Cyclic Voltammetry of the Pyridine Nucleotides and a Series of Nicotinamide Model Compounds*

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ABSTRACT: On the basis of polarographic studies, it had been postulated that the first step in the electrochemical reduction of the pyridine nucleotides involved a reversible electron transfer leading to a free radical; this was followed by an irreversible chemical process believed to be dimerization. Much more conclusive evidence for the existence of this radical has now been obtained by cyclic voltammetry using asymmetric voltage sweeps. Following a scan in the negative direction, a fairly fast, reverse voltage sweep discloses the reversible reoxidation of a transient intermediate, as well as the oxidation of the chemical product at a much more anodic potential. Similar behavior is observed with oxidized diphosphopyridine nucleotide, oxidized triphosphopyridine nucleotide, and 1-methyl-, 1-ethyl-, 1-*n*-propyl-, and 1 benzyl-3-carbamidopyridinium chlorides. Pronounced adsorption effects at the electrode surface complicate the theoretical treatment of the data and lead to uncertainty in calculated dimerization rate constants.

In previous papers, polarographic and controlled potential coulometric studies of 1-methyl-3-carbamidopyridinium chloride (Burnett and Underwood, 1965a) and DPN[1] (Burnett and Underwood, 1965b) were described. More recently, similar work has been completed on several other 1-alkyl-3-carbamidopyridinium salts (A. L. Underwood and M. T. Barnhill, 1965, unpublished observations) and TPN (Cunningham and Underwood, 1966). Reduction patterns for all of these compounds are essentially the same. The first of the two polarographic waves appears to represent a reversible, one-electron transfer process; this is followed by an irreversible chemical step, the product of which is reoxidized only at a much more positive potential. A mechanism involving free-radical formation followed by dimerization of the radical was postulated (Burnett and Underwood, 1965b).[2] The two-electron reduction product (1-alkyl-3-carbamido-1,4-dihydropyridine, DPNH, or TPNH) is formed on the second polarographic wave; with the 1-methyl compound, this is the only product, whereas with DPN^+ and TPN^+ a mixture of dihydro compound and dimer is obtained. Admittedly, rigorous structure work on the product of the first wave has not been completed. It is referred to here as "dimer" for convenience; the data suggesting that it is a 4,4′ dimer have been summarized (Burnett and Underwood, 1965b).

Free-radical formation on the first wave was actually only inferred in the previous work, although it appeared to be entirely reasonable. The voltage sweep in classical polarography is much too slow to permit detailed study of transient intermediates whose lifetimes are of the order of perhaps milliseconds or even less. The technique of cyclic voltammetry at suitable voltage sweep rates promised much more information. Using this method, clear evidence for a radical intermediate has been obtained. Also, the adsorption characteristics of oxidized and reduced species on the mercury electrode surface have been seen more clearly than before. Although this work is entirely nonphysiological and no conclusions about *in vivo* processes can be drawn from it, the existence of a radical intermediate stable enough to detect electrochemically at least opens to question whether the pyridine coenzymes possess unelucidated metabolic functions.

In cyclic voltammetry a triangular voltage sweep is applied to a quiet electrode. Thus the product of a cathodic process is available at the electrode surface for reoxidation as the voltage sweeps back in an anodic

* From the Department of Chemistry, Emory University, Atlanta, Georgia 30322. *Received March 31, 1966.* This investigation was supported by the U. S. Public Health Service Research Grant No. GM 08282 from the Division of General Medical Sciences, National Institutes of Health. TPN was purchased through a grant from the McCandless Fund, awarded by the Emory University Research Committee. This paper is based upon a dissertation presented by A. J. Cunningham in partial fulfillment of the requirements for the Ph.D. degree, Emory University, 1966.

[1] Abbreviations used in this work: DPN, diphosphopyridine nucleotide; DPN^+, oxidized DPN; DPNH, reduced DPN; TPN, triphosphopyridine nucleotide; TPN^+, oxidized TPN; TPNH, reduced TPN; sce, saturated calomel electrode.

[2] In our first paper on the electrochemistry of the methylnicotinamide model compound (Burnett and Underwood, 1965a), it was unfortunately stated that the first wave reduction process was irreversible. Although, to be sure, the over-all process is irreversible (*i.e.*, the dimer is reoxidized only at a much more positive potential than that required for its formation), the electron-transfer step itself is reversible by the usual polarographic criteria. The behavior of all of the compounds studied is the same in this regard. The wording in this paper was misleading, since some readers have supposed that the methyl model compound behaves differently from DPN^+ and TPN^+, which is not the case.

direction. Current–voltage curves obtained by cyclic voltammetry exhibit peaks rather than the more familiar polarographic plateaus because the area of the stationary electrode is constant and the electrode does not expand into the solution as does the growing drop with the usual dropping mercury electrode. In most of the work in this field, the triangular wave has been symmetric, and sweep rates have been relatively slow, say, from 0.1 to perhaps 10 v/min. X-Y recorders are commonly employed for recording the cyclic voltammograms. However, it is easily possible to employ an asymmetric sweep; for example, one can scan slowly in a cathodic direction, and then complete the anodic portion of the scan very much more rapidly. Also, much higher sweep rates than the customary ones are readily employed, say, several hundred volts per second. With very fast sweep rates, oscillographic recording becomes necessary. Cyclic voltammetry was probably first described by Sevcik (1948). Kemula and his co-workers developed the technique extensively (*e.g.*, Kemula and Kublik, 1958), and many applications have been described by Adams (*e.g.*, Mizoguchi and Adams, 1962).

Experimental Section

Materials. β-DPN$^+$ was obtained from P-L Biochemicals, Inc., Milwaukee, Wis., and TPN$^+$ from Nutritional Biochemicals Corp, Cleveland, Ohio. Tetra-*n*-butylammonium hydroxide was obtained from Southwestern Analytical Chemicals Co., Austin, Texas. 1-Alkyl-3-carbamidopyridinium iodides were prepared by the method of Karrer *et al.* (1936) and converted to the corresponding chloride salts by shaking aqueous solutions with freshly precipitated silver chloride. After filtration of silver iodide and excess silver chloride, the chloride salts were separated from the solution by the addition of ethanol and ether. Frequently in this step, even with very slow addition of the organic solvents, the salts came out as oils rather than solids. In this event, the salt was separated from the water–ethanol–ether phase and heated to about 110° to boil out the solvents, after which the oil crystallized nicely upon cooling. The salts were recrystallized from ethanol; in some cases, a little acetone was added in the latter stages to improve the filterability. The salts melt with decomposition; thus melting points are poorly reproduced, but fairly good checks with literature values were obtained. Argentimetric titration of the chloride indicated a purity of at least 99.5% in all cases.

Apparatus. The circuit is shown schematically in Figure 1. The controlled voltage source was a Heath EUW-19A operational amplifier system with a plug-on sweep generator as described by Weir and Enke (1964). The generator provides a choice of symmetric or asymmetric wave form with sweep rates of 1 mv/sec to 500 v/sec. The cyclic voltammograms were displayed and recorded using a Tektronix type 564 oscilloscope equipped with type 2A63 differential amplifier and type 3A72 dual trace amplifier plug-in units and a Tektronix C-12 oscilloscope camera with a Polaroid back.

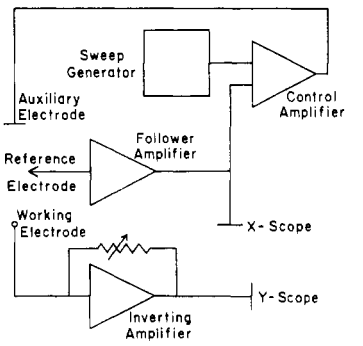

FIGURE 1: Schematic diagram of circuit for cyclic voltammetry.

The working electrode was a stationary hanging mercury drop with a surface area of 0.02 cm^2 which could be introduced into the solution reproducibly by means of a Metrohm type E-140 micro-feeding apparatus. A mercury pool and an sce were employed as auxiliary and reference electrodes, respectively. The water-jacketed electrolysis vessel was held at 25.0 ± 0.1° by means of a Haake Model F constant temperature circulator.

pH measurements were performed with a Leeds and Northrup pH meter, Model 7401, equipped with 124,138 miniature electrodes. All solutions were prepared using deionized water and reagent chemicals.

Procedure. Solutions which were generally about 1×10^{-3} M were prepared by dissolving solid DPN$^+$, TPN$^+$, or 1-alkylnicotinamide in 0.2 M acetate (pH 5), phosphate (pH 7), or tetra-*n*-butylammonium carbonate (pH 9) buffers which were 0.5 M in potassium chloride. A 5-ml portion of the solution was purged with prepurified nitrogen for about 10 min, after which a nitrogen blanket was maintained for the duration of the experiment. A portion of mercury was expelled from the capillary, and then a fresh drop was quickly hung and the voltage sweep was initiated. (A fresh drop was used in all cases except those where repetitive cycling to study adsorption was required, as noted below.) Each solution was examined with a wide range of sweep rates and sweep limits. Replicate measurements were performed by observing the oscilloscope trace utilizing the storage facility of the scope, although only a minimal number of photographs were made. Attention was focussed upon the first of the two reduction processes, because the major goal was evidence for free-radical formation.

Results

Broadly, neglecting details to be noted below, at pH values of 5 and 7, all of the compounds exhibit a cathodic peak at about −1 v *vs.* sce and, at slow sweep rates, one anodic peak at a much more positive

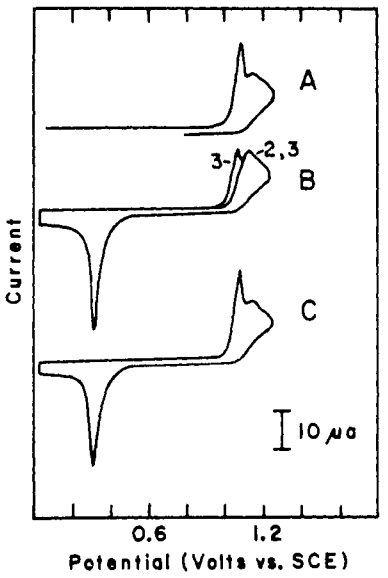

FIGURE 2: Cyclic voltammograms for 1-*n*-propyl-3-carbamidopyridinium chloride. A solution 1.0×10^{-3} M in phosphate buffer of pH 7.0; scan rate 2 v/sec in each direction; curve C is a typical complete single cycle; for explanation of curves A and B, see text.

potential (−0.35 v *vs.* sce for the 1-alkylnicotinamides and −0.15 v for DPN and TPN). At pH 9, a second cathodic process is seen as a shoulder on the large current representing breakdown of the background electrolyte solution. The two cathodic processes and the anodic peak correspond to the now-familiar polarographic waves of the system which have been described previously (Burnett and Underwood, 1965a,b; Cunningham and Underwood, 1966). Only the first cathodic peak was studied in detail, and hereafter mention of *cathodic peak* implies this one.

With ethyl-, propyl-, and benzylnicotinamides, closer inspection shows that the cathodic process actually comprises two peaks which are very close together. A typical single cycle for the propyl compound is shown at C in Figure 2. A possible interpretation may be given on the basis of the following experiment. Curve A shows a scan with a fresh mercury drop where both peaks may be seen, the second less prominent than the first. As shown, the anodic portion of the scan was terminated at about −0.8 v *vs.* sce; *i.e.*, the electrode did not become positive enough to reoxidize the product of the cathodic scan. Beginning at this point, a second cathodic scan was initiated, which yielded the peak designated 2 in curve B of Figure 2; the first of the two original cathodic peaks had disappeared. Now the anodic sweep was allowed to proceed through the potential for oxidation of the reduction product, and the next cathodic scan (labeled 3 in curve B of Figure 2) showed once again the two cathodic peaks as in the original scan.

This seemingly strange behavior can be explained by the following hypothesis. The first of the two cathodic peaks represents the reduction of oxidized species which is adsorbed at the electrode surface. (This adsorption occurs fairly rapidly, because a fresh mercury drop was hung and the first scan was initiated within a few seconds.) The second cathodic peak represents the reduction of so-called soluble species supplied to the electrode by diffusion from the solution. Now suppose that the reduction product is much more strongly adsorbed than the oxidized form. After a cathodic sweep which has formed this product, and in the absence of an anodic sweep sufficiently positive to remove it, the adsorbed oxidized species is no longer present and its peak is absent from the next cathodic scan, where only reduction of the soluble species is seen. After another anodic sweep which does remove the reduction product, both adsorbed and soluble oxidized species are once again observed. The process as described here would be referred to by electroanalytical chemists as an AR–SR mechanism: adsorbed material reduced first, then soluble material. (The term "soluble" in this connection simply distinguishes a substance in solution from an adsorbed solute.)

The data in this paper do not unequivocally prove this mechanism. It is possible, for example, that the earlier peak represents reduction of soluble species to form a strongly adsorbed product through which a hampered electron transfer leads to the second, slightly more negative peak. However, much stronger evidence for the AR–SR mechanism has been obtained with the same compounds by Epple and Wilson (1966), using current reversal chronopotentiometry. Our data are compatible with this, and the interpretation given here is the simplest one which is consistent with all of the facts. Polarographic observations (Burnett and Underwood, 1965b) had suggested that adsorption at the electrode surface was prominent in the case of DPN. It has turned out, not unexpectedly, that the details of the adsorption phenomena have best been studied by chronopotentiometry, while cyclic voltmanmetry as reported here has provided the clearest evidence for the one-electron free-radical intermediate (see below).

With 1-methylnicotinamide, only one of the two cathodic peaks is observed. As best the potentials can be measured, it appears that this peak corresponds to the more negative of the two observed with the ethyl, propyl, and benzyl compounds. From the interpretation of the two peaks given above, it follows that absorption of the oxidized species is much less pronounced in this case. With DPN and TPN, again only one peak is observed, but its potential corresponds much more nearly to the reduction of adsorbed material.

Peak potentials for the processes described above are given in Table I for a sweep rate of 1.15 v/sec. Actually, as expected, the peak potential is a function of sweep rate for all of the compounds studied. Indeed, in ideal cases, the rate constant for a chemical reaction following the electron-transfer step can be calculated from the effect of sweep rate upon peak potential (Nicholson, 1965a). In the present case, dimerization rate constants of 2.24×10^{-2}, 8.49×10^{6}, and 5.45

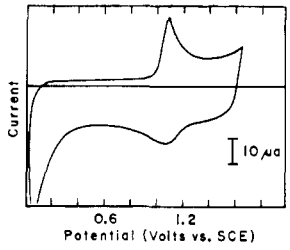

FIGURE 3: Cyclic voltammogram for TPN with asymmetric sweep, showing free-radical oxidation. A solution 1.0×10^{-3} M in phosphate buffer of pH 7.0; forward (cathodic) sweep rate 5.7 v/sec, reverse sweep rate 57.3 v/sec.

TABLE I: Peak Potentials for Cathodic Peaks.[a]

Compound	Peak Potential (v *vs.* sce)	
1-Methylnicotinamide	—	−1.140
1-Ethylnicotinamide	−1.100	−1.140
1-*n*-Propylnicotinamide	−1.090	−1.160
1-Benzylnicotinamide	−1.000	−1.150
DPN	−0.995	—
TPN	−1.000	—

[a] pH 7; sweep rate 1.15 v/sec.

$\times 10^{10}$ l. mole^{-1} sec^{-1} were obtained for methylnicotinamide, DPN, and TPN, respectively. These values are probably inaccurate, both absolutely and relatively; it is likely that complications introduced by adsorption vitiated the assumptions of the theory which treats more straightforward cases.

The ratio of the peak heights for these two peaks is a function of sweep rate. Some typical data for 1-*n*-propylnicotinamide are shown in Table II, where it may be seen that the current for reduction of adsorbed oxidant increases relative to that for the soluble species as the sweep rate increases. Possibly this behavior

TABLE II: Typical Data on the Effect of Voltage Sweep Rate upon Relative Peak Currents for Reduction of Adsorbed and Soluble Species for 1-*n*-Propyl-3-carbamidopyridinium Chloride, pH 7.

Sweep Rate (v/sec)	$i_{P_{adsorbed}}/i_{P_{soluble}}$
0.57	0.44
1.15	0.67
2.29	1.20
11.50	*a*

[a] Second peak nearly disappeared.

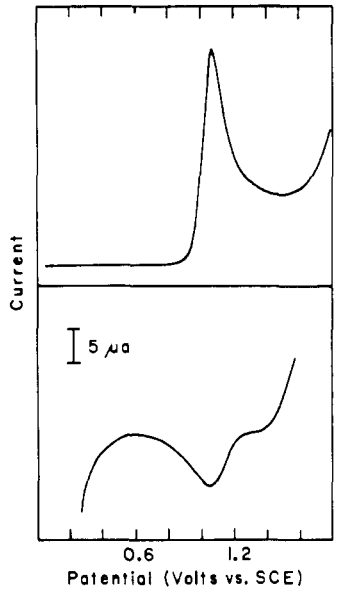

FIGURE 4: Cyclic voltammogram of DPN with asymmetric sweep, showing free-radical oxidation. A solution 1.0×10^{-3} M in phosphate buffer of pH 7.0; forward (cathodic) sweep rate 5.7 v/sec, reverse sweep rate 115 v/sec.

simply implies that at fast sweeps the electrolysis terminates before the soluble species has interacted appreciably with the electrode through an adsorbed layer which tends to "block" the surface.

The sharpness of the peaks is sometimes described as the difference between the peak potential and the potential where the current is one-half of the peak height. The theory for uncomplicated cases involving the dimerization of radicals following electron transfer predicts a value of 39 mv for this difference (Nicholson, 1965a). The values obtained in this study were 52 ± 3 mv for 1-methylnicotinamide, 54 ± 5 mv for DPN, and 48 ± 5 mv for TPN. For the ethyl, propyl, and benzyl compounds, the value was about 50 mv for the first of the two peaks; the second was difficult to measure because of the small potential separating the two peaks.

The results described above were obtained at fairly slow sweep rates. Now, when the anodic portion of the cyclic scan was speeded up sufficiently, a hitherto unobserved anodic peak appeared in the vicinity of −1 v *vs.* sce, along with the more positive anodic peak noted previously. There can be little doubt that this peak represents the reversible reoxidation of a free radical which, at the faster voltage sweep, has had insufficient time to disappear *via* the chemical step following electron transfer. Figures 3 and 4 show typical scans in which the radical peak is readily seen.

Even for a truly reversible system, the cathodic and anodic peaks do not occur at exactly the same potential in cyclic voltammetry. On the basis of certain assumptions, the separation of the peaks is supposed to be

$59/n$ mv for a reversible case, employing a symmetric wave form (Matsuda and Ayabe, 1955). Actually, this prediction has been verified only for fairly low sweep rates, say, 2 or 3 v/min, and it is probably not exactly correct for high rates. Nicholson and Shain (1964) and Nicholson (1965b) have extended the theoretical treatment of the separation of anodic and cathodic peak potentials to show the effects of sweep-rate and switching potential. However, it is still difficult to predict what the separation should be for an asymmetric sweep; also, strong adsorption phenomena place the system beyond the purview of the theory which is based upon a simpler model. Thus the separation of the peaks in the present case may not be used as a rigorous test of true reversibility. However, from a practical standpoint, we may view our peak separations of 10–50 mv as evidence that we are observing a process which is essentially reversible.

By plotting anodic peak current for the free-radical oxidation *vs.* time (taken as the time between the switching potential and the anodic peak potential) for a series of cases with the same cathodic sweep rate and sweep limit, one might hope to approximate the half-life of the free radical. Using this technique, half-lives of the order of 2 msec were obtained, but the data were so scattered that the values are probably no better than an order of magnitude. Nor are the values consistent with the dimerization rate constants mentioned above.

TABLE III: Typical Data for the Cathodic Peak with 1-Methyl-3-carbamidopyridinium Chloride, DPN, and TPN.

Solution	Sweep Rate (v/sec)	Peak Current, i_p (μa)	$i_p/v^{1/2}$
1-Methyl-3-carbamidopyridinium chloride (1.0×10^{-3} M in 0.2 M phosphate buffer and 0.5 M KCl, pH 7)	0.57	11.40	15.2
	1.15	21.00	19.7
	2.29	30.75	20.4
	5.73	43.50	18.2
	11.50	60.50	17.9
	22.90	93.00	19.5
DPN^+ (1.0×10^{-3} M in 0.2 M phosphate buffer and 0.5 M KCl, pH 7)	0.57	9.40	12.5
	1.15	14.00	13.1
	2.29	19.80	13.1
	5.73	29.25	12.2
	11.50	38.50	11.4
	22.90	57.00	11.9
	57.30	98.00	13.0
TPN^+ (1.0×10^{-3} M in 0.2 M phosphate buffer and 0.5 M KCl, pH 7)	1.15	10.00	9.4
	2.29	16.75	11.1
	5.73	25.50	10.7
	11.50	37.00	10.9
	22.90	48.50	10.2

Cathodic and anodic peak currents cannot be compared directly in the case of asymmetric sweeps, but it is possible to normalize results through the function $i_p/v^{1/2}$, where i_p is the peak current and v is the voltage sweep rate. For methylnicotinamide, DPN, and TPN, this function was constant over a wide sweep-rate range at a given concentration, and exhibited a linear concentration dependence at a given sweep rate, for the cathodic process and for the reversible reoxidation of the dimer. Table III contains some typical data for the cathodic process with the methyl compound, DPN, and TPN. The value of $i_p/v^{1/2}$ for the reversible free-radical reoxidation was never more than about 10% of the cathodic value. As the sweep rate was decreased, the radical reoxidation peak became smaller while the peak representing oxidation of the dimer grew. However, the latter was always so much larger than the former that good measurements of i_p values for the two processes could not be obtained in the same experiment with the apparatus employed. In certain electrode mechanisms, the rate constant of a chemical step intrudes into the $i_p/v^{1/2}$ function in a manner dependent upon the sweep rate, but Nicholson has shown that this is not the case for simple dimerization following electron transfer.

Discussion

Because comparison of radical half-lives through the series of compounds would be interesting, it is disappointing that strong adsorption phenomena so alter the properties of the electrode–solution interface as to preclude quantitative deductions based upon the standard simple models. Similar problems were encountered by Broman and Murray (1965) in their electrochemical study of the phenylmercuric ion, and they too were forced to restrict their interpretations to qualitative commentary.

There has been much interest in the mechanism of the enzymatic reduction of DPN^+ to DPNH by various substrates. The available evidence appears to favor transfer of a hydride ion as contrasted with a radical mechanism (*e.g.*, Westheimer, 1959). Some writers have concluded that reduction of DPN^+ in reactions involving radicals, including electrolytic reduction, leads to a dihydropyridine isomeric with DPNH (*e.g.*, see Pullman and Pullman, 1963). However, the two-electron electrochemical reduction leads to the 1,4-dihydropyridine (Burnett and Underwood, 1965a,b); the comments in the literature are based upon an erroneous interpretation of reduction upon the first of the two polarographic waves as a two-electron process whereas it actually involves one. In our view, it may be desirable to reconsider the idea that hydride reduction leads to the 1,4-dihydropyridine while reduction *via* a radical mechanism leads to other dihydropyridines. The evidence that one-electron electrochemical reduction leads to a dimer is substantial, and it seems clear that some writers have confused this dimer with one of the isomeric dihydropyridines.

The electrochemical generation of free radicals of

the pyridine coenzymes of course does not establish their importance or even their presence in metabolic reactions. However, DPN and TPN participate in biological electron-transport systems where radicals of other species such as flavins have been recognized. Commoner *et al.* (1957) reported the detection of an electron spin resonance (esr) signal during enzymatic reduction of DPN^+ with ethanol as well as in the reoxidation with acetaldehyde. Mahler and Brand (1961) were unable to find such a signal, but they obtained a stable esr signal in a model reaction involving DPNH and riboflavin which was not clearly attributable to the flavin. Pullman (1964) has reviewed the evidence for the participation of free-radical intermediates in biological redox systems involving DPN and TPN. Schellenberg and Hellerman (1958) found that DPNH was oxidized much more efficiently by reagents that function as one-electron acceptors than by equally powerful oxidants considered to be obligate two-electron acceptors; a DPN radical intermediate was postulated for these nonenzymatic oxidations.

Sometimes an esr spectrum can be obtained for an electrolytically generated free radical by performing the electrolysis in a special cell mounted in the microwave cavity of an esr spectrometer. A diligent search for such a spectrum using apparatus which yielded excellent spectra with electrolytically generated free radicals of nitrobenzene failed to disclose an esr signal. Modulation techniques involving electrolysis with an impressed square wave voltage were unsuccessful. Of course failure to obtain an esr signal does not disprove the existence of free radicals, but shows only that a sufficient steady-state radical concentration has not been achieved. Hence this experiment, while disappointing, does not reflect unfavorably upon the conclusions from the cyclic voltammetric study.

Acknowledgment

The authors are grateful to Professor A. K. Garrison of the Emory University Department of Physics for his generous help in the esr experiments. Helpful discussions with Professors R. A. Day, Jr., and A. M. Wilson are gratefully acknowledged.

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