V. I. (1979) *JETP Lett. (Engl. Transl.) 29*, 717–720.
Rubinov, A. N., & Tomin, V. I. (1970) *Opt. Spectrosc. (Engl. Transl.) 29*, 578–580.
Rudik, K. I., & Pikulik, L. G. (1971) *Opt. Spectrosc. (Engl. Transl.) 30*, 147–148.
Spencer, R. D., & Weber, G. (1969) *Ann. N.Y. Acad. Sci. 158*, 361–376.
Stryer, L. (1965) *J. Mol. Biol. 13*, 482–489.
Weber, G., & Farris, F. J. (1979) *Biochemistry 18*, 3075–3078.

Double Integration and Titration of the Electron Paramagnetic Resonance Signal in the Molybdenum Iron Protein of *Azotobacter vinelandii*[†]

W. B. Euler,[‡] J. Martinsen,[‡] J. W. McDonald, G. D. Watt,* and Z.-C. Wang[§]

ABSTRACT: The electron paramagnetic resonance (EPR) signal from the MoFe protein component of nitrogenase was doubly integrated over the temperature range 2.2–15 K. Comparison of the protein double integral with that from the spin standard copper ethylenediaminetetraacetate demonstrated that two $S = {}^3/_2$ spin centers are responsible for the observed EPR signal. The zero-field splitting parameter was found to be 15 ± 1 cm^{-1} from variation of the double integral with temperature. The double integral varied with the Mo content of the protein, suggesting direct Mo involvement in the $S = {}^3/_2$ spin centers. EPR titrations using methylene blue, thionine, or dichlorophenolindophenol as oxidants under a wide variety of solution conditions support previous results of three "P clusters", two EPR centers, and a single center of a third but unknown type.

Electron paramagnetic resonance (EPR) spectroscopy has played an important role in developing the present ideas and understanding of biological nitrogen fixation. Early EPR studies (Orme-Johnson et al., 1972; Smith et al., 1973; Mortenson et al., 1973) of the two component proteins under fixing conditions demonstrated that the EPR signal of the MoFe protein nearly disappears during steady-state conditions but reappears after reductant depletion. However, quite the opposite occurs with the Fe protein. Its EPR signal is present during steady-state conditions but is absent under reductant-depleted conditions. These results were interpreted mechanistically to indicate that during nitrogenase catalysis, electron flow occurs from the ATP-binding Fe protein to the MoFe protein, creating a further reduced (EPR silent) form of the latter protein which then induces substrate reduction. This view has been extended recently (Hageman & Burris, 1978a,b), and the conclusion has been drawn (Hageman & Burris, 1979) that two electrons are accumulated per EPR center under hydrogen evolution conditions.

A more detailed EPR and Mössbauer spectroscopic examination of the isolated MoFe protein component was reported (Münck et al., 1975; Orme-Johnson et al., 1977; Zimmerman et al., 1978). In these studies, the number of centers giving rise to the EPR signal of $S_2O_4^{2-}$-reduced MoFe protein was determined by double integration of the EPR signal in the temperature range 7–20 K. The results showed that 0.91 spin center was present per molybdenum atom or 2 spin centers per protein molecule which contains two molybdenum atoms per gram molecular weight. The presence of two spin centers per protein molecule was verified by Orme-Johnson et al. (1977) and Zimmerman et al. (1978) by oxidative titrations of the reduced $S_2O_4^{2-}$-free MoFe protein. These titrations showed that 4 equiv of thionine oxidant reacted with the protein without diminishing the EPR signal but, as predicted from the EPR integration experiments, the next 2 equiv of oxidant eliminated the EPR signal. These EPR results indicate that two independent EPR centers exist which can be separately silenced by a one-electron oxidation and that four other oxidizable, but EPR silent centers, are also present in the MoFe protein.

EPR titration results from this laboratory (Watt et al., 1981) and those of Stephens et al. (1981) differ from those just discussed. The main difference is that the latter studies report that only three electrons are removed from the protein before the EPR signal is affected.

In order to investigate more fully this titration behavior and perhaps to understand the cause of these differences, we initiated both EPR titration and EPR double-integration studies of MoFe protein samples prepared by different procedures and studied under different solution conditions. The results of these studies are presented here.

Experimental Procedures

MoFe Protein. Protein samples for both EPR titrations and EPR double integration were prepared by three separate procedures. The first method was that of Shah & Brill (1973), and the second was that of Burgess et al. (1980). The third method consisted of first isolating the purified nitrogenase complex according to Bulen & LeComte (1972) and then fractionating this complex on DEAE-cellulose. The resulting MoFe protein was concentrated and then crystallized by NaCl dilution. At least one sample prepared by each of the above methods was recrystallized and used for EPR titration; others were used only after a single crystallization. Fully reduced but $S_2O_4^{2-}$-free MoFe protein was prepared and characterized as previously described (Watt et al., 1980). Three-electron-oxidized MoFe proteins were prepared (Watt et al., 1981) by reacting MoFe protein with excess indigodisulfonate (IDS) for 20 min followed by anaerobic G-25 Sephadex chroma-

[†] From the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received October 19, 1983.* Contribution No. 836. This research was supported by the U.S. Department of Agriculture Competitive Research Grants Office, Project No. 81-CRCR-1-0675 (to J.W.M.) and 82-CRCR-1-1172 (to G.D.W.).
[‡] Present address: Department of Chemistry, Northwestern University, Evanston, IL.
[§] Present address: Institute of Botany, Peking, The People's Republic of China.

0006-2960/84/0423-3021$01.50/0 © 1984 American Chemical Society

tography. A fully developed EPR signal was present, and coulometric reduction indicated that the protein was oxidized by three electrons.

EPR Titrations. EPR titrations of reduced but $S_2O_4^{2-}$-free MoFe protein and MoFe protein oxidized by three electrons were carried out by using IDS, methylene blue (MB), thionine, and dichlorophenolindophenol (DCPIP) as oxidants in calibrated 3-mm i.d. quartz EPR tubes. The EPR spectra were recorded at constant temperature in the range 9–14 K at a power level of 1–5 mW, a value determined to be nonsaturating. The following protein solution variables were investigated: pH (7.0–8.5); buffer components [0.05 M tris(hydroxymethyl)aminomethane (Tris), *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES), or *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES)]; NaCl concentration (0.1, 0.15, 0.25, and 0.5 M); incubation temperature (20–35 °C); and gas phase (argon, N_2, He, and 10% H_2 in argon). For all samples prepared, the oxidant and protein were reacted for 20–30 min at the desired temperature before freezing in liquid nitrogen. Protein samples used in these titrations contained 1.8–2.0 Mo and 25–29 Fe and possessed activities of 2200–2800 nmol of $H_2 \cdot min^{-1} \cdot mg^{-1}$. Biuret protein or the absorbance at 400 nm for the reduced protein ($\epsilon_{400}^{red} = 6.23 \times 10^4$ cm^{-1} M^{-1}) and a molecular weight of 230 000 for the *A. vinelandii* MoFe protein were used to quantitate the amount of protein (Watt et al., 1980). The $S_2O_4^{2-}$-free MoFe protein was verified by coulometry to be fully reduced before being used in the EPR titrations.

EPR Double Integration. Protein samples for EPR integration were of two types. The first type consisted of carefully screened MoFe protein preparations low in molybdenum. The second type consisted of samples high in activity with nearly a full complement of Mo (>1.7) and Fe (>27). Some samples of this latter type were also used for the EPR titrations described above. A relative comparison of two identical protein samples, one as isolated and the other modified by one of the solution variables discussed above, was used to assess the effect of the chosen variable on the EPR signal intensity. These measurements were made on the Kettering Varian 4502 EPR spectrometer at a constant temperature near 10 K at power levels of 0.5–2 mW. This temperature is above that for optimal EPR signal development (see below), and consequently, absolute spin concentration was less than one (0.75–0.90) spin per Mo. We assumed that comparison of the EPR signal intensity of the native protein with the modified protein at the same temperature and power would reveal any effect that the variable induced in the spin intensity of the protein.

Absolute spin concentration of highly active, fully constituted MoFe protein was carried out in precision-bore quartz EPR tubes in the temperature range 2.2–4.2 K by using a modified Varian E-4 EPR spectrometer at Northwestern University. Spectra were recorded at power levels between 0.01 and 20 mW, and no saturation effects were noted. Temperatures below 4.2 K were calculated from the vapor pressure above the pumped helium in which the EPR sample was immersed. The Kettering EPR spectrometer was used for measurements in the 7.0–15 K temperature range. An iron–gold thermocouple immersed in glycerol contained in an EPR tube and calibrated in boiling He (4.2 K) was used to measure the temperature in those samples integrated between 7 and 15 K. With both sets of measurements, a 5×10^{-4} M copper ethylenediaminetetraacetate (CuEDTA) (EDTA/Cu > 10) spin standard was used to evaluate the spin concentration of the MoFe samples. In some cases, rubredoxin ($S = {}^5/_2$) from *Clostridium thermoaceticum* and $[Et_4N]_3[Fe(WS_4)_2]$ ($S = {}^3/_2$) (Friesen et al., 1983) were also used as spin standards. The spin concentration was calculated by using the method outlined by Aasa & Vanngard (1970, 1975).

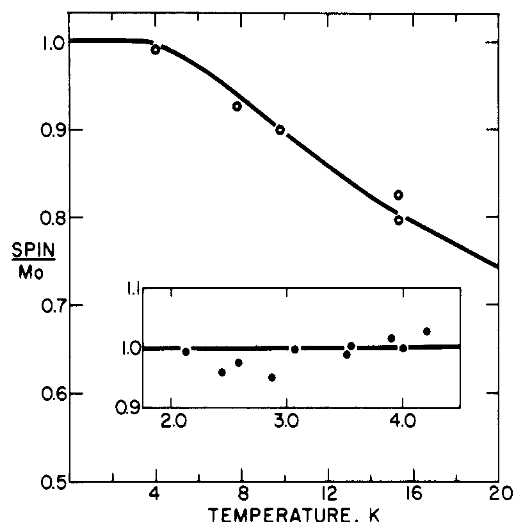

FIGURE 1: Spin concentration per molybdenum atom of sample 1 at 7.8, 9.8, and 15.3 K plotted against the absolute temperature. The vertical axis can also be viewed as the spin density in the lower doublet of the $S = {}^3/_2$ envelope of the EPR center in the MoFe protein. The solid line is drawn from the Boltzmann equation by using 15 cm^{-1} as the zero-field splitting energy. The inset is the spin concentration per molybdenum atom of sample 2 in the temperature range 2.12–4.2 K.

Table I: Results of EPR Double Integration at 4.2 K of Five (Samples 2–6) Separately Prepared MoFe Protein Samples[a]

EPR sample	concn (mg/mL)	Mo/mol	Fe/mol	spin/MoFe	spin/Mo	sp act.
1	28.7	1.88	27.5	1.86	0.99	2446
2	27.4	1.71	26.6	1.75	1.03	2595
3	24.8	1.70	25.8	1.67	0.98	2251
4	21.2	1.66	26.50	1.56	0.94	1990
5	18.4	1.50	21.91	1.22	0.81	2025
6	24.7	1.47	24.13	1.27	0.86	1987

[a] Sample 1 was doubly integrated at temperatures above 4.2 K and corrected to this temperature as outlined in the text. The units of specific activity are nanomoles of H_2 evolved per minute per milligram.

Results

Figure 1 is a plot of the doubly integrated EPR signal of the reduced MoFe protein, corrected for Curie temperature dependence, plotted against the absolute temperature. The line of zero slope between 2.12 and 4.2 K (see insert Figure 1) indicates that the EPR signal is completely developed in this temperature region and that two spins are present per MoFe protein. This same result was obtained at several power levels, demonstrating that power saturation of the signal was not occurring at these low temperatures. Having established that the signal is fully developed at 4.2 K, the EPR spectra of MoFe protein samples 2–6 in Table I were then recorded and doubly integrated at this temperature. Sample 1 in Table I was used to evaluate the apparent spin concentration of the MoFe protein at temperatures greater than 4.2 K (Figure 1). Figure 1 shows results at 7.8, 9.8, and 15.3 K indicating the apparent spin concentration decreases with increasing temperature. This spin concentration variation with temperature was fit with the Boltzmann equation, yielding a zero-field splitting energy of 15 cm^{-1}. With the use of this calculated

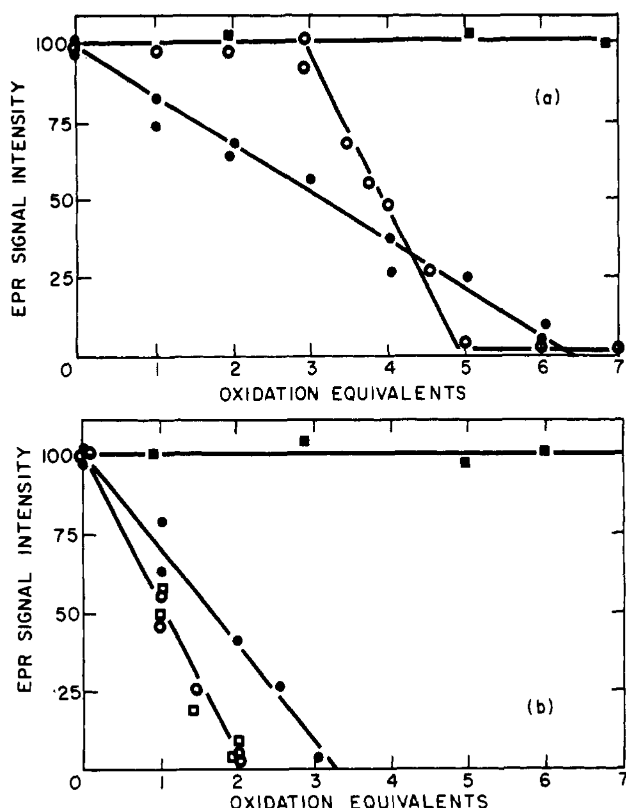

FIGURE 2: (a) EPR titrations of reduced but $S_2O_4^{2-}$-free MoFe protein in 0.05 M Tris–0.25 M NaCl, pH 8.0, with standardized indigodisulfonate (■), methylene blue (○), and dichlorophenolindophenol (●). (b) EPR titrations of three-electron-oxidized MoFe protein in 0.05 M Tris–0.25 M NaCl, pH 8.0, with standardized thionine (□) and the same oxidants used in (a). The vertical axis in both (a) and (b) is the signal intensity at the $g = 3.65$ resonance relative to the fully $S_2O_4^{2-}$-reduced state of the MoFe protein.

zero-field splitting energy, the apparent spin concentrations at 7.8, 9.8, and 15.3 K were corrected to 4.2 K, averaged, and entered as sample 1 in Table I.

It is clear from Table I that as the molybdenum content of the protein approaches two, the spin concentration of the MoFe protein approaches two. The spin concentration per MoFe protein decreases with Mo content, but when the spin concentration is normalized per Mo atom, a constant value of 0.94 ± 0.13 spin/molybdenum is found. This result implicates molybdenum atom involvement in the EPR signal of the MoFe protein and corroborates the recent electron nuclear double resonance (ENDOR) studies of Hoffman et al. (1982) on ^{95}Mo-enriched MoFe protein which strongly indicate that the molybdenum atoms are part of the center giving rise to the EPR signal.

Figure 2a is an EPR titration of reduced MoFe protein with indigodisulfonate (IDS), methylene blue (MB), and dichlorophenolindophenol (DCPIP) as oxidants. Three types of reactivity are clearly evident. IDS has no effect in diminishing the EPR signal intensity although coulometry and optical spectroscopy clearly establish that oxidation by three electrons has occurred. With MB and thionine [results for thionine not shown in Figure 2a but see Watt et al. (1981)], 3 equiv of oxidant reacts with the fully reduced MoFe protein with no change in the EPR signal intensity. The fourth and fifth equivalents then decrease the signal intensity by 50% and 90–100%, respectively. The reaction of DCPIP with reduced MoFe protein shows a monotonic decrease of the EPR signal with added oxidant until after slightly more than 6 equiv the EPR signal is completely eliminated. As was noted earlier (Watt et al., 1980), reaction times of 15–30 min are required for these oxidants to fully react with the EPR centers.

Figure 2b is an EPR titration of the three-electron-oxidized MoFe protein initially possessing a fully developed EPR signal with the three oxidants discussed above. The addition of IDS does not alter the EPR signal intensity even at a 10-fold excess whereas MB and thionine induce signal loss after the addition of 2 equiv. A linear decrease in signal intensity with added DCPIP occurs with 3 equiv of DCPIP at which point the EPR signal declines to zero.

Experimental errors in the data presented in Figure 2 arise from uncertainty in protein and oxidative titrant standardization as well as in measurement of the EPR signal intensities. The MoFe protein measurements are based upon amino acid content and dry weight protein measurements [see Watt et al. (1980)], and the organic titrants were purified and standardized as previously described (Watt et al., 1981). Calibrated EPR tubes were used in all measurements, and the temperature was maintained constant (±1 K) during the entire series of samples constituting the titrations shown in Figure 2. A total uncertainty of 10–13% at each point is estimated from those sections of the titration curve where the EPR signal is not affected by added oxidant and from those points where replicate measurements were performed.

EPR titrations carried out in an atmosphere of 10% H_2 in argon showed that the EPR signal is still present even after 30 or more equiv of oxidant is added. Evidence presented elsewhere (Wang & Watt, 1984) suggests that in the presence of the MoFe protein, H_2 reduces the methylene blue oxidant before oxidation of the EPR centers can occur.

Discussion

The EPR double-integration measurements from 2.2 to 4.2 K clearly establish the presence of two EPR centers per MoFe protein. The results in Table I also demonstrate that there is a closely observed relationship of one spin per Mo atom even as the Mo content of the MoFe protein varies. These results agree completely with those of Münck et al. (1975). The variation of the spin concentration with temperature (Figure 1) suggests a zero-field splitting energy of 15 ± 1 cm^{-1}, a value somewhat larger than that reported by Münck et al. (1975).

The EPR titrations reported here using thionine or MB as oxidants are in agreement with results of Stephens et al. (1981) and those previously reported from this laboratory (Watt et al., 1981). Three equivalents of oxidant per mole of protein (M_r 230 000) reacts with the protein with no diminution of the EPR signal. The addition of the next 2 equiv of oxidant then attenuates the signal. None of the variables examined (except the presence of H_2) caused deviation from the results just described. It was anticipated that the variables examined might alter the redox properties of a sixth EPR-silent redox center (Watt et al., 1980) and cause it to undergo oxidation with the first three EPR-silent centers. This result would reconcile the different EPR titration results from our laboratory and those of Zimmerman et al. (1978). However, no evidence was found in this study for four EPR-silent centers reacting with oxidant prior to the two EPR centers undergoing oxidation.

The EPR double-integration experiments reported here have confirmed the presence of two spin centers (Münck et al., 1975) in the MoFe protein. However, EPR titrations using three different oxidants with quite different redox potentials have demonstrated that a considerable variation exists in the reactivity of the EPR centers and the other redox centers known to be present in the MoFe protein. For example, IDS ($E_{1/2}$ = −125 mV, pH 7) rapidly oxidizes three non-EPR centers as evidenced by optical and CD spectroscopy and

coulometry but has no effect on the EPR signal intensity even when in >10-fold excess. Methylene blue ($E_{1/2}$ = 11 mV, pH 7) and thionine ($E_{1/2}$ = 64 mV, pH 7) sequentially and specifically oxidize three non-EPR centers and then the two EPR centers followed by a single non-EPR center. Both kinetic and thermodynamic factors were found to be operative in causing the three types of oxidation reactivity to be clearly separated and observable (Watt et al., 1981) with the MB or thionine oxidants. A final type of reactivity is that displayed by DCPIP ($E_{1/2}$ = 217 mV, pH 7), the strongest oxidant studied. There appears to be no selectivity in the reaction of this oxidant with any of the centers of the MoFe protein. The increased thermodynamic tendency for reaction associated with this oxidant apparently overcomes any kinetic barriers and renders all centers on the MoFe protein equally susceptible to oxidation. This effect is further demonstrated by the report that DCPIP easily oxidizes the MoFe protein beyond the six-electron state to a 9- and a 12-electron-oxidized species (Watt et al., 1978).

Preoxidation of the MoFe protein with excess IDS followed by Sephadex G-25 chromatography forms the stable three-electron-oxidized, fully EPR active MoFe protein species (Watt et al., 1981). Titration of this protein form with methylene blue, thionine, or DCPIP gives results quite similar to those just discussed for the fully reduced protein except that the EPR decrease begins immediately with added oxidant (Figure 2b). Two equivalents of thionine or methylene blue silences the EPR signal whereas DCPIP requires 3 equiv to attain an EPR-silent state. These two sets of titration results considered along with the reports (Watt et al., 1980, 1981) that excess methylene blue oxidizes the MoFe protein by six electrons clearly indicate that an EPR-silent center exists which is oxidized after the two EPR centers by the 6th (3rd) equiv of thionine or methylene blue added to the reduced (three-electron-oxidized) MoFe protein. With DCPIP, this center reacts with equal probability as the EPR centers toward this oxidant. The detailed nature of this center is of interest, and further study is warranted.

The presence of H_2 in the atmosphere alters the course of reaction between the MoFe protein and oxidant such that under the conditions of the EPR titrations reported here no oxidation of the EPR centers occurs (Wang & Watt, 1984). Up to 60 equiv of oxidant has reacted with the MoFe protein with no detectable decrease in the EPR signal intensity. A more detailed study of this phenomenon is under way, and preliminary results have been reported (Wang & Watt, 1984). It appears that the MoFe protein catalyzes the reduction of the methylene blue oxidant by hydrogen faster than this oxidant can react with the EPR centers of the protein. The net effect of these competitive reactions is the disappearance (reduction) of oxidant but with full retention of the EPR signal intensity even with a significant excess of added oxidant. The relationship of this hydrogen reaction and the EPR centers of the MoFe protein is presently being investigated.

Registry No. DCPIP, 956-48-9; Mo, 7439-98-7; methylene blue, 61-73-4; thionine, 581-64-6.

References

Aasa, R., & Vanngard, T. (1970) *J. Chem. Phys. 52*, 1612.

Aasa, R., & Vanngard, T. (1975) *J. Magn. Reson. 19*, 308.

Bulen, W. A., & LeComte, J. (1972) *Methods Enzymol. 24*, 456.

Burgess, B. K., Jacobs, D. B., & Stiefel, E. I. (1980) *Biochim. Biophys. Acta 614*, 196.

Friesen, G. D., McDonald, J. W., Newton, W. E., Euler, W. B., & Hoffman, B. M. (1983) *Inorg. Chem. 22*, 2202.

Hageman, R. V., & Burris, R. H. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2699.

Hageman, R. V., & Burris, R. H. (1978b) *Biochemistry 17*, 4117.

Hageman, R. V., & Burris, R. H. (1979) *J. Biol. Chem. 254*, 1189.

Mortenson, L. E., Zumft, W. G., & Palmer, G. (1973) *Biochim. Biophys. Acta 292*, 422.

Münck, E., Rhodes, H., Orme-Johnson, W. H., Davis, L. C., Brill, W. J., & Shah, V. K. (1975) *Biochim. Biophys. Acta 400*, 32.

Orme-Johnson, W. H., Hamilton, W. D., Jones, T. L., Tso, M.-Y. T., Burris, R. H., Shah, V. K., & Brill, W. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3142.

Orme-Johnson, W. H., Davis, L. C., Henzl, M. T., Averill, B. A., Orme-Johnson, N. R., Münck, E., & Zimmerman, R. (1977) in *Recent Developments in Nitrogen Fixation* (Newton, W. E., Postgate, J. R., & Rodriguez-Barrueco, C., Eds.) Academic Press, New York.

Shah, V. K., & Brill, W. J. (1973) *Biochim. Biophys. Acta 305*, 445.

Smith, B. E., Lowe, D. J., & Bray, R. C. (1973) *Biochem. J. 135*, 331.

Stephens, P. J., McKenna, C. E., McKenna, M. C., Nguyen, H. T., Morgan, T. V., & Devlin, F. (1981) in *Current Perspectives in Nitrogen Fixation* (Gibson, A. J., & Newton, W. E., Eds.) Australian Academy of Sciences, Canberra, Australia.

Wang, Z.-C., & Watt, G. D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 376.

Watt, G. D., Burns, A., & Tennent, D. L. (1981) *Biochemistry 20*, 7272.