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INTRINSIC AND RADIATION INDUCED STABLE MELANIN FREE RADICALS IN COMPLEXES WITH SERUM ALBUMIN

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INTRINSIC AND RADIATION INDUCED STABLE MELANIN FREE RADICALS IN COMPLEXES WITH SERUM ALBUMIN.

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Short running title: ESR of irradiated melanoprotein

Key index words: Melanin- Serum albumin- Melanoprotein- Radiation induced free radicals.

Abreviations:
L-dopa: L-dihydroxyphenylalanine
BSA: Bovine serum albumin
LNT: Liquid Nitrogen Temperature
RT: Room Temperature

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Abstract

An ESR study was performed on systems composed by Bovine Serum Albumin and synthetic melanin (melanin/BSA weight ratio between 0.5 and 5%) Due to the interaction with the protein, both the intensity and the saturation behaviour of the melanin ESR signal changed, suggesting a shift of the ox-red equilibrium of the intrinsic semiquinone free-radicals. The number of X-ray induced free-radicals in the protein is reduced by the binding with melanin, suggesting the accurrence of energy or radical transfer from BSA toward the pigment. After irradiation at 77K, the radical evolution with increasing temperature leads to ESR spectra showing almost only the melanin singlet. These data are compared with the results obtained with irradiated pure melanin and melanosomes, and interpreted on the bases of a radioprotective property of melanin at molecular and subcellular level.

INTRODUCTION

Melanins or melanosomes inside the cells are in an environment containing many different macromolecules including proteins. Melanosomes theirselves have a high protein content. It has been observed that the presence of proteins may alter electron transfer properties of eumelanins [1] and pheomelanins [2]. More recently, increasing attention has been devoted to the interaction between melanins and dyes like porphirins [3] ou neurotoxic metabolytes [4]. These observations suggest the possibility that the function of the pigment at cellular level could be thought in terms of its interaction with other cellular components. In this sense it has been observed for example that there is a decrease in radiosensitivity of melanized cell cultures when compared to unmelanized ones [5,6]. In order to attain a better understanding of radiation effects on pigment-protein complexes, we performed investigations on model systems composed of synthetic melanin obtained from autoxidation of L-dihydroxyphenylalanine (L-dopa) and Bovine Serum Albumin (BSA). ESR spectroscopy has been used to monitor the melanin intrinsic free radicals and their variations resulting from interactions with BSA as well as new radicals induced by X-ray irradiation.

MATERIALS AND METHODS

Sample preparation. Synthetic autoxidized L-Dopa melanin (referred in the following simply as melanin) was prepared by autoxidation of L-dopa (Merck, Darmstadt) at pH 9.0 in the presence of NaOH, followed by dialysis. Powder was obtained after drying the final black suspension in air at 40° C. The synthetic melanin thus obtained was rather

soluble in water at concentrations lower than 30 μg/ml and pH greater than 6.0. BSA from Sigma Co. (St. Louis, MO, USA) was utilized without additional purification. Aqueous solutions of BSA and melanin (melanin/BSA weight ratio between 0.5 and 5%) were prepared with bidistilled water and dried in air at 40° C. The powder was pressed and put in ESR sample tubes that were sealed in vaccum (less than 10^{-3} mm Hg). All experiments were done with dry material.

Melanosomes were isolated from bovine eye choroids following the methods described by Cope et al [7]. Isolated and purified melanosomes were liophylized and sealed under vacuum in ESR sample tubes.

Sample irradiation. Sealed samples were irradiated with a Phillips X-ray tube operating at 80 kV peak and 15 mA, keeping equivalent irradiation conditions for all samples.The exposure rate, corrected for the quartz tube absorption, was 520 Roentgen/min or 2.2×10^{-3} $C.kg^{-1}.s^{-1}$. Irradiations were performed at RT and LNT.

ESR measurements. ESR spectra were obtained with a Varian E-4 spectrometer operating in the X-band at temperatures ranging from 120K to 300K.

Progressive Saturation. The dependence of ESR signal intensity on the excitation microwave power was monitored by progressive saturation measurements. The melanin ESR signal shows inhomogeneous broadening characteristics and in this case, as developed by Castner [8], Bowman [9] and Rupp [10], the signal intensity I_{ESR} can be related to the microwave power P through

$$I_{ESR} \alpha \frac{\sqrt{P}}{(1 + P/P^*_{\frac{1}{2}})^{b/2}} \qquad (1)$$

In this relation b is the so called inhomogeneity parameter and $P^*_{\frac{1}{2}}$ is the value of microwave power such that the intensity of the signal with inhomogeneus broadening is half of the value that would be attained in the absence of saturation.

RESULTS

Unirradiated samples.

ESR spectra were obtained for the following samples: melanin; melanin-BSA complexes; melanosomes. All samples showed the typical melanin free radical signal, without hyperfine structure and with g-values between 2.0030 and 2.0037. Linewidth, measured as peak-to-peak width in first derivative curve, ranged between 4.5 and 5.0 G. The melanosome signal was the largest and more assymetric one; in a blank test BSA, as expected, did not show any ESR signal.

For melanin-BSA complexes, although lineshape remains unchanged, the intensity of the pigment signal measured as peak-to-peak height in first derivative curves was not proportional to the melanin content of the samples. The values of these intensities normalized per unit mass of melanin-content in the complex are shown in Table 1. These results indicate that the number of stabilized free radicals in melanin increases with complexation with BSA.

Progressive saturation measurements were performed at room temperature and at 120 K. Normalized values of EPR signal intensities I_{ESR} and the corresponding values of power P were used to plot $\log(I_{ESR}/\sqrt{P})$ as a function of log(P), following equation 1; values of parameters b and $P^*_{\frac{1}{2}}$ can thus be obtained (Rupp et al [10]) and are presented in Table 2. Values of b lower than 1 may indicate that saturation processes are slow compared to relaxation times. On the other hand the parameter $P^*_{\frac{1}{2}}$ is proportional to $(T_1T_2)^{-1}$ where T_1 and T_2 are spin-lattice and spin-spin relaxation times respectively. There is a consistent decrease of $P^*_{\frac{1}{2}}$ with lowering temperature. However, comparative $P^*_{\frac{1}{2}}$ values for melanin alone and in complexes with BSA, (at a given temperature) show a significative

alteration: with temperature decreasing, $P^*_{\frac{1}{2}}$ for the pigment alone becomes higher than the value for the pigment complexed with BSA.

The dependence of ESR signals on temperature was analysed from the plots of 1/A versus T, where A is the area under the absorption curve, which resulted in straight lines intercepting the origin. This indicates that the ESR signal comes from free radicals that behaves as pure paramagnetic centers.

Irradiated samples. Room temperature.

ESR spectra obtained after X-ray irradiation of melanin-BSA complexes showed the characteristic radiation induced protein doublet due to a peptide radical [11] superimposed to the free radical singlet from L-dopa melanin, as illustrated in Figure 1. The protein doublet has g value $g = 2.0034 \pm 0.0005$ and hyperfine constant $A = 14.8 \pm 0.25$. It is important to observe that irradiation in this dose range has no effect on lineshape and intensity of the ESR signal of pure melanin or melanosomes. Dose response curves for melanin in complexes with BSA are illustrated in Figure 2, where the evolution of singlet signal intensity is plotted as a function of irradiation exposure time. It can be seen that the number of induced free radicals increases with the exposure time, i.e., with absorbed dose.

The intensity of protein free radical signal was monitored by the height of the first maximum of the doublet, corrected for the contribution of the melanin signal. The values shown in Table 3 are normalized per unit mass of BSA and have as reference the intensity of the induced protein signal in absence of melanin. For a given exposure, the number of induced protein free radicals decreases with increasing melanin content in the complex.

Progressive saturation measurements on irradiated melanin-BSA complexes show that the melanin singlet has basically the same behavior as for unirradiated samples, suggesting that X-ray induced and intrinsic signals are subjected to the same relaxation mechanisms. Alterations in $P^{*}_{\frac{1}{2}}$ parameter however are observed in irradiated melanin and melanosomes, as shown in Table 2.

After exposure to air ESR spectra show the disappearing of the protein signal without alteration of melanin singlet intensity, suggesting that oxygen interacts with radiation induced radicals on BSA and not with those induced on the pigment.

Irradiated samples. Low temperature.

BSA irradiated at LNT in vacuum shows a large unresolved signal at 160 K that evolves to the typical peptide radical doublet observed at room temperature, as reported by Libby [12] and Aripova [13]. LNT irradiation of melanin and melanosomes does not induce new free radicals and the values of g and ΔH of the usual singlet do not change under heating to room temperature. LNT irradiation of melanin-BSA complexes results in ESR spectra qualitatively similar to the superposition of the effects in melanin and protein irradiated separately. However, as observed for irradiation at RT, the contribution of the large BSA signal is proportionally less significant with the increase of melanin concentration in the complex. It was also noticed that with increasing temperature, BSA signal strongly decreases and doesn't evolve to the protein doublet. At room temperature almost exclusively the melanin free radical becomes stabilized (figure 3).

DISCUSSION

Melanin-BSA complexes.

Results of ESR measurements in melanin-BSA complexes show that the number of free radicals stabilized in the complex per unit mass of melanin depends on the relative concentration of its constituents. This behavior differs from that observed by Arnaud et al [14] on L-dopa melanin diluted in KBr matrix, where the ESR signal intensity per unit mass of melanin is constant. It is known that when paramagnetic centers are diluted in a diamagnetic matrix, the changes in magnetic interactions may cause modifications in the lineshape of ESR signal, such that the area under absorption curve remains constant. This is not the present case, where we observed that the intensity of ESR signal is modified without alteration in lineshape. As we can see in Table 1, there are more free radicals stabilized in the pigment complexed to BSA than what would be expected for the same quantity of the pigment without complexation with BSA. If one assumes that the melanin free radical comes from a semiquinone form stabilized between hydroquinones and quinones [15], it is possible that the interaction between melanin and BSA shifts the equilibrium in the direction of the semiquinone free radical.

Differences between ESR signal from melanin and from complexes with BSA are also verified by progressive saturation measurements (Table 2). The value $P^{*}_{\frac{1}{2}}$ is proportional to $(T_1T_2)^{-1}$ [16]. As shown in Table 2, $P^{*}_{\frac{1}{2}}$ at room temperature is higher in melanin-BSA complexes than in melanin or melanosomes. This means that in the complexes the values of (T_1T_2) are smaller, i.e., relaxation processes are faster. According to Sarna and Hyde [17], T_1 does not vary significantly among several kinds of melanins and so the decreasing of (T_1T_2) can be attributed mainly to a decrease

in T_2 . In this sense, the complexes with BSA show more efficient processes of spin-spin energy transfer. However these processes are temperature dependent and the relative increase in (T_1T_2) at 120 K for the complexes with BSA is more significant than for melanin or melanosomes. Thus at low temperature spin-spin energy transfer processes seem less efficient in the complexes.

Radiation effects.

Irradiation of melanin complexed with BSA at room temperature induces the formation of free radicals. We observed both an increase in the intrinsic melanin free radical ESR singlet and the induction of the typical irradiated BSA doublet. The total area under absorption curve increases with exposure time, indicating the stabilization of new paramagnetic centers created by irradiation in vacuum.

As shown in figure 2, the growth in the singlet intensity is higher in the samples with higher melanin content. At the same time it may be observed that at higher pigment concentration the number of free radicals induced in the protein is lower, suggesting that in presence of melanin the free radicals are preferentially stabilized in the pigment rather than in the protein.

As shown in Table 2, the unchanged values of $P^*_{\frac{1}{2}}$ for melanin in BSA complexes suggest that relaxation processes are the same as for unirradiated samples. On the other hand, irradiated pure melanin and melanosomes, that do not show new free radical stabilization, have different $P^*_{\frac{1}{2}}$ values, indicating that radicals observed after interaction with ionizing radiation may have different relaxation mechanisms.

From the point of view of radiation damage as studied by ESR, these results indicate that melanin exerts a protective effect on the protein moiety. Irradiation at room

temperature shows that the induced BSA doublet decreases with the percentual increase of melanin content, concomitantly with a growth of the singlet induced in the pigment.

Moreover, it should be noticed that the induced melanin radicals are stable even after exposure to O_2 . The induced BSA doublet, on the contrary, interacts with oxygen as seen by the decay of its intensity after opening to air.

Irradiation at LNT allows the investigation of primary and intermediate radicals. LNT irradiation of BSA results in a large ESR spectra that evolves to the doublet signal when the sample temperature is raised to RT. In melanin-BSA complexes the spectra obtained after heating to RT are somewhat different from those obtained by direct irradiation of these complexes at RT. In fact the protein doublet almost disappears and free radicals are stabilized preferentially on the pigment. This suggests that radicals induced in BSA at low temperature are partially subjected, during heating, to mechanisms of internal re-arrangements and energy transfer from protein to the pigment.

Melanin-protein interaction promotes radical scavenging properties of the pigment through the occurrence of two mechanisms of radical stabilization: one, coming directly from increased energy deposition in the pigment and the other from increased stabilization of the radicals signal due to the interaction between pigment and irradiated protein (Fig. 4) .

Melanosomes, in spite of protein content around 40% of their dry weight(18), when irradiated at LNT do not present any protein contribution to the ESR signal as observed in melanin-BSA complexes. The evolution of the intensity of the signal with temperature in melanosomes irradiated at LNT is the same as for unirradiated melanosomes and pure melanin. In melanosomes, primary induced free radicals are probably stabilized directly in the structure of the melanin inside the organelle, and direct effects over the proteins are not

detectable. The radiobiological implications of such a behaviour is not fully understood at present.

These studies show that, due to interaction with BSA, the ESR characteristics of melanin are modified, both in intensity and in saturation behaviour. Even if the present results are not completely conclusive some points can be established: a) interaction with BSA modifies the equilibrium of the intrinsic melanin free radicals and consequently their number per unit mass of pigment; b) stabilized free radicals, even with similar lineshape, have not a common nature in melanin-protein complexes and in pure melanin or melanosomes, as seen by relaxation results; c) concerning the relaxation processes, radiation induced radicals in complexes with BSA have the same behaviour as before irradiation, while in pure melanin and melanosomes these mechanisms are altered, meaning that ionizing radiation may have effects not directly detectable by measuring signal intensities only. These results are complementary to others observed by one of us [19] with optical methods, suggesting that the interaction between melanin and serum albumin leads to changes in pigment and protein properties. In that work it was also observed an efficient quenching of protein fluorescence due to excitation energy transfer form protein to pigment.

The existence of processes involving free radical migration were also observed in the present work. After irradiation, free radical damage induced in the protein is reduced through mechanisms that allow migration of free radicals from the protein towards the pigment. The free radical and energy transfer mechanisms from proteins to melanin could be related to the protective action of the pigment at cellular level and in this sense deserves further investigation.

ACKNOWLWDGMENTS

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REFERENCES

1. Gan ,E.V.; Haberman, H.F. and Menon, I.A. (1974). Biochim. Biophys. Acta 30, 62-69.

2. Ito, A.S. and Crippa, P.R. (1985). Phisiol. Chem. Phys. Med. NMR 17, 205-209.

3. Bielec, J.; Pilas, B. and Sarna, T. (1986). J. Chem. Soc., Faraday Trans. 2, in press.

4. D Amato, R.J.; Lipman, Z.P. and Snyder, S.H. (1986). Science 231, 987-989.

5. Prasad, N.K. and Johnson, H.A. (1968). Rad. Res. 33, 403-416.

6. Luckiewicz, S.; Pilas, B.; Nowicka, J.; Cleszka, K. and Gurbiel, R. (1981). in: Phenotypic Expression in Pigment Cell,pp 647-653, (M. Seiji ed.), Univ. Tokyo Press, Tokyo.

7. Cope, F.W.; Sever, R.J. and Polis, B.D. (1963). Arch. Bioch. Bioph. 100, 171-177.

8. Castner Jr, T.G. (1959). Phys. Rev. 115, 1506-1515.

9. Bowman, M.K.; Hase, H. and Kevan, L. (1976). J. Magn. Res. 22, 23-32.

10. Rupp, H.; Rao, K.K.; Hall, D.O. and Cammack, P. (1978). Biochim. Biophys. Acta 537, 255-269

11. Crippa, P.R. and Vecli, A. (1972). Int. J. Rad. Biol. 22, 105-113.

12. Libby, D.; Ormerod, M.G.; Charlesby, A. and Alexander, P. (1962). Int. J. Rad. Biol. 4, 21-26.

13. Aripova, D.F. (1967). Biophysics 12, 1131-1140.

14. Arnaud, R.; Perbet, G.; de Flandre, A. and Lang, G. (1983). Photochem. Photobiol. 38, 161-168.

15. Sealy, R.C.; Felix, C.C.; Hyde, J.S. and Swartz, H.M. (1980). In: Free radicals in Biology,. vol. IV (E. Pryor, ed.), pp. 209-259.

16. Zimbrick, J. and Kevan, L. (1967). J. Chem. Phys. 47, 5000-5009.

17. Sarna, T. and Hyde, J.S. (1978). J. Chem. Phys. 69, 1945-1948.

18. Borovansky, J.; Melezinck, I. and Budesinska, A. (1986). Anal. Biochem. 159, 249-252.

19. Kawamura, M.R. (1986). Ph. D. Thesis, University of São Paulo.

TABLE 1- Intensity of ESR signal of autoxidized L-dopa melanin.

	Intensity a
Mel.[b]	1.0
BSA + 0.5% Mel.	1.9
BSA + 1.0% Mel.	2.8
BSA + 3.0% Mel.	3.1
BSA + 4.0% Mel.	3.5
BSA + 5.0% Mel.	4.0
BSA + 10.0% Mel.	4.3

a- Values normalized per unit mass of melanin, mean values obtained from microwave power between 0.05 and 0.2 mW.

b- In this and in the other tables, Mel. means autoxidized L-dopa melanin. Percent values refer to the percentual mass of melanin present in the complex.

TABLE 2- Parameters b and $P^*_{1/2}$ obtained from plots of equation 1. First two columns refer to unirradiated samples.

	RT		120 K		Irradiated(RT)	
	b	$P^*_{1/2}$	b	$P^*_{1/2}$	b	$P^*_{1/2}$
BSA + 1%,2%,3%,4% Mel.	0.85	0.98	1.1	0.14	0.94	0.96
L-Dopa Melanin	0.76	0.40	1.1	0.34	0.81	0.64
Melanosomes	0.72	0.42	1.2	0.36	0.78	0.60

TABLE 3- Intensity of ESR doublet induced in BSA, normalized per protein unit mass.

	Intensity
BSA	1.0
BSA + 0.5% Mel.	0.96
BSA + 1.0% Mel.	0.89
BSA + 2.0% Mel.	0.81
BSA + 3.0% Mel.	0.74
BSA + 4.0% Mel.	0.71

Figure Captions

Figure 1- ESR spectra of samples irradiated at RT: a) BSA; b) BSA + 0,5% melanin; c) BSA + 1% melanin.

Figure 2- Increase in ESR singlet intensity with exposure time to X-ray. Values are normalized to unit mass of melanin. Percentual mass of melanin: (●) 4%, (Δ) 3%, (O) 2% and (□) 1%.

Figure 3- ESR spectra of BSA-5% melanin complex irradiated at LNT and measured at increasing temperatures. a) 125 K; b) 150 K; c) 174 K; d) 204 K; e) 245 K; f) 274 K.

Figure 4- Possible kinetic scheme for the evolution of X-ray induced radicals in BSA-Melanin complexes. $(BSA)^{\bullet}$ indicates every kind of free radicals in the protein moiety and $(Mel)^{\bullet}$ indicates only new free radicals in the pigment.

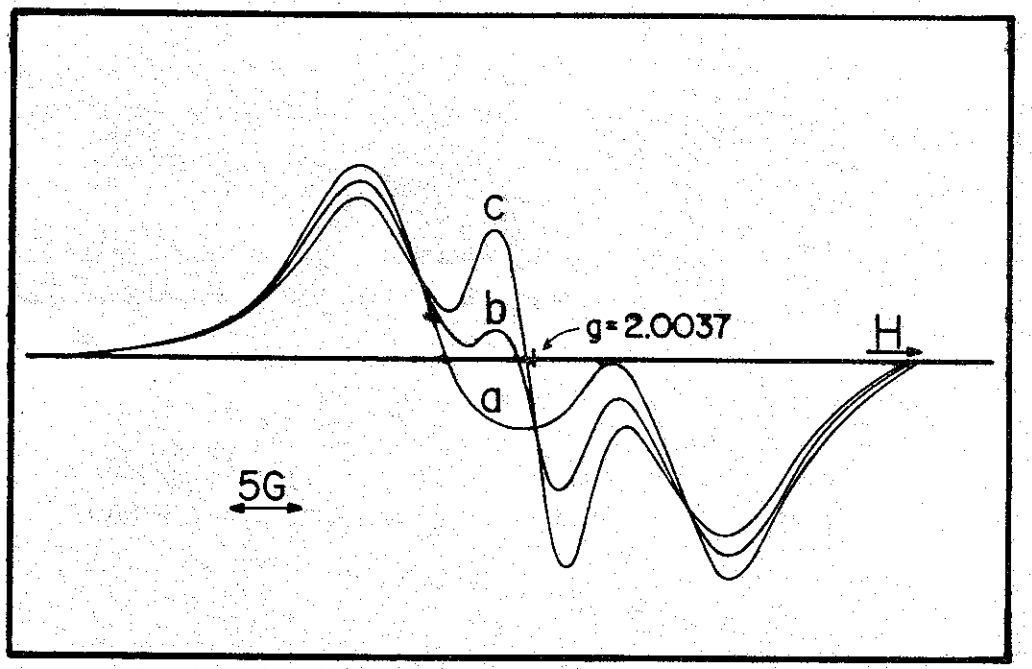

FIGURE 1

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"Intrinsic and Radiation Induced Stable Melanin Free Radicals in Complexes with serum Albumin"

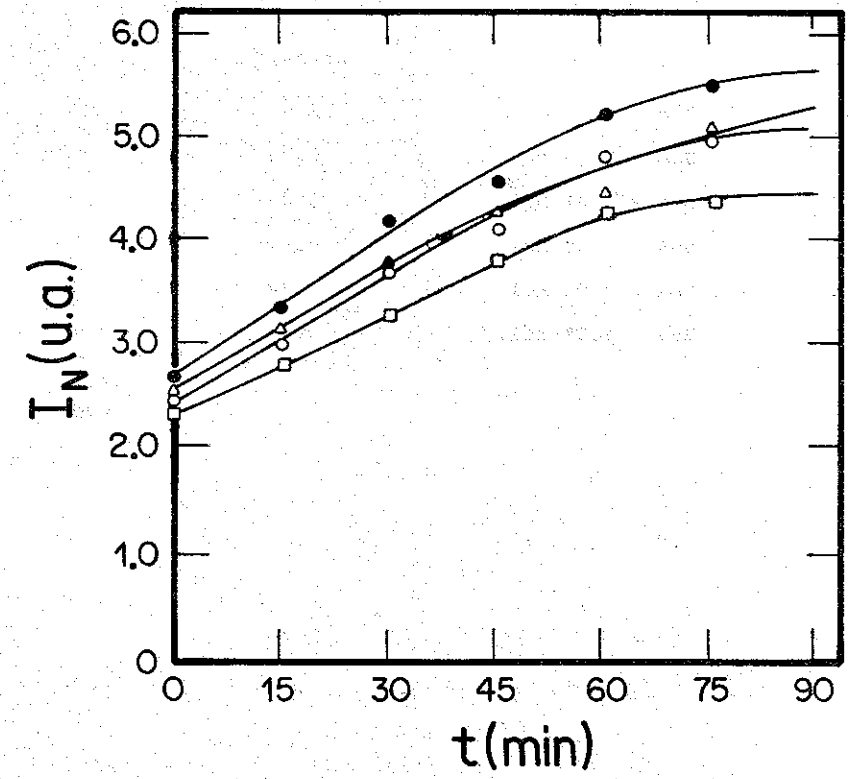

FIGURE 2

"Intrinsic and Radiation Induced Stable Melanin Free Radical in Complexes with serum Albumin"

M.R.D. Kawamura, A.S. Ito & P.R. Crippa

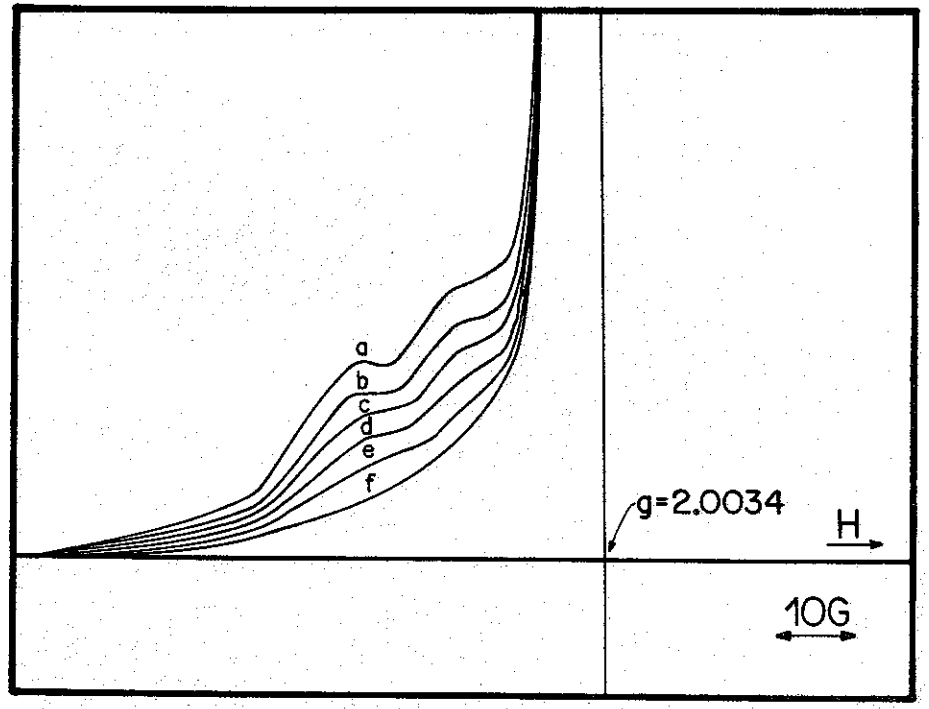

FIGURE 3
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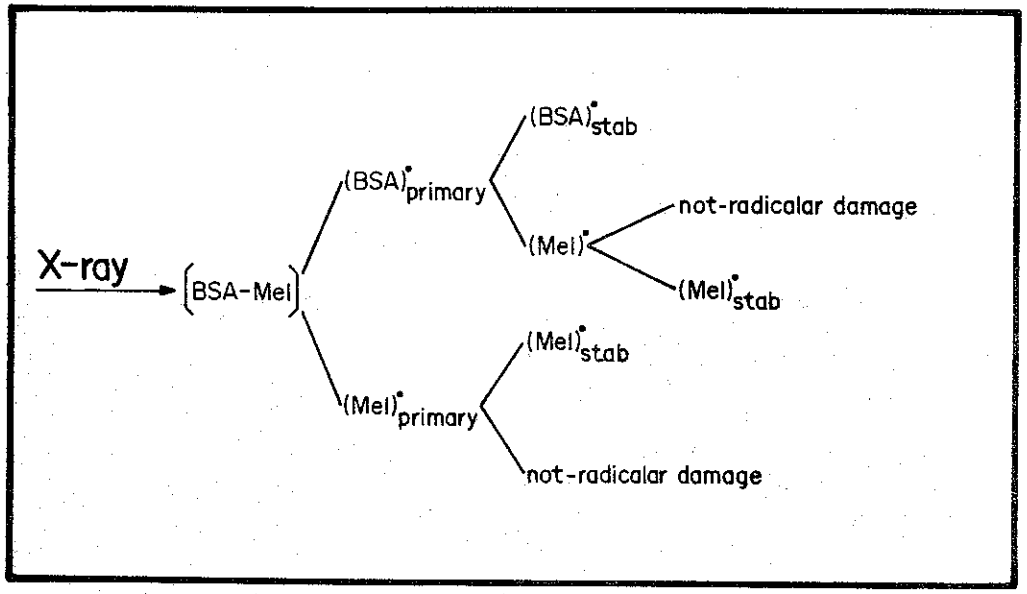

FIGURE 4
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