60. *The Reduction of Diphosphopyridine Nucleotide and Some Model Compounds by X-Rays.*

By Gabriel Stein and A. J. Swallow.

Aqueous solutions containing diphosphopyridine nucleotide and ethanol, in the absence of oxygen, when irradiated with *X*-rays, produce acetaldehyde, hydrogen, and a reduced form of the nucleotide.[1,2] Adenosine 5′-phosphate is not a good model for diphosphopyridine nucleotide in this reaction, but 1-alkylnicotinamide chlorides give reduction products. In the case of diphosphopyridine nucleotide, the value of the reduction yield, together with the number of reducing equivalents of the product, strongly suggests that the product is a dimer. The reaction mechanism is discussed and free-radical equations are put forward.

Diphosphopyridine nucleotide can be reduced by irradiating its solutions in oxygen-free water containing excess of ethanol, with *X*- or γ-rays.[1,2] The reaction proceeds through the agency of free hydroxyethyl radicals, and the product differs from the normal enzymic reduction product. The reaction is of interest in connection with the mechanism of dehydrogenase action,[1,2] because of its implications for radiobiology,[3] and because of

[1] Swallow, *Biochem. J.*, 1953, **54**, 253.
[2] *Idem*, *ibid.*, 1955, **61**, 197.
[3] *Idem*, in "Progress in Radiobiology," ed. by Mitchell, Holmes, and Smith, Oliver and Boyd, Edinburgh, 1956, p. 317.

the novel nature of the method of reduction and of the product. This method of reduction and ordinary chemical methods have already been compared,[4] but we have investigated it further in order to ascertain the nature of the reduced product and the reaction mechanism. Some of this work has been briefly reported elsewhere.[3, 5]

FIG. 1. *Absorption curves of 1-methylnicotinamide chloride solution after irradiation with* 78,000 r; ●, *control*; +, *presence of air*; ○, *absence of oxygen.*

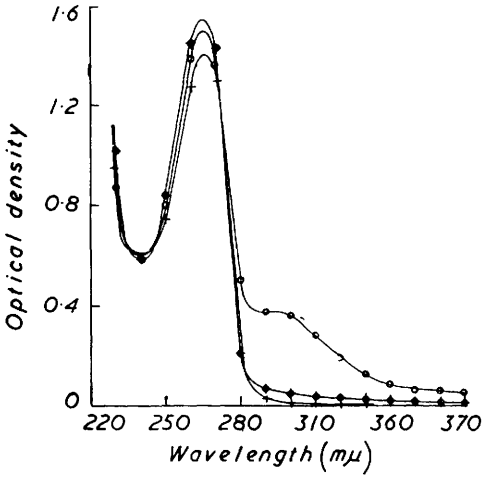
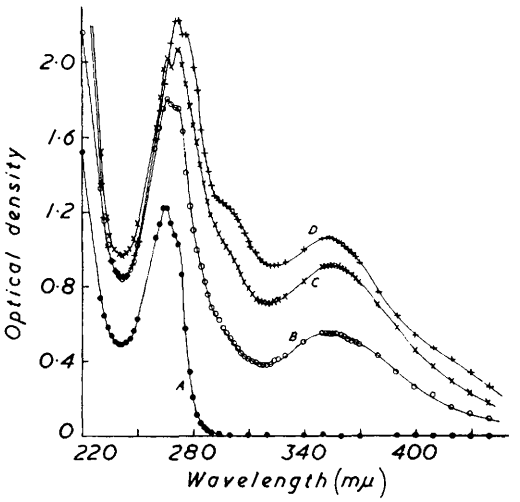


FIG. 2. *Absorption curves of oxygen-free 1-methylnicotinamide chloride solutions irradiated in the presence of* 0·5M-*ethanol*; *A*, *control diluted* 4 ⟶ 3; *B*, 39,000 r; *C*, 78,000 r; *D*, 156,000 r.



EXPERIMENTAL

Irradiation Arrangements.—Two Victor Maximar *X*-ray sets were used, one operated at 220 kvp, with the radiation filtered through 1 mm. of aluminium, and the other at 190 kvp, the radiation being unfiltered. Dosimetry was by the ferrous sulphate method,[6] the yield being taken as $G = 15{\cdot}5$ molecules of ferrous ion oxidised per 100 ev absorbed. The temperature of irradiation was normally 20—30° but during the large-scale reduction of 1-methylnicotinamide chloride the temperature rose to about 40°.

Spectrophotometric Measurements.—1 cm. quartz spectrophotometer cuvettes were used. The absorption curves shown in Figs. 1 and 2 were on undiluted solutions, except where stated otherwise.

Materials.—Diphosphopyridine nucleotide (from the Sigma Chemical Company, U.S.A., and from C. F. Boehringer and Soehne, Germany), crystalline yeast alcohol dehydrogenase (from C. F. Boehringer and Soehne), and adenosine 5′-phosphate (from Schwarz Laboratories, U.S.A.) were used as received. Methylene-blue was a commercial product, recrystallised three times from water. 1-Methyl- and 1-propyl-nicotinamide and chloride were prepared from nicotinamide by the method of Karrer *et al.*[7] Water was distilled from alkaline permanganate. Other chemicals were of " AnalaR " purity.

Spectrophotometric Measurements without Exposure to Oxygen.—The ultraviolet absorption curves given previously [1, 2] were obtained after tipping of irradiated solutions into spectrophotometer cuvettes for measurement. As it seemed possible that exposure to air at this stage could affect the results, the experiments have been repeated in an enclosed system. Air-free aqueous solutions of diphosphopyridine nucleotide (3×10^{-4}M) were irradiated with a dose of 39,000 r. The results obtained were as before, *i.e.*, a product absorbing at 340 mμ was formed

[4] Stein, *J. Chim. phys.*, 1955, **52**, 634; Stein and Stiassny, *Nature*, 1955, **176**, 734; Ke, *Arch. Biochem. Biophys.*, 1956, **60**, 505.
[5] Stein and Swallow, *Nature*, 1954, **173**, 937.
[6] Donaldson and Miller, *J. Chim. phys.*, 1955, **52**, 578; Haybittle, Saunders, and Swallow, *J. Chem. Phys.*, 1956, **25**, 1213.
[7] Karrer, Schwarzenbach, Benz, and Solmssen, *Helv. Chim. Acta*, 1936, **19**, 811.

when solutions were 0·5M in ethanol, but there was no product in absence of ethanol. Measurement for Tables 1 and 2 were made without exposure to air.

Enzymic Determination of Yield.—*ca.* 3×10^{-4}M-Diphosphopyridine nucleotide solutions, 0·5M in ethanol and at pH 7·8, were irradiated with an *X*-ray dose of 20,000 r. The irradiated solutions were diluted and sodium hydroxide added. The optical density at 340 mμ was measured, alcohol dehydrogenase was added to convert unchanged diphosphopyridine nucleotide into the dihydro-form, and the optical density was again measured. Final pH's were about 9. The difference between the first and the second optical density reading is a measure of unchanged diphosphopyridine nucleotide, and the initial concentration was calculated from control experiments with unirradiated solutions. The yield of diphosphopyridine nucleotide altered by radiation was calculated to be $G = 6{\cdot}9$ molecules/100 ev. This value was independent of the source of the nucleotide and should be correct to about 10%. If all the diphosphopyridine nucleotide had been converted into a dihydro-compound, then from the optical density of irradiated solutions the molar extinction coefficient would be $3{\cdot}2 \times 10^3$ If the product were a dimer, then the molar extinction coefficient would be $6{\cdot}4 \times 10^3$. To avoid possible uncertainty due to the enzyme method, these experiments were repeated, using however the spectrophotometric method based on the formation of a cyanide complex by the unchanged nucleotide ion (DPN^+). The results obtained gave a value for $-G_{DPN^+}$ in good agreement with that from the enzymic method.

Determination of the Number of Reducing Equivalents of the Product.—In irradiations as above, if the product were a dihydro-compound with two reducing equivalents, the optical density found would correspond to a concentration of $2{\cdot}4 \times 10^{-4}$N. For a dimer with two reducing equivalents, the concentration would be $1{\cdot}2 \times 10^{-4}$N. 0·6 ml. of this solution was mixed *in vacuo* with 3 ml. of $4{\cdot}6 \times 10^{-5}$N-methylene-blue, and reduction was followed by the decrease in optical density at 655 mμ. From the amount of methylene-blue reduced the irradiated solution was calculated to be $1{\cdot}1 \times 10^{-4}$N. This method gave the correct result when tested on dihydrodiphosphopyridine nucleotide of known concentration, confirming the view that the product is a dimer.

Adenosine 5′-Phosphate.—It has been shown elsewhere [2] that the product from diphosphopyridine nucleotide, which absorbs at 340 mμ, is a reduced form. Reduction at the nicotinamide end of the molecule seems probable, but to eliminate the possibility that the adenine end is concerned we irradiated $2{\cdot}8 \times 10^{-4}$M-solutions of adenosine 5′-phosphate in 0·01M-pyrophosphate–hydrochloric acid buffer at pH 7·8 with an *X*-ray dose of 78,000 r. The optical density of the solution at 260 mμ was decreased by irradiation whether dissolved oxygen was present or not. Ethanol (0·5M) prevented the decrease in the presence of air, but in the absence of air there was a large decrease in optical density at 260 mμ and an increase below 235 mμ and at 290—300 mμ. However, in no case was there an increase near 340 mμ of the type found for diphosphopyridine nucleotide. It is concluded that the appearance of the absorption band at 340 mμ cannot be ascribed to a change at the adenine moiety, although it is possible that the small decrease at 260 mμ which is also observed [2] may be partly due to a change at this part of the molecule.

1-*Alkylnicotinamide Chlorides.*—Methylnicotinamide chloride was chosen as a model for the nicotinamide end of diphosphopyridine nucleotide. The absorption curve of $3{\cdot}9 \times 10^{-4}$M-solutions, irradiated with 78,000 r in the presence of air at pH 7·8, is little affected (Fig. 1), but in absence of oxygen (Fig. 2) there is an increase in absorption at 290—300 mμ which is found also when the solution is saturated with hydrogen. Fig. 2 shows also that irradiation of oxygen-free solutions 0·5M in ethanol increases absorption at 355 mμ, this resembling the increase at 340 mμ obtained with diphosphopyridine nucleotide under these conditions. In the present case, the optical density increases near 275 mμ, behaviour not observed with diphosphopyridine nucleotide. However, many of the properties of our irradiated chloride resemble those of irradiated diphosphopyridine nucleotide. In particular, when 0·1 ml. of N-hydrochloric acid was added to 3 ml. of an irradiated solution, the absorption bands at 355 and 275 mμ disappeared and a new band appeared at 295 mμ: this change occurred slowly even on storage without addition of acid, but was less pronounced at pH 10·4 than at pH 7·8. Irradiated solutions did not fluoresce under Wood's light (pH 7·8 or 10·4) but a pale blue fluorescence appeared on addition of 1 : 4-dihydro-1-methylnicotinamide, prepared from 1-methylnicotinamide chloride and sodium dithionite. Irradiated solutions were bright yellow; solutions of 1 : 4-dihydro-1-methylnicotinamide with the same optical density at 360 mμ were paler.

Isolation of the Reduction Product of 1-*Methylnicotinamide Chloride.*—A 0·034% aqueous solution of the methochloride containing 3% of ethanol was prepared in a carbonate–bicarbonate buffer at pH 10·2. 250 ml. of the solution in a round-bottomed flask were deaerated by evacuation, filling with oxygen-free nitrogen (British Oxygen Company), and two repetitions of this cycle. The solution was given a dose of 260,000 r (5·5 hr.), becoming bright yellow. The aqueous solution was evaporated to about 10 ml. under reduced pressure of nitrogen. 1 ml. of N-sodium hydroxide was added, and the solution extracted six times under nitrogen with butan-1-ol (previously washed with N-sodium hydroxide). The butanol extract was dried (Na_2SO_4) and evaporated under reduced pressure of nitrogen to leave an orange oil. This was left overnight in a vacuum-desiccator, then extracted, under nitrogen, with chloroform. The chloroform extract was evaporated under nitrogen, and a solid which separated was filtered off. The residual oil was dissolved in a minimum of methanol and evaporated to dryness. This was repeated and the oil finally dried at room temperature *in vacuo*. The final product was a golden-brown sticky solid.

It seems from the experiments described that 1-methylnicotinamide chloride in many ways resembles diphosphopyridine nucleotide whereas adenosine 5′-phosphate does not. The irradiation product of the methochloride was too labile to be recrystallised. It did not fluoresce under Wood's light whereas 1 : 4-dihydro-1-methylnicotinamide exhibited a brown fluorescence in the solid state and was less soluble in chloroform. An aqueous solution of the irradiation product (1 mg./ml.) had pH ~6. It reduced silver nitrate and did not contain chloride. It decolorised methylene-blue solutions at the same rate as 1 : 4-dihydro-1-methylnicotinamide. A solution at pH 11 exhibited absorption maxima at 278 and 355 mμ.[5] Wallenfels and Schüly [8] also obtained a reduced nicotinamide derivative possessing two absorption bands. The solution showed only a slight pale green fluorescence which was very much less than that of 1 : 4-dihydro-1-methylnicotinamide. On addition of acid, the absorption maxima shifted irreversibly to give a single peak at 295 mμ, like that given by 1 : 4-dihydro-1-methylnicotinamide.

The properties of the irradiation product show it to be a reduced form of the methochloride different from 1 : 4-dihydro-1-methylnicotinamide. Reduction at the amide group cannot explain the properties; the product might be a dihydro-compound (I) or (II) [7] or a dimer, *e.g.*, (III; R = Me).[9]

As the methochloride did not crystallise, we examined the propylochloride. This behaves on irradiation in a similar manner to the methochloride, except that it gives a crystalline product,[4] whose properties differ from those of the dithionite reduction product; the elementary analysis is consistent with a dihydro-structure and the previous titrations [4] support this. The structure of the product is therefore probably (I or II; R = Pr^n). Further work on this compound is in progress.

On irradiation of solutions of the propylochloride at various concentrations in the presence of various amounts of ethanol at pH 9·2 the optical density at 360 mμ rose linearly with dose up to 37,500 r in every case except for $3·9 \times 10^{-5}$M-solutions which were 0·5M in ethanol (for which the optical density reached a maximum at 10,000 r) and $3·9 \times 10^{-4}$M-propylochloride solutions 10^{-2}M or 2×10^{-2}M in ethanol (for which a slight downward curve was noted). As a measure of relative yield under the various conditions, the small optical density of a control solution was subtracted from the optical density resulting after 10,000 r. Results given in Tables 1 and 2 show that the yield is little affected by the concentration of the reactants, and therefore here there is no chain reaction.

TABLE 1. *Effect of concentration of* 1-*propylnicotinamide chloride on yield of substance absorbing at* 360 mμ. (0·5M-EtOH.)

Propylochloride concn. (M)	$3·9 \times 10^{-1}$	$3·9 \times 10^{-2}$	$3·9 \times 10^{-3}$	$3·9 \times 10^{-4}$	$3·9 \times 10^{-5}$
Optical density at 360 after 10,000 r *minus* initial optical density	0·22	0·27	0·275	0·23	>0·15

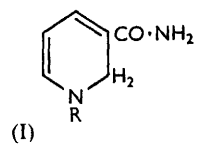
TABLE 2. *Effect of concentration of ethanol on yield of substance absorbing at* 360 mμ. ($3·9 \times 10^{-4}$M-1-*propylnicotinamide chloride.*)

EtOH concn. (M)	3·0	1·0	0·5	0·1	0·05	0·02	0·01
Optical density (as Table 1)	0·24	0·235	0·225	0·21	0·20	0·19	0·17

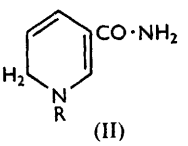
[8] Wallenfels and Schüly, *Angew. Chem.*, 1955, **67**, 517.
[9] Tompkins and Schmidt, *Univ. Calif. Pub. Physiol.*, 1943, **8**, 247.

Discussion

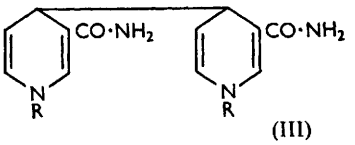
Nature of the Reduction Product of Diphosphopyridine Nucleotide.—As Karrer and his collaborators recently emphasised,[10] the reduction of *N*-substituted nicotinamide derivatives is highly complex, and in particular is strongly dependent on the nature of the substituent. The reduction of diphosphopyridine nucleotide itself does not, therefore, necessarily give rise to the same type of product as is obtained from the model compounds. Information bearing on the nature of the product as been obtained from a determination of the yield of diphosphopyridine nucleotide reduced by irradiation in aqueous solution containing ethanol. Approximate values have been estimated previously from the optical density of irradiated solutions at 340 mμ on the assumption that the product has the same extinction coefficient as dihydrodi-



(I)



(II)



(III)

phosphopyridine nucleotide.[11] This method can only give a very rough estimate because the product is *not* dihydrodiphosphopyridine nucleotide. Moreover, previous spectrophotometric results have been only semi-quantitative. The yield has now been measured enzymically and found to be $G = 6{\cdot}9 \pm 0{\cdot}6$ molecules of diphosphopyridine nucleotide reduced per 100 ev absorbed. In view of the general agreement that the yield of radicals in irradiated water is about $G = 6$ radicals per 100 ev absorbed [12] it is difficult to explain a reduction yield as high as G 6·9 without postulating either that the reduction is a one-equivalent one, in which case the product cannot be a dihydro-compound, or that the reaction involves a chain mechanism. However, the reaction does not show the features of a chain reaction, and, in particular, the yield of acetaldehyde on irradiation of ethanolic $2{\cdot}7 \times 10^{-3}$M-diphosphopyridine nucleotide is the same as that from $2{\cdot}7 \times 10^{-4}$M-solutions.[1] To explain the high yield we therefore conclude that the reduction is a one-equivalent one, so that the product does not possess structure (I) or (II; R = ribose-pyrophosphate-ribose-adenine). Additional weight is lent to this argument from related work with methylene-blue,[13] *p*-benzoquinone,[14] and riboflavin.[15] In the case of methylene-blue, where reduction involves two equivalents, the yield has recently been found to be G 3·1. From benzoquinone (irradiated in the presence of excess of formic acid) the yield of quinol was 2·7—2·8, except at high quinone : formic acid ratios where different effects (which could not explain the diphosphopyridine nucleotide results) were evident. For riboflavin, where only one reducing equivalent is needed to produce the stable semiquinone free radical of riboflavin, the yield has been found to be G 6·6.

The value of the yield of diphosphopyridine nucleotide reduced strongly supports the idea that the reduction product is a dimer such as (III), and this is confirmed by measurements of the number of reducing equivalents of the product, measured against methylene-blue. It is therefore highly probable that the product is a dimer, though not necessarily (III), and the two halves of the molecule may be joined elsewhere than at position 4 in the pyridine rings.

Mechanism of the Reduction of Diphosphopyridine Nucleotide.—The principal facts to be accounted for are: (1) the yield of dimer formed by irradiation is G 3·4 molecules/100 ev (*i.e.*, half the yield of diphosphopyridine nucleotide reduced); (2) the acetaldehyde yield [1] in the system is G 5·9 molecules formed/100 ev; (3) the hydrogen yield is G 3·2 molecules formed/100 ev.[2]

We consider the available evidence to exclude participation of sub-excitation electrons [16] in the reaction. The starting point of this discussion must then be the reaction: [17]

$$H_2O \longrightarrow\!\!\!\!\sim\!\!\!\!\rightarrow H + OH \qquad \ldots\ldots\ldots \quad (I)$$

[10] Brook, Blumer, Krishna, Schnell, and Karrer, *Helv. Chim. Acta*, 1956, **39**, 667.
[11] Barron, Johnson, and Cobure, *Radiation Res.*, 1954, **1**, 410.
[12] Hart, *Ann. Rev. Phys. Chem.*, 1954, **5**, 139.
[13] Day and Stein, unpublished work.
[14] Baxendale and Smithies, *Z. phys. Chem.* (*Frankfurt*), 1956, **7**, 242.
[15] Swallow, *Nature*, 1955, **176**, 793.
[16] Weiss, *J. Chim. phys.*, 1955, **52**, 539; Platzman, *Radiation Res.*, 1955, **2**, 1.
[17] Weiss, *Nature*, 1944, **153**, 748.

This reaction is essentially true for "ideal" *X*-rays,[18] but with ordinary *X*- and γ-rays it is usually necessary to allow for reactions such as the formation of molecular hydrogen and hydrogen peroxide.[19] However, the accuracy of the experiments discussed here does not merit a detailed discussion of minor reactions and we therefore feel justified at first in discussing only the principal reaction, *i.e.*, reaction (1).

When aqueous ethanol is irradiated in the absence of oxygen, the products include acetaldehyde, hydrogen, and butane-2 : 3-diol.[20] The yield of hydrogen is high (G = 2·8 molecules/100 ev) [2] and is probably due to the reaction:

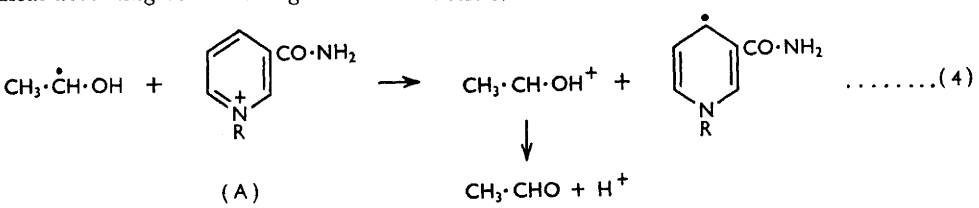
$$H + CH_3{\cdot}CH_2{\cdot}OH \longrightarrow H_2 + CH_3{\cdot}\dot{C}H{\cdot}OH \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

There is growing evidence that this type of reaction occurs, and in particular a similar reaction has recently been put forward for methanol.[21] The view that the free radicals resulting from the oxidation of alcohols are hydroxyalkyl rather than alkoxyl is supported by the detection of glycols in irradiated solutions.[20, 21]

The hydroxyl radicals produced in reaction (1) react with ethanol according to the well-established reaction:

$$OH + CH_3{\cdot}CH_2{\cdot}OH \longrightarrow H_2O + CH_3{\cdot}\dot{C}H{\cdot}OH \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

The experiments with diphosphopyridine nucleotide were conducted with ethanol concentrations of 5×10^{-1}M and diphosphopyridine nucleotide concentrations of 3×10^{-4}M, so that ethanol would be likely to protect the nucleotide from the free radicals formed in reaction (1). It seems that no part of diphosphopyridine nucleotide is sufficiently "radiosensitive," compared with ethanol, for this expectation to be improbable,[22] and further the results with air-saturated solutions confirm the protection afforded by ethanol to diphosphopyridine nucleotide. The reduction of tetrazolium salts by Fenton's reagent in the presence of ethanol [23] has confirmed the reducing action of hydroxyethyl radicals and it is therefore highly probable that diphosphopyridine nucleotide is being reduced by such radicals, probably to give a resonance-stabilised free radical according to the charge-transfer reaction:



The diphosphopyridine nucleotide radicals so formed then dimerise to give the final reduction product represented tentatively as (III).

From these equations, starting with a yield of G = 3 for reaction (1), we can calculate the yield of the various products. The molecular yield of hydrogen (G_{H_2} 0·5), which has so far been neglected, must now be added to the yield of hydrogen calculated from reaction (2). The total reaction is then given by reaction (5).

$$H + OH + 2C_2H_5{\cdot}OH + 2\,(A) \longrightarrow 2CH_3{\cdot}CHO + 2H^+ + H_2 + H_2O + (III) \quad (5)$$

$-G_{calc} = 6$	$G_{calc} = 6$	$G_{calc} = 3 + 0{\cdot}5 = 3{\cdot}5$	$G_{calc} = 3$
$-G_{found} = 6{\cdot}9$	$G_{found} = 5{\cdot}9$	$G_{found} = 3{\cdot}2$	$G_{found} = 3{\cdot}4$

It will be seen that the experimental value for $-G_{DPN}$ is greater than $+G_{CH_3{\cdot}CHO}$, whilst the values calculated for reaction (5) are equal. On the other hand, less hydrogen is evolved than the calculated G_{H_2}. This may be due to some of the hydrogen atoms' being used up in reducing the ion DPN^+.

[18] Stein and Swallow, in "Advances in Radiobiology," Oliver and Boyd, Edinburgh, 1957, p. 16.
[19] Allen, *Radiation Res.*, 1954, **1**, 85.
[20] Scholes, *J. Chim. phys.*, 1955, **52**, 640.
[21] McDonell, *ibid.*, p. 208.
[22] Swallow, Ph.D. thesis, Cambridge, 1954.
[23] Mackinnon and Waters, *J.*, 1953, 323.

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DEPARTMENT OF RADIOTHERAPEUTICS, UNIVERSITY OF CAMBRIDGE,
DOWNING STREET, CAMBRIDGE.
DEPARTMENT OF PHYSICAL CHEMISTRY, THE HEBREW UNIVERSITY,
JERUSALEM, ISRAEL.
[Present address (A. J. S.): TUBE INVESTMENTS RESEARCH LABORATORIES,
HINXTON HALL, CAMBRIDGE.]

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