A LOW MOLECULAR WEIGHT RUTHENIUM COMPLEX INHIBITORY TO MITOCHONDRIAL Ca^{2+} TRANSPORT

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1. Introduction

Moore [1] and Vasington et al. [2] have demonstrated a specific inhibition of mitochondrial reactions associated with active Ca^{2+} transport by commercial preparations of Ruthenium Red. However, such preparations generally contain less than 20% of the pure compound [3]. Recent studies in this laboratory [4, 5], which concentrated on the mechanism of inhibition of the Ca^{2+} carrier by pure Ruthenium Red ($[(NH_3)_5$ Ru-O-Ru$(NH_3)_4$-O-Ru $(NH_3)_5]^{6+}Cl_6^-$, $4H_2O$; [6]), suggested that a colourless contaminant of the crude preparations has inhibitory properties similar to those of Ruthenium Red itself.

Identification of the compound would be of some interest as it should provide further information on the nature of the sites involved in non-competitive inhibition of the Ca^{2+} carrier. A second feature, inherent in its lack of absorption in the visible spectrum, is that it would not interfere with optical measurements of mitochondrial redox components and thus would be more useful than Ruthenium Red in determining the relation between non-competitive carrier inhibition and energy transduction.

This report describes preliminary attempts to isolate and identify this compound.

2. Materials and methods

Mitochondria were isolated from rat liver and assayed for protein as described previously [7].

Mitochondrial volume changes were monitored semi-quantitatively by measuring E_{520} in a Varian-Techtron split-beam recording spectrophotometer; the same instrument was used to record absorbance spectra of the ruthenium complexes. Oxygen uptake was measured polarographically [8].

Scintillation counting was carried out as described previously [5].

Thin-layer chromatography media were products of the Gelman Instrument Corp. (Michigan, USA); silicic acid (for column chromatography) was from E. Merck A.-G. (Darmstadt, Germany).

^{103}Ru was purchased from the Radiochemical Centre (Amersham, U.K.) and $RuCl_3, 3H_2O$ from Hopkin and Williams (Essex, UK); $[Ru(NH_3)_5Cl]Cl_2$ and $[Ru(NH_3)_6]Cl_3$ were kindly provided by Dr. J. Broomhead (Chemistry Dept., Australian National University).

3. Results and discussion

The first approach adopted was to synthesize Ruthenium Red from Ru^{3+} with the inclusion of $^{103}Ru^{3+}$ to enable detection of all ruthenium complexes. Since commercial Ruthenium Red is probably synthesized under similar conditions, the inhibitory impurities of commercial preparations should be present and labelled in the resulting mixture of products.

Two g of $RuCl_3, 3H_2O$ and 34 μCi ^{103}Ru were used as the starting materials in the synthetic procedure described by Fletcher et al. [6]. The supernatants from successive Ruthenium Red crystallizations were combined, and this solution was used as the source of material in subsequent analyses.

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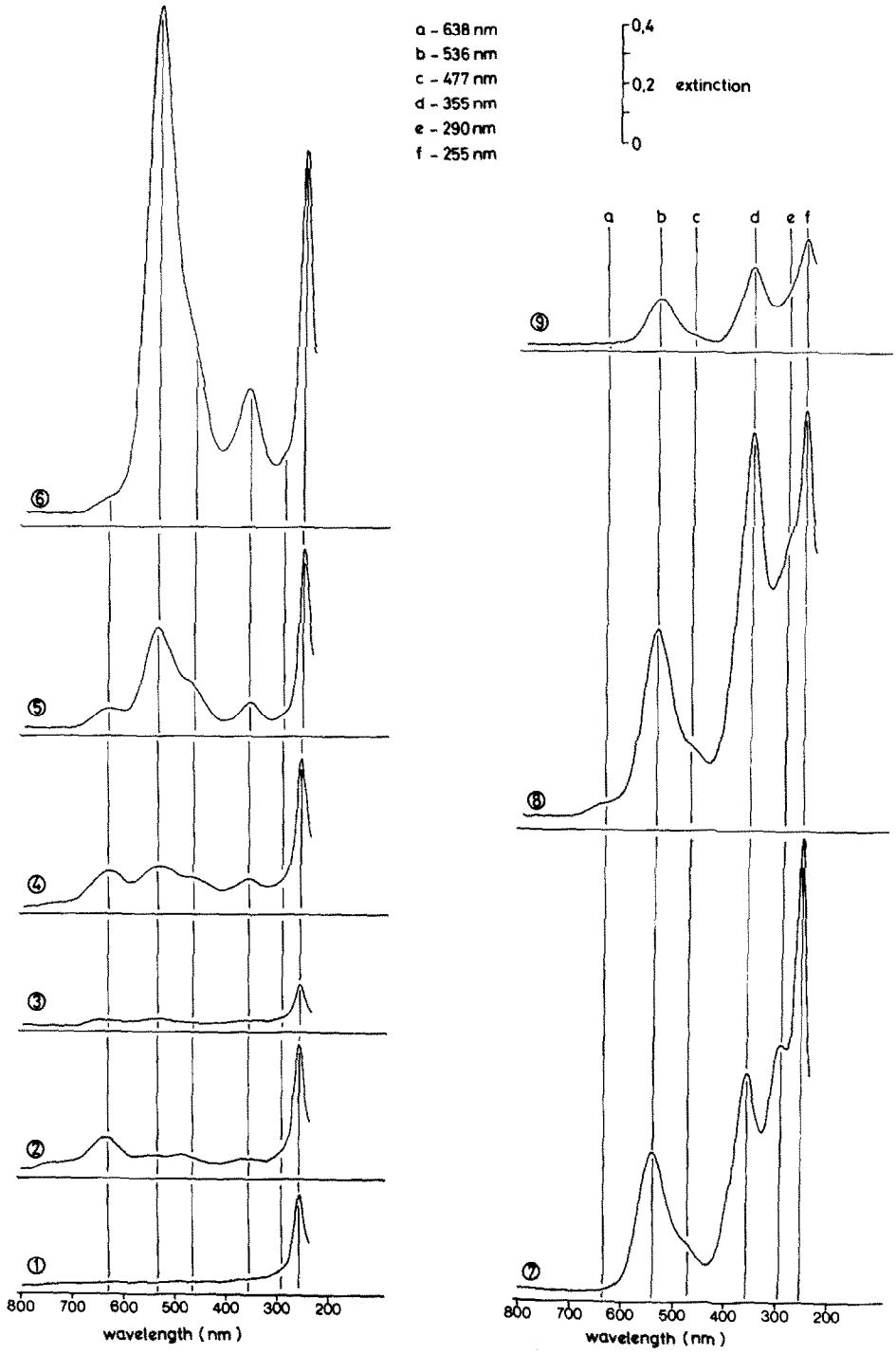

Fig. 1. Absorbance spectra of fractions resolved by thin-layer chromatography. A sample of the crude mixture (see text) was chromatographed on Gelman ITLC (Type SG) with 75% methanol, 25% ammonium acetate (0.1 M final concentration). The nine visible bands were cut into thin strips and eluted in small volumes of 0.1 M ammonium acetate (pH 5.0). Spectra of centrifuged eluates were recorded against a solvent blank.

Partial separation of the components of this mixture was achieved by using sheets of cellulose, DEAE-cellulose and various thin-layer silica and alumina gel media as the supporting adsorbant phase, with combinations of methanol and ammonium acetate [3] as the solvent. The best resolution of visible components was attained on cellulose impregnated with silica gel (Gelman ITLC: Type SG) with a solvent of 95% methanol: 25% ammonium acetate (0.1 M final concentration). The nine visible bands from such a chromatogram were eluted immediately with 0.1 M ammonium acetate (pH 5) and the eluates were centrifuged to remove suspended material. Each of these nine fractions was then analysed for visible- and ultraviolet-absorbing material, for radioactivity, and for its ability to inhibit mitochondrial Ca^{2+} transport.

Fig. 1 shows the absorbance spectra of the nine fractions. Six distinct peaks are apparent, three in the visible region and three in the ultraviolet. The peak at 536 nm and the shoulder at 477 nm are due to Ruthenium Red and Ruthenium Brown respectively [3]; the peak at 355 nm is partially attributable to these complexes [3,6] but is due also to an additional component since its height is not related to the height of the visible peaks for Ruthenium Red and Brown.

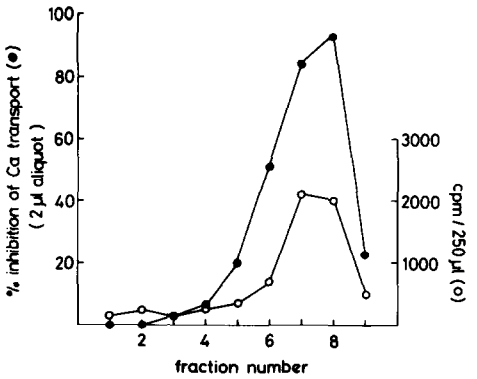

Fig. 2. Radioactivity and inhibitory activity of fractions resolved by thin-layer chromatography. Samples of the nine fractions (fig. 1) were counted for radioacitivity (○) and for their ability to inhibit the initial rate of respiration-supported Ca^{2+} transport (●). The latter effect was assessed by measuring the initial rate of mitochondrial swelling (E_{520}) when 333 µM Ca^{2+} was added to 3 ml of medium containing 4.2 mg of mitochondrial protein in 250 mM sucrose, 5 mM HEPES–Tris (pH 7.4), 5 mM succinate–Tris, 1 µM rotenone, and 10 mM acetate–Tris.

The radioactivity due to ^{103}Ru, and hence the proportion of total ruthenium, is greatest in fractions seven and eight (fig. 2); so too is the potency of inhibition of Ca^{2+} transport (fig. 2). Since Ruthenium Red is most concentrated in fraction six (fig. 1) the inhibitory activity of the latter fractions is due to some other component.

The properties of the ruthenium complexes revealed by the thin-layer separation and elution procedures, together with a series of qualitative adsorption tests, suggested that chromatography on a column of silica under mild acid conditions would be more successful in resolving the components of the mixture. The column adsorbent was prepared by extensive washing and filtration of 70-325 mesh silicic acid in 0.1 M ammonium acetate (pH 5.0) which was used to pack a 1.5 × 50 cm column. The sample was prepared by adsorbing the crude mixture of ^{103}Ru complexes to a small amount of silicic acid at pH 8.5 in 0.1 M ammonium acetate which was carefully layered on top of the column. This was eluted with 0.1 M ammonium acetate (pH 5.0) under nitrogen pressure and 10 ml fractions were collected until the first coloured band reached the bottom of the column (at least six coloured components were visible).

Fig. 3 shows the distribution of radioactivity (^{103}Ru) in the eluate and the activity of each fraction in causing inhibition of Ca^{2+}-stimulated respiration. Two peaks of inhibitory activity were obtained: the first corresponded to the initial peak of radioactivity, but this was less potent than the material concentrated in the second peak (fig. 3). This second region of inhibitory material was associated with only a small shoulder in the radioactivity profile (fig. 3).

Ultraviolet spectra of the column fractions (fig. 4) revealed the presence of four separate absorbing species, none of which had been completely resolved. There is no obvious correlation between any one of these species and the inhibition profile with the possible exception of the small peak at 355 nm (fig. 4); the material absorbing at 255 nm may also show a reasonable correlation.

The second approach was to test ruthenium ammine complexes for inhibitory activity. The rationale behind this search was provided by three separate lines of evidence: firstly, since the unknown compound probably inhibits at the same site as does Ruthenium Red, it would be expected to be structurally related.

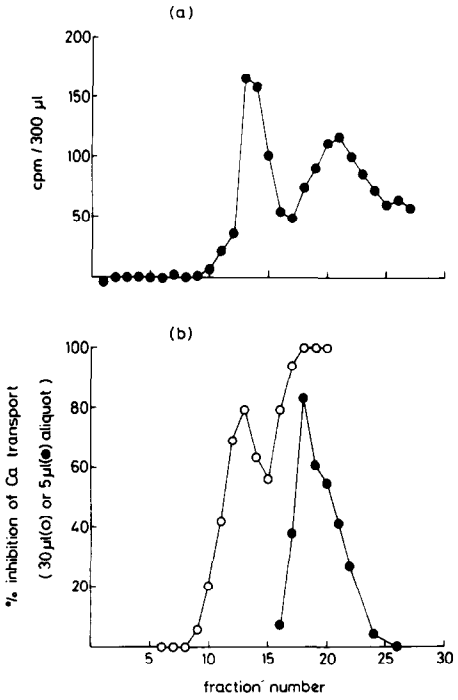

Fig. 3. Radioactivity and inhibitory activity of fractions resolved by silicic acid chromatography. A sample of the crude mixture was chromatographed on a silicic acid column as described in the text. The fraction volume was 10 ml and the flow rate was 85 ml/hr. Room temperature: (a) Radioactivity in 300 µl aliquots; (b) per cent inhibition of the initial rate of respiration-supported Ca^{2+} transport caused by 30 µl (○) or 5 µl (●) samples of each fraction. The initial rate of Ca^{2+} transport was measured as the respiratory stimulation induced by the addition of 500 µM Ca^{2+} to 5.6 (○) or 2.8 (●) mg of mitochondrial protein in 2 ml of 250 mM sucrose, 5 mM HEPES–Tris (pH 7.4), 5 mM succinate–Tris and 10 mM acetate–Tris at 30°C.

The mononuclear analogues are $[Ru(NH_3)_6]^{3+}$, $[Ru(NH_3)_5OH]^{2+}$, and $[Ru(NH_3)_4(OH)_2]^{+}$. Secondly, Tashmukhamedov et al. [9] have reported that 'hexamine cobaltichloride' ($[Co(NH_3)_6]^{3+}$) inhibits mitochondrial Ca^{2+} transport. The ammine complexes of trivalent cobalt are similar in many respects to those of Ru^{III} (e.g. ref. [10]), suggesting that inhibition by $[Co(NH_3)_6]^{3+}$ and the unknown ruthenium complex may be due to structurally-similar compounds.

Finally, the ammoniacal reaction mixture from which Ruthenium Red is synthesized contains a large proportion of ruthenium ammine complexes (J. Broomhead, personal communication).

$[Ru(NH_3)_6]^{3+}$ was tested by dissolving $[Ru(NH_3)_6Cl_3$ in water (λ_{max} = 275 nm [11]) and using the solution immediately. It caused no inhibition of Ca^{2+}-stimulated respiration at concentrations up to 0.5 mM. Neither did $[Ru(NH_3)_5OH]^{2+}$ (λ_{max} = 295 nm), which was prepared as a fresh solution of $[Ru(NH_3)_5Cl]Cl_2$ in 0.1 M NH_3 (the chloro-complex (λ_{max} = 327 nm) undergoes very rapid base-catalysed hydrolysis [10]). However, when a 10 mM solution of ruthenium pentammine chloride in 0.1 M NH_3 was allowed to stand overnight at room temperature it developed inhibitory activity. The resulting solution was dark brown, and in addition to the hydroxypentammine absorption at 295 nm its ultraviolet spectrum showed a shoulder at 355 nm. The ratio of the absorbance at 355 nm to the inhibitory activity of the solution was different from the similar relations for the active fractions from the chromatography procedures described above.

A sample of the aged solution was applied to a microcolumn of silicic acid and eluted with 0.1 M ammonium acetate (pH 5). The only spectral component in the inhibitory fractions of eluate was a strong symmetrical peak at 295 nm due to $[Ru(NH_3)_5OH]^{2+}$. The shoulder at 355 nm in the original spectrum had disappeared and was apparently due to Ruthenium Red and/or a brown complex, both of which adsorbed more strongly to the column than did the major inhibitory compound. There was no evidence of components absorbing at 255 nm.

This work, although incomplete, conclusively demonstrates the existence of a colourless ruthenium complex inhibitory to Ca^{2+} transport, but provides little positive evidence of its identity. That its molecular weight is considerably lower than 800 and that it is a major contaminant of commercial Ruthenium Red were established in earlier studies [5]. Its weak adsorption to silica at pH 5.0 implies a lower cationic charge than Ruthenium Red. It may absorb in the ultraviolet, but if so it has a low extinction coefficient; there is insufficient information available in the literature to assign all spectral peaks to specific complexes, or to establish the products arising from treatment of $[Ru(NH_3)OH]^{2+}$ with ammonia.

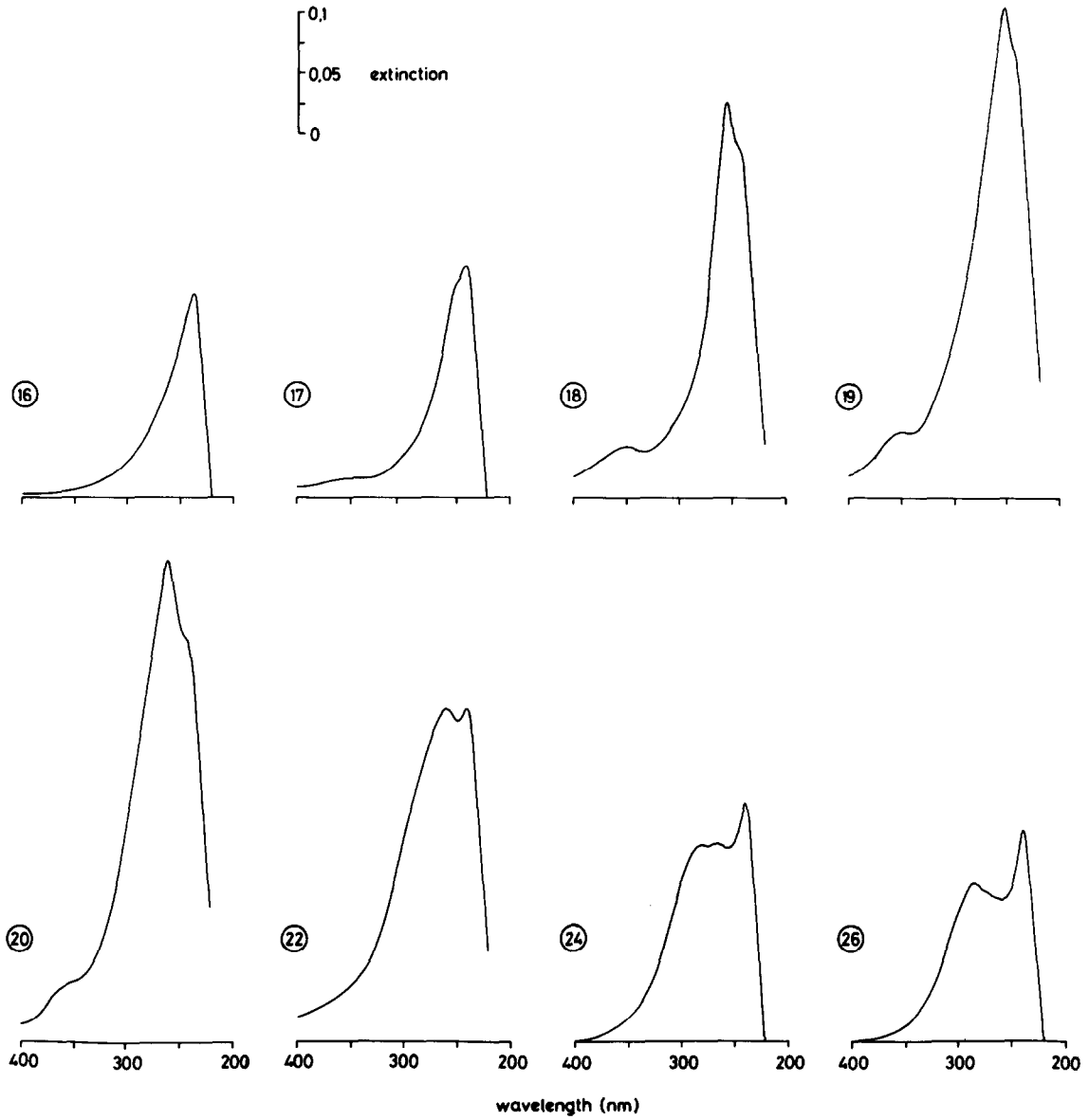

Fig. 4. Ultraviolet absorbance spectra of fractions resolved by silicic acid chromatography. The spectra of the inhibitory fractions shown in fig. 3 were recorded at room temperature against a solvent blank.

Acknowledgements

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