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Vitamin A as an Enzyme That Catalyzes the Reduction of MTT to Formazan by Vitamin C

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Abstract The tetrazolium salt 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to formazan by the succinate dehydrogenase system of active mitochondria, and hence, specifically used to assay for the viable cells, such as measurement of cell proliferation, cytotoxicity, and cell number. However, in the present study we have shown that some component specifically present in M199 but not in RPMI 1640 media can reduce MTT to formazan in the absence of a living system. Further study revealed that ascorbic acid reduced MTT to formazan, which was profoundly increased by a very small amount of retinol, whereas retinol alone had no effect. Oxidation of ascorbic acid by H_2O_2 destroyed its ability to reduce MTT. The rate of MTT reduction was directly proportional to the concentration of MTT in the absence of retinol, but approached a zero-order state beyond a certain concentration of MTT in the presence of retinol. Furthermore, retinol remained unchanged after the completion of the reaction. Taken together, these results showed that retinol acts as a reductase that catalyzes the reduction of MTT to formazan using ascorbic acid as the cosubstrate (electron donor). J. Cell. Biochem. 80:133–138, 2000.

Key words: formazan; MTT; vitamin A; vitamin C; cell viability

MTT is a yellow water-soluble tetrazolium salt (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide). The dye is converted to water-insoluble purple formazan on the reductive cleavage of its tetrazolium ring by the succinate dehydrogenase system of the active mitochondria [Slater et al., 1963]. Thus, the amount of formazan formed is directly proportional to the number of metabolically active cells in the culture, which can be quantitated colorimetrically/spectrophotometrically simply after dissolving the formazan in an organic solvent. The conversion of MTT to formazan by the active mitochondria has been used to assay for the viable cells, such as measurement of cell proliferation cytotoxicity and cell number [Mossman, 1983; Carmichael et al., 1987]. The use of MTT to assay for viable cells was based on the current knowledge that this dye is reduced to formazan exclusively by the mitochondrial succinate dehydrogenase system. However, in our routine MTT assay we consistently observed significant amount of formazan formation in some, but not in all culture media in the absence of any living system. This indicates that some components of the culture media might react with MTT.

The aim of the present study was to detect the component(s) of the culture media that can reduce MTT to formazan. We found that MTT is reduced to formazan by ascorbic acid (vitamin C). More importantly retinol (vitamin A) acts as a reductase to catalyze this reaction.

MATERIALS AND METHODS

Materials

MTT, horse heart cytochrome c, catalase (EC 1.11.1.6), and Hank's Balanced Salt solution (HBSS) were purchased from Sigma Aldrich, MO. Vitamin C (ascorbic acid), Medium 199 (M199), and Roswell Park Memorial Institute 1640 medium (RPMI 1640) were from Himedia, Mumbai, India. Vitamin A (retinol) was from USV Limited, Mumbai, India. H_2O_2 (30% wt/vol) was from E. Mark Limited, Mumbai, India. Fetal bovine serum (FBS) was purchased from

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Gibco BRL, Grand Island, NY. The horseradish peroxidase was bought from Boehringer Mannheim, Indianapolis, IN. The multivitamin mixture, containing in each milliliter 8,000 IU retinol, 666 IU cholecalciferol, 1.6 mg thiamine, 1.6 mg riboflavin, 1.6 mg pyridoxin, 5 mg Napantothenate, 16 mg niacinamide, and 83 mg ascorbic acid, was from Pharmpak (P) Ltd., Mumbai, India.

Measurement of Formazan Formation

Unless otherwise mentioned, in 0.1-ml reaction volumes, MTT was incubated with culture media, or with retinol and ascorbic acid in HBSS at 37°C for 4 h in flat-bottom wells of 96-well plates. After that, formazan crystals were washed very gently with HBSS and solubilized by adding equal volumes of acidic isopropanol (0.04 N HCl). The absorbance of the dissolved formazan was measure at 490 nm (A_{490}) with a multiwell plate reader (Molecular Devices, Sunnyvale, CA). The MTT backgrounds in media (MTT incubated with culture media for zero times) or in HBSS (MTT alone in HBSS) were subtracted from the respective values.

Photography of Formazan Crystals

One-tenth milliliter RPMI 1640 or M199 was incubated with 2.5 mM MTT at 37°C for 4 h as drops in the confined areas of the inner side of the lids of a 96-well plate. After incubation, the solution from the top of the drop was removed gently, and the settled formazan was mounted with a coverslip and photographed at 50× magnification with a Nikon UFX2 bright-field light microscope.

Measurement of Cytochrome C Reduction by Ascorbic Acid

Various amounts of horse heart cytochrome c were incubated with various amounts of ascorbic acid in the presence or absence of retinol for 20 min at 37°C. After that, absorption spectra of the samples from 250- to 600-nm wavelengths (λ) were measured with a Beckman DU640B Spectrophotometer. The red-shift of the λmax from 410 (oxidized cytochrome c) to 416 nm (reduced cytochrome c) and the appearance of α and β peaks at 524 and 550 nm of the reduced cytochrome c were analyzed.

Reductive Degradation of H_2O_2 by Ascorbic Acid

Five-tenths millimolar H_2O_2 was incubated with various concentration of ascorbic acid (0–0.5 mM) in the presence or absence of retinol for 30 min at 37°C. H_2O_2 in the samples and in the standard was measured by the horseradish peroxidase method as we described previously [Kumar and Chakrabarti, 2000]. The H_2O_2 concentration in the sample was quantitated by comparing with the standard curve of the known H_2O_2 concentrations.

RESULTS AND DISCUSSION

The initial impetus to this study came from our observation that during a cell viability test using MTT, a significant amount of formazan is formed in the absence of cells in M199 but not in RPMI 1640 medium. As shown in Figure 1, 4-h incubation of 2.5 mM MTT in M199 resulted in the formation of huge numbers of purple-colored needlelike formazan crystals, which on solubilization with acidic isopropanol showed an absorbance at 490 nm λ (A_{490}) of ~0.5, whereas under the same condition, no formazan crystal was observed in RPMI 1640 and A_{490} was ~0.05. These results clearly showed that some component(s) present in M199, but not in RPMI 1640, can cause the reduction of MTT to formazan in the absence of any living system.

In the course of the study, we also observed that FBS, commonly used for cell culture, dose-dependently inhibited the formation of formazan crystals and corresponding decrease in A_{490}, such that a 50% decrease was observed between 5% and 10% FBS (Fig. 2a). The decrease in formazan crystal formation in the presence of FBS was not caused by the inhibition of formazan formation, but by the dispersion of formazan in the solution instead of being settled as crystals. The suspended formazan was lost during washing, resulting in the decrease in A_{490}. The clue to this came from our observation that the reaction mixture becomes increasingly darker, even though the amount of microscopically visible formazan crystals decreases, with an increasing amount of FBS. As shown in Figure 2b, the amount of formazan present in the top half of the reaction mixture was approximately two times higher in the presence of 10% FBS (A_{490} = 0.83) than

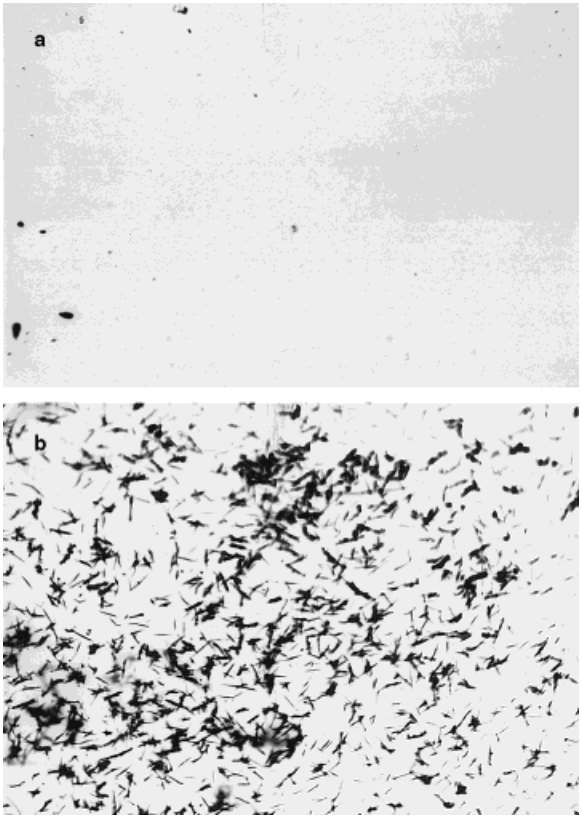

Fig. 1. Reduction of MTT to formazan in M199 in the absence of a living system. One-tenth milliliter RPMI 1640 or M199 were incubated with 2.5 mM MTT at 37°C for 4 h as a drop in the confined areas of the inner side of the lid of a 96-well plate (for photography) or in flat-bottom wells of 96-well plates (for quantitation). Photography and quantitation of formazan crystals were as described in the Materials and Methods section. The A_{490} values are shown on the top of the photographs (n = 3).

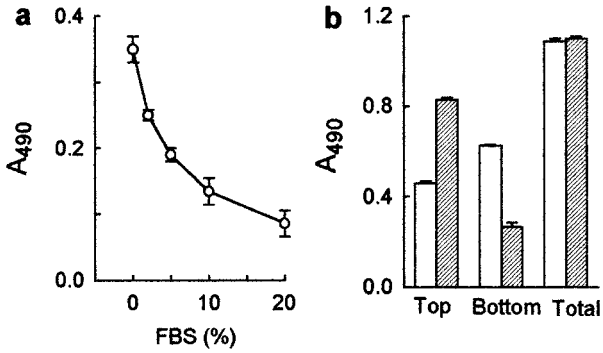

Fig. 2. Serum interference of formazan crystal formation. **a:** One-tenth milliliter M199 was incubated with 2.5 mM MTT in the presence or absence of various amounts of fetal bovine serum (FBS) and A_{490} measured as described in Materials and Methods. **b:** Two-tenths milliliter M199 was incubated with MTT in the presence (hatched bars) or absence (open bars) of 10% FCS as in Figure 1. After that, the reaction mixture was divided into two equal portions, by removing 0.1 ml very gently from the top of the reaction mixture. Both the top and bottom portions were spun at 13,000 rpm for 15 min in a microfuge. The formazan pallet was resuspended in 0.1 ml HBSS, solubilized, and A_{490} was measured (n = 3).

in its absence (A_{490} = 0.46). The reverse happened in the bottom half of the reaction mixture, which contained approximately two times more formazan in the absence (A_{490} = 0.62) than in the presence of FBS (A_{490} = 0.26) (Fig. 2b). However, the total amount of formazan (top + bottom half of the reaction mixture) was the same both in the presence (A_{490} = 1.1) or absence of FBS (A_{490} = 1.08). These results showed that serum contains some factor(s) that can bind specifically to the component(s) of the M199 responsible for the reduction of MTT to formazan. That is why, in the presence of FBS, a greater amount of formazan remained suspended, instead of being settled as large crystals.

Of all the components of M199 and RPMI 1640, only vitamins have specific binding factors in the serum. Thus, the above results indicate that the compound, which reduced MTT to formazan, is a vitamin(s) specifically present in M199 but not in RPMI 1640. Thus, we examined the reduction of MTT to formazan by a multivitamin mixture, whose composition is closer to the vitamin composition of M199 than RPMI 1640. To our excitement, we found that incubation of MTT in this multivitamin mixture resulted in the formation of formazan crystals whose amount was dependent on the dilution of the multivitamin mixture, as based on the measurement of A_{490} of the dissolved formazan. At 2.5×10^3 dilution, the A_{490} was ~0.5, which decreased with increasing dilution of the multivitamin mixture (Fig. 3a). Comparing the vitamin contents of the multivitamin mixture, RPMI and M199, retinol and ascorbic acid appeared to be the possible factors responsible for the reduction of MTT to formazan. Thus, in the next step these two vitamins were tested for their ability to reduce MTT to formazan. As shown in Figure 3b, ascorbic acid reduced MTT to formazan linearly in a dose-dependent way. However, retinol (10 μM) did not cause any detectable reduction of MTT, but dramatically enhanced (approximately three times) the MTT reduction by ascorbic acid (Fig. 3b). The enhancement was dependent on the

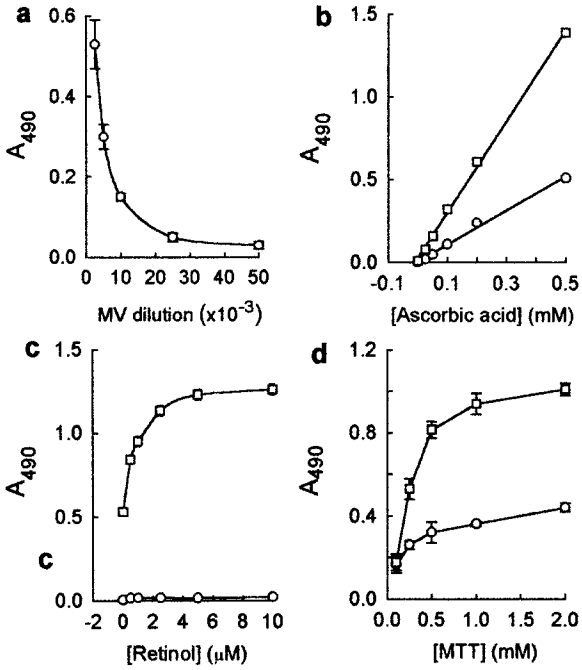

Fig. 3. Reduction of MTT to formazan by retinol and ascorbic acid. 2.5 mM MTT was incubated with various dilutions of a multivitamin (MV) mixture **(a)**, with various concentrations of ascorbic acid in the presence (squares) or absence (circles) of 10 μM retinol **(b)**, and with various concentrations of retinol in the presence (squares) or absence (circles) of 0.5 mM ascorbic acid **(c)**. **d:** Five-tenths millimolar ascorbic acid was reacted with various concentrations of MTT in the presence (squares) or absence (circles) of 2.5 μM retinol. In all experiments, the reaction was carried and A_{490} was measured (n = 4).

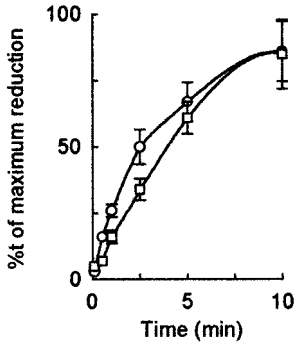

Fig. 4. Temporal pattern of MTT reduction. Five-tenths millimolar MTT was incubated with 0.5 mM ascorbic acid in the presence (circles) or absence (squares) of 2.5 μM retinol for various periods and A_{490} was measured. The amount of MTT reduction at a given time point with respect to its maximum reduction under a given condition (i.e., ±retinol) was expressed as percent of maximum reduction = 100 × A_{490} at a given time/maximum A_{490}.

concentration of retinol. At 0.5 mM ascorbic acid alone, A_{490} was ~0.5, which was enhanced by 60% with as low as 0.5 μM retinol (Fig. 3c). A maximum enhancement (~120%) was obtained with 2.5 μM retinol. These results showed that ascorbic acid spontaneously reduced MTT to formazan, which was dramatically enhanced by comparatively very little amount of retinol. Next, we determined the stoichiometry of reaction between ascorbic acid and MTT. With 0.5 mM ascorbic acid and 2.5 μM retinol, the amount of formazan formation increased with increasing doses of MTT, which become maximum at 0.5 mM MTT (Fig. 3d). Addition of more ascorbic acid at this point did not increase the amount of formazan any further (data not shown), thus giving a stoichiometry of 1 ascorbic acid:1 MTT. However, under the same reaction condition, but in the absence of retinol, maximum formazan was formed at 0.25 mM MTT, which was much less in amount than in the presence of retinol (Fig. 3d). Titration of 0.25 mM MTT by various concentrations of ascorbic acid revealed that it is completely reduced by 0.25 mM ascorbic acid (1:1 ratio) in the presence of retinol, but by 1 mM ascorbic acid (4:1 ratio) in the absence of retinol (data not shown). This shows that to reduce a given amount of MTT completely, a concentration of ascorbic acid that is four times higher is required in the absence of retinol than in its presence.

We have noted that formazan formation begins immediately after starting the reaction, but no formazan crystals are found. A longer incubation time (~4 h) was required for the formazan to be crystallized. Thus, we sought to determine the time required for the complete reduction of 0.5 mM MTT by an equimolar amount of ascorbic acid. In the presence of retinol, significant reduction (17% of maximum) was detectable as early as 30 s, which increased exponentially up to 5 min when the reaction became 70% of maximum (Fig. 4). After that, the reaction began to enter a plateau, reaching 90% of maximum by 10 min and maximum by 20 min (A_{490} = 0.98 ± 0.14). Similarly, in the absence of retinol, a maximum MTT reduction also took place by 20 min; however, at lower time points (30 s to 5 min) the amount of MTT reduction, relative to the maximum reduction, was much less (Fig. 4). These results clearly showed that retinol, besides reducing the amount of ascorbic acid required for

complete reduction of a given amount of MTT, also increases the speed of the reaction.

It is conceivable that ascorbic acid reduced MTT to formazan spontaneously, because this vitamin is known to spontaneously reduce many compounds such as H_2O_2, cytochrome c, and tetramethyl-*p*-phenylenediamine. Thus, to establish firmly the reductive role of ascorbic acid, we examined the effect of H_2O_2 on MTT reduction. Prior oxidation of ascorbic acid by H_2O_2 resulted in the loss of its ability to reduce MTT with a maximum loss at 2.5 mM H_2O_2 (Fig. 5a), whereas similar treatment of retinol did not affect its ability to catalyze MTT reduction by ascorbic acid (Fig. 5a), showing that ascorbic acid acts as the reductant in MTT reduction. The role of retinol would be either to synergize with or to catalyze this reaction. The synergistic role was ruled out, because of lack of any detectable MTT reduction by retinol alone. The first indication of the catalytic role of retinol in this reaction came from the rate of MTT reduction (initial velocity, v_0) by ascorbic acid in the presence and absence of retinol. In the absence of retinol, the v_0 increases linearly with increase in MTT concentration (Fig. 5b), typical of an uncatalyzed reaction. However, in the presence of retinol the v_0 gradually approached a zero-order state beyond a certain concentration of MTT (Fig. 5b), typical of a catalyzed reaction. The rate of the catalyzed reaction was approximately five times higher than the uncatalyzed one. These results show that retinol catalyzes the reduction of MTT to formazan, using ascorbic acid as the electron donor.

Another fundamental property of a catalyst or an enzyme (biological catalyst) is that it remains unchanged after completion of the reaction it catalyzed. To show this, an equimolar amount of ascorbic acid and MTT (0.5 mM each) was incubated for 20 min in the presence of 2.5 μM retinol. After that, the reaction mixture was incubated for another 20 min on addition of vehicle (control), initial concentrations of ascorbic acid + retinol, retinol + MTT, or ascorbic acid + MTT. In control, A_{490} was ~1.1, as usually observed under the same condition (Fig. 5c). Addition of ascorbic acid + retinol during the second reaction did not alter the A_{490} (1.3) significantly, indicating that no detectable MTT was left after the first reaction. Similarly, addition of retinol + MTT did not

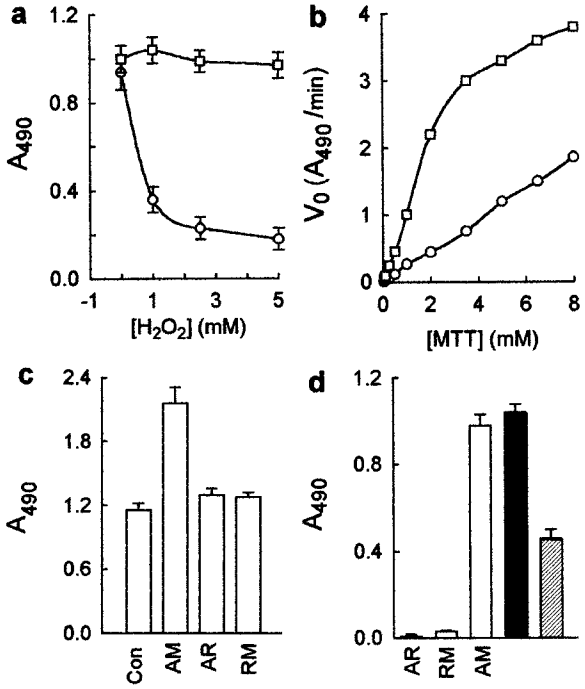

Fig. 5. Retinol catalyzes the reduction of MTT by ascorbic acid. **a:** Five-tenths millimolar ascorbic acid (circles) or 2.5 μM retinol (squares) was incubated with different concentrations of H_2O_2 for 30 min and the excess H_2O_2 was degraded by another 10-min incubation with 2×10^3 U/ml catalase. The complete oxidation of the ascorbic acid was confirmed from the disappearance of absorbance of ascorbic acid at 245 nm. The H_2O_2-treated ascorbic acid and retinol were incubated with untreated retinol (2.5 μM) and ascorbic acid (0.5 mM), respectively, in the presence of 0.5 mM MTT for 20 min (n = 3). **b:** Five-tenths milliliter ascorbic acid was incubated with various concentration of MTT for various times in the presence (squares) or absence (circles) of 2.5 μM retinol and the initial reaction velocity v_0 (A_{490}/min) for each MTT concentration was determined. **c:** Five-tenths millimolar MTT was reacted with 2.5 μM retinol and 0.5 mM ascorbic acid for 20 min. After this, the reaction mixture was incubated for an additional 20 min on addition of vehicle only (control, Con), 0.5 mM ascorbic acid + 2.5 μM retinol (AR), 2.5 μM retinol + 0.5 mM MTT (RM), and 0.5 mM ascorbic acid + 0.5 mM MTT (AM) (n = 4). **d:** Five-tenths millimolar MTT, 2.5 μM retinol and 0.5 mM ascorbic acid was incubated for 20 min and heated in boiling water for 30 min. The resultant clear solution was removed gently, without disturbing the formazan adhered to the wall of the tube, and incubated for another 20 min on addition of ascorbic acid + retinol (AR), retinol + MTT (RM), and ascorbic acid + MTT (AM). As controls, MTT reaction was done with ascorbic acid alone (hatched bar) or in combination with retinol (solid bar) for 20 min (n = 4). In all experiments, formazan was solubilized and A_{490} was measured as in the Materials and Methods section.

change A_{490} (~1.3) significantly, indicating that no detectable ascorbic acid was left after the completion of the first reaction. However, addition of ascorbic acid + MTT increased A_{490} twice (~2.2) that obtained in controls, showing that after the completion of the first reaction, retinol was able to catalyze another complete reaction that is quantitatively equal to the first reaction. We did another experiment, which was same as the above experiment except that formazan formed from the first reaction was removed before the second reaction started. Formazan was removed by letting it adhere to the wall of the tube on incubation of the reaction mixture in boiling water for 30 min. Such heat treatment did not destroy retinol and MTT activity, and destroyed ascorbic acid (0.5 mM) only partially, so that it can cause significant reduction of MTT (~50% as compared to 0.5 mM untreated ascorbic acid) both in the presence or absence of retinol (data not shown). As shown in Figure 5d, the amount of formazan formed on addition of ascorbic acid + MTT (A_{490} = 0.98) is quantitatively higher than the amount formed from a similar fresh reaction in the absence of retinol (A_{490} = 0.4), but equal to the amount formed from a similar fresh reaction containing 2.5 μM retinol. Addition of ascorbic acid + retinol or retinol + MTT during the second reaction did not cause any formazan formation, indicating that no detectable MTT or ascorbic acid was left after the completion of the first reaction. It may be argued that MTT and ascorbic acid were destroyed during heating after the first reaction. However, as mentioned above, such heat treatment does not destroy MTT and destroys ascorbic acid only partially. Thus, a large amount of formazan would have been formed during the second reaction on addition of ascorbic acid + retinol or retinol + MTT, if ascorbic acid and MTT remained unchanged after the completion of the first reaction. Thus, the results of Figure 5c and 5d show that retinol remained unchanged after catalyzing the reduction of MTT by ascorbic acid so that it could catalyze the equal amount of same reaction again. Finally, we determined the specificity of retinol action, another important property of an enzyme, by showing that it does not catalyze the reduction of cytochrome c and H_2O_2 by ascorbic acid (data not shown).

Ascorbic acid is known to reduce many compounds. In the present work, we showed that ascorbic acid also reduces MTT to formazan, which calls for a renewed look into the specificity of the succinate dehydrgenase system in reducing MTT and its implication in the assay for viable cells. However, the novelty of the findings is that retinol acts as a reductase in catalyzing this reaction specifically. To our knowledge, this is the first report showing that a nonprotein, non-RNA biomolecule acts as an enzyme.

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