine coordination predominates, the optical absorbance was measured near the maximum of the G → Ru(III) LMCT band at 550 nm. A plot of A_{550} arising from DNA-coordinated ruthenium vs. [Ru] as detrmined by XRF is shown in Figure 3. Scanning the visible spectra over the concentration ranges shown revealed that approximately the same relative amounts of ruthenium were coordinated to adenine and guanine sites: however, since this ratio changes from one series to another, the total

absorbance at any one wave length is not an accurate measure of the amount or type of ruthenium present.

At relatively low [Ru]/nucleotide ratios, guanine sites are preferentially bound. Since the reduction potentials of Ru-adenine complexes are fairly negative the DPV scans are sensitive only to Ru-guanine residues. A significant background current results in a positive intercept in the plot of DPV current vs. [Ru]XRF as shown in Figure 4.

DISCUSSION

Since atomic fluorescence lines between elements are usually very well resolved, XRF is normally free of matrix interferences and so requires little, if any, sample preparation. The MDL by XRF (10 ng) is substantially better than that by routine OA (3 μg) or flame AA (1 μg) (2). While having approximately the same sensitivity as DPV (30 ng), there are fewer interferences. Although somewhat less sensitive than a flameless AA technique utilizing a graphite furnace (0.5 ng), this technique is often difficult to carry out reproducibly owing to the high boiling point of Ru (4423 ^{o}K).

Both the sensitivity and the MDL far exceed the requirements of the present work and appear to exceed the estimated requirements for therapeutic levels of Ru. Moreover, the linearity of the XRF signal versus ruthenium concentration is superior to that of other methods. Since XRF measures total ruthenium and does not discriminate between that bound in different ways to the biological material, its use must be considered complementary to other techniques, such as OA and DPV, that respond to the ligand environment of the metal. The reduced correlations between OA, DPV and XRF for native specimens in Figs. 3 and 4, as compared to those obtained with denatured samples, is being studied at present.

It is expected that the LMCT bands arising from DNA coordination exhibit corresponding changes in molar absorptivities within the DNA environment relative to the monomeric nucleotide complexes free in solution. Measurements of the total ruthenium content by XRF will then allow for determinations of the molar absorptivities of each Ru-DNA nucleotide component and their individual quantitation. Because of the energies of the Ru characteristic energies it is conceivable to apply XRF technique to monitor Ru build-up in superficial tumors in vivo.

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FIGURE CAPTIONS

Fig. 1 Calibration of Ru x-ray yield versus Ru concentration.

Fig. 2 XRF spectra of two Ru standards.

Fig. 3 Comparison of Ru measurements by optical absorptiometry and XRF.

Fig. 4 Comparison of Ru measurements by differential pulse voltemmetry and XRF, the same samples as in Fig. 3.

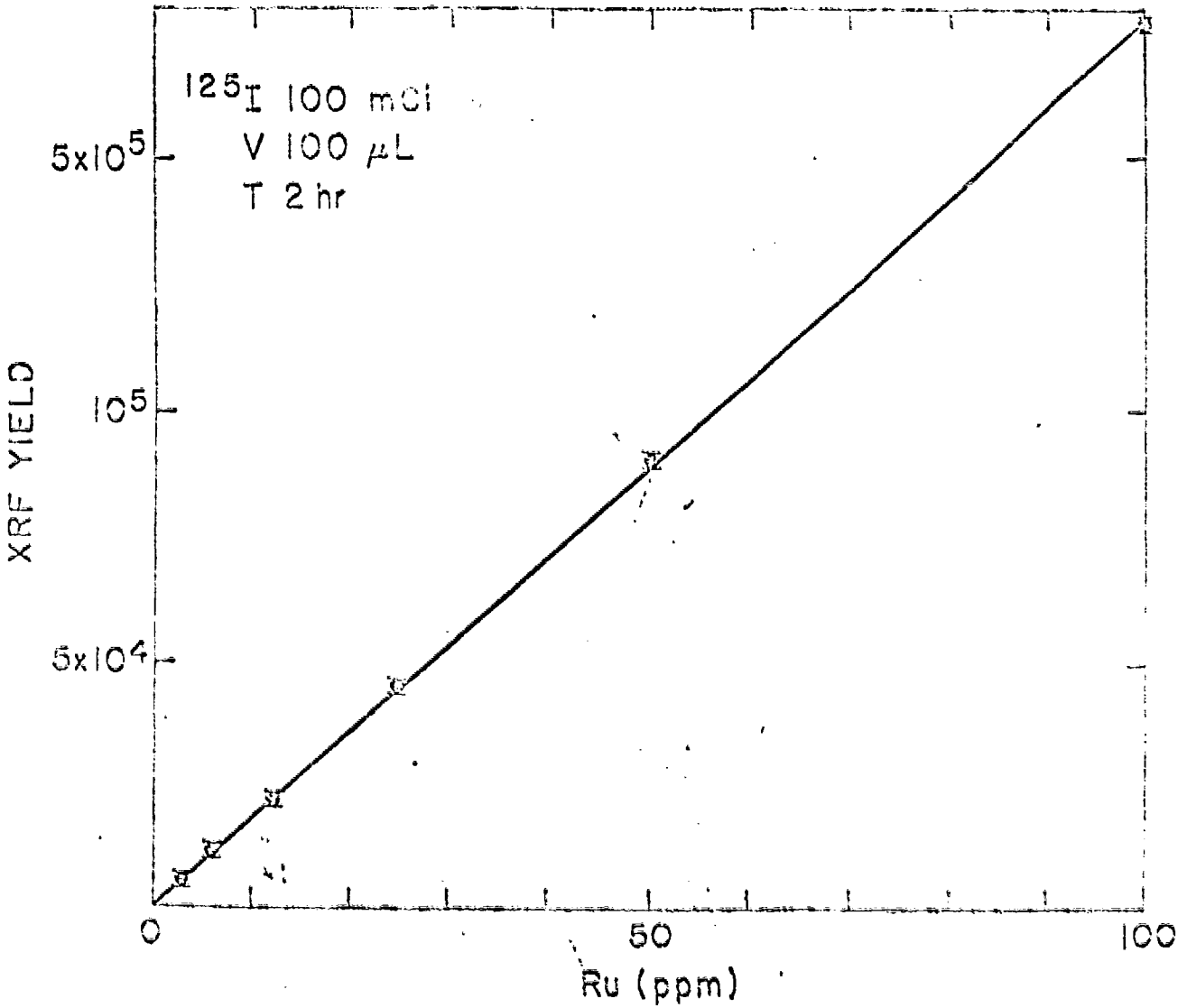

Fig. 1. Calibration of Ru x-ray yield versus Ru concentration.

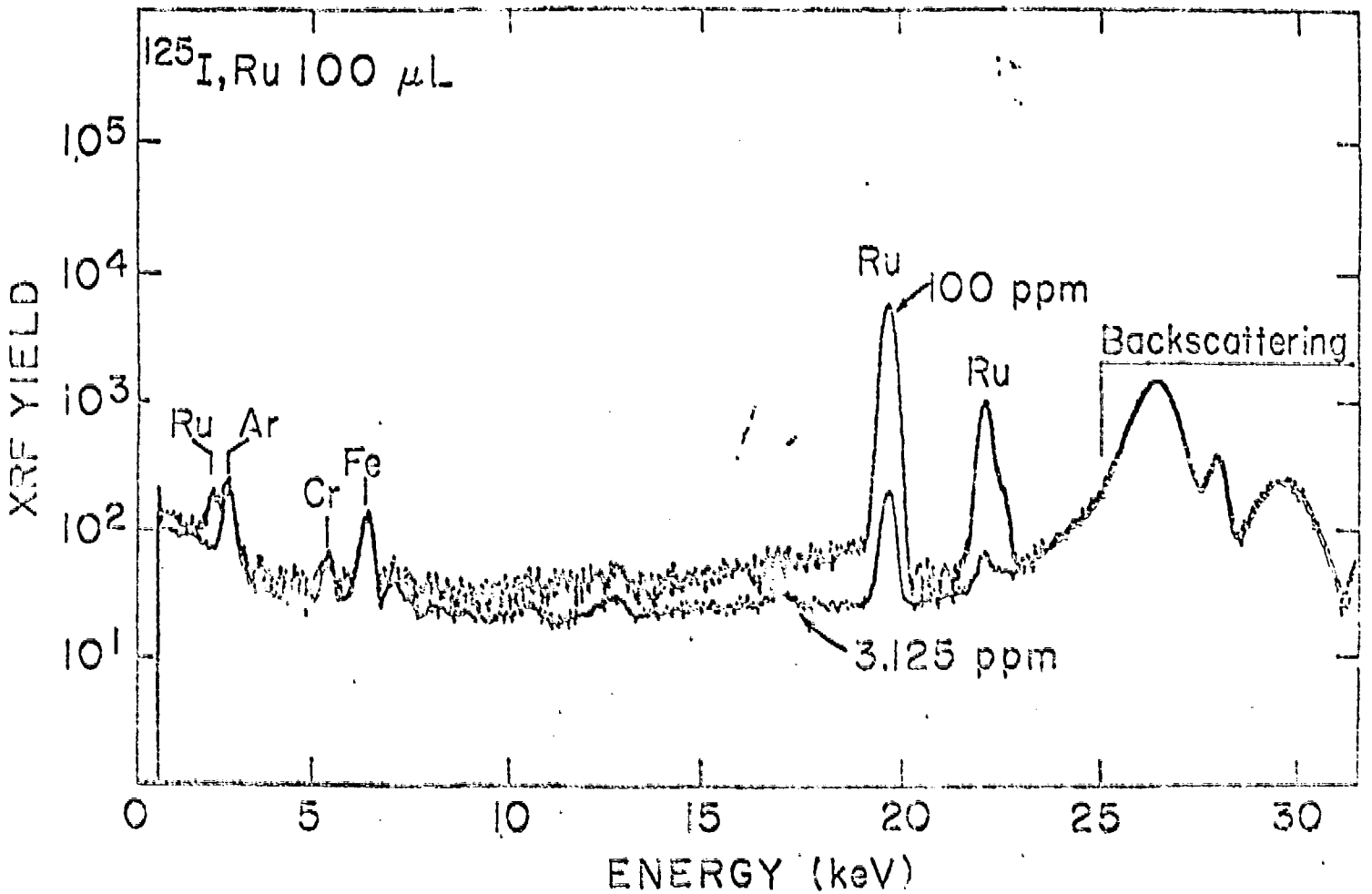

Fig. 2. XRF spectra of two Ru Standards

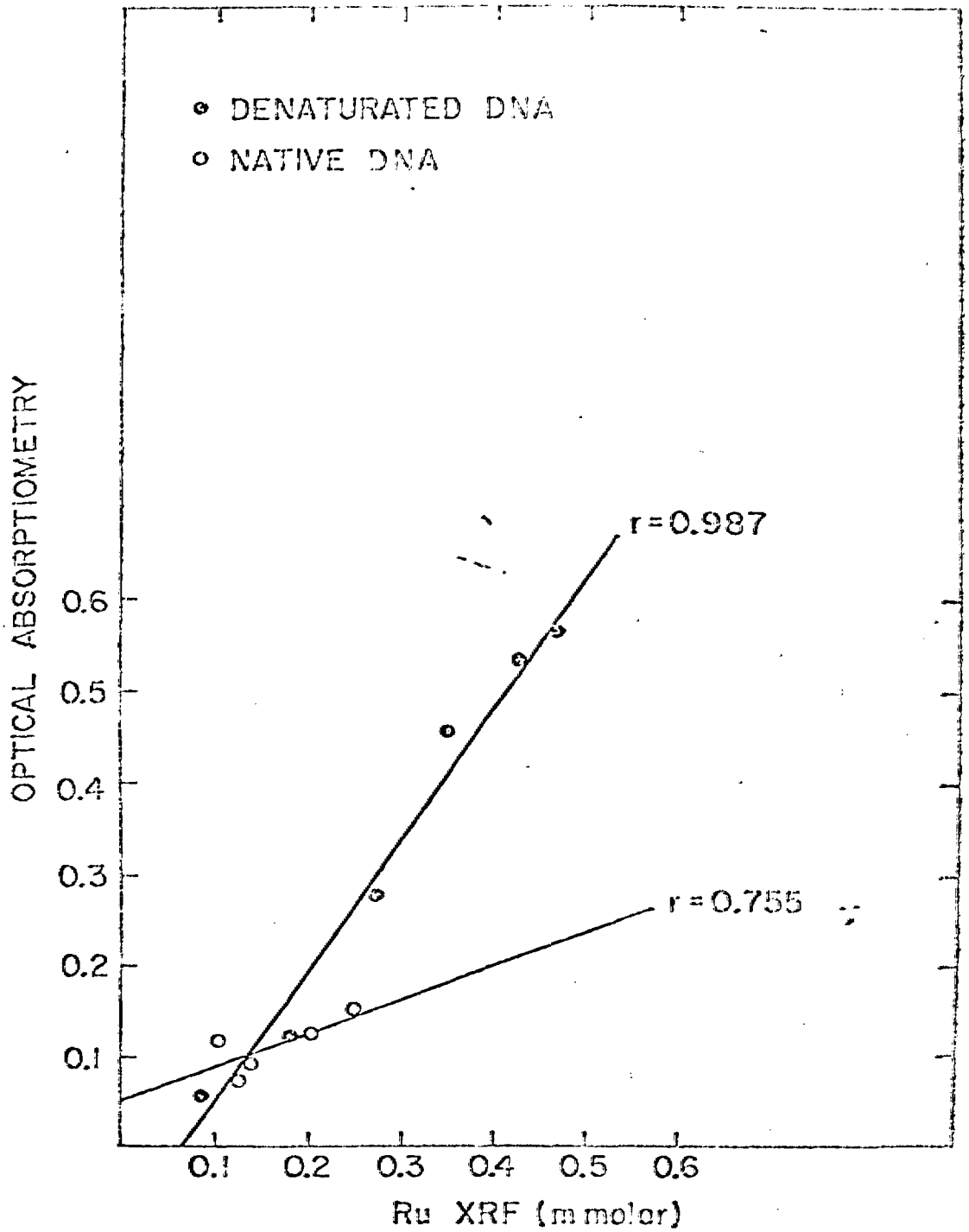

Fig. 3. Comparison of Ru measurements by optical absorptiometry and XRF.

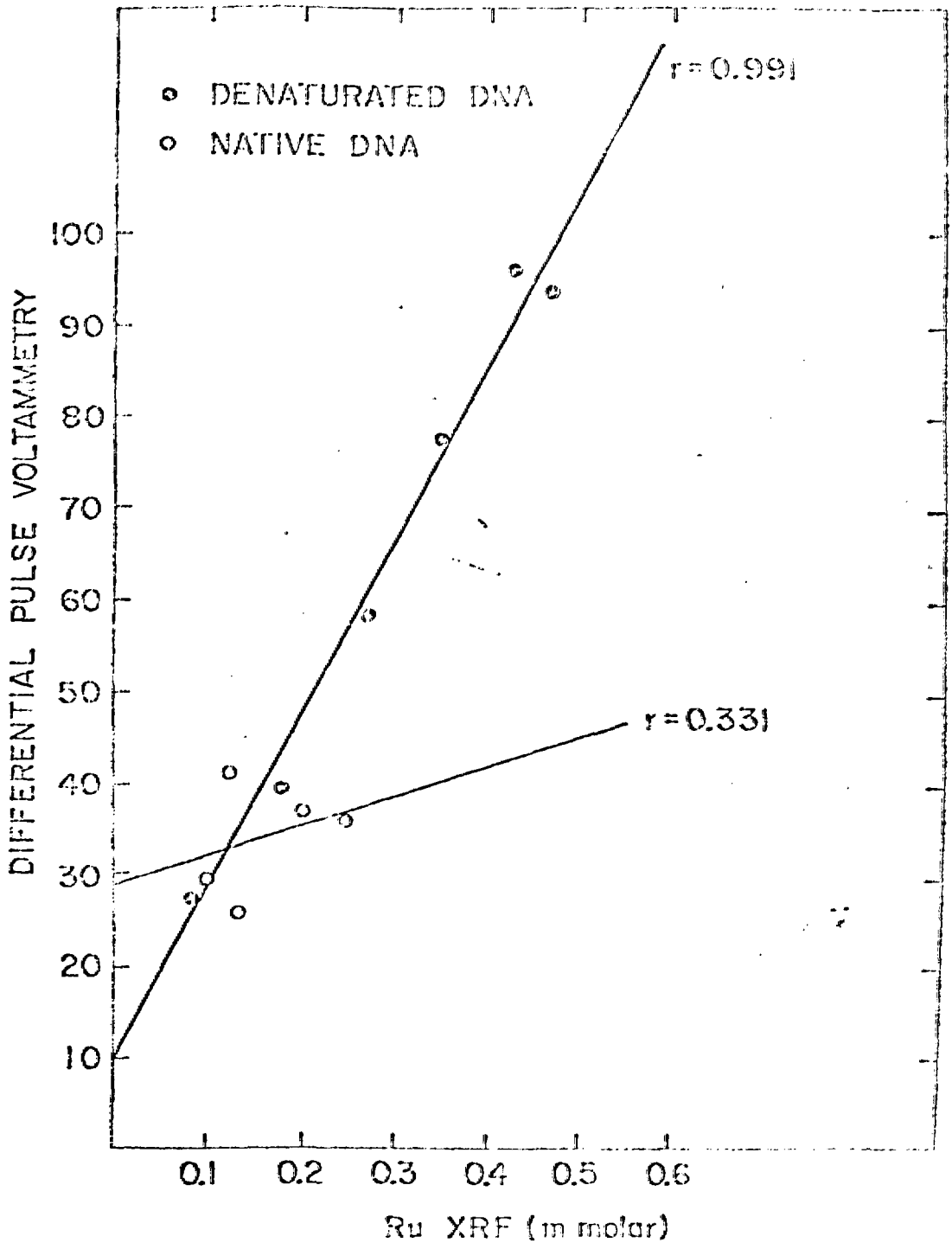

Fig. 4. Comparison of Ru measurements by differential pulse voltammetry and XRF, the same samples as in Fig. 3.