Melanogenic Regulatory Factors in Coated Vesicles from Melanoma Cells

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Coated vesicles have been found to contain much higher tyrosinase and γ-glutamyl transpeptidase activities than premelanosomes. This indicates that similar to tyrosinase, γ-glutamyl transpeptidase, an enzyme responsible for pheomelanogenesis, is highly concentrated in coated vesicles after its maturation in Golgi associated endoplasmic reticulum (GERL). Furthermore, in the pre- and post-dopaquinone melanogenic pathway, coated vesicles convert dopachrome to colorless indole compounds more quickly than in premelanosomes because of their higher dopachrome conversion factor activity. Melanosomes have been found to exhibit indole conversion factor activity, while coated vesicles show indole blocking factor activity. In moderately tyrosinase-rich premelanosomes, the levels of dopachrome conversion factor and indole blocking factor are lower than in coated vesicles or melanosomes. High levels of indole blocking factor in coated vesicles may indicate why melanin polymer formation does not occur there in vivo despite their high tyrosinase activity. *J Invest Dermatol 93:616–620, 1989*

Original concepts of the initial stages of melanogenesis envisioned the joining of active tyrosinase with premelanosomes in a specialized functional segment of the Golgi apparatus [1]. However, work from our laboratory has indicated that newly synthesized tyrosinase is activated through glycosylation steps that occur along with other processing events in the GERL and coated vesicle (CV) system [2,3] and eventually lead to the formation of active melanin polymers in vivo [4,5]. We have also reported much higher tyrosinase activity in the GERL and CV than in the premelanosome (PMS) fractions of the pigment cells [2]. Thus, the transfer of activated tyrosinase into PMS by CV is presumed to be necessary for the initiation of melanization in vivo, which can be observed by electron microscopy [6].

Another glycoprotein, γ-glutamyl transpeptidase (γGTP), an enzyme involved in pheomelanogenesis, has been shown to co-exist with tyrosinase in premelanosomes [7,8]. Our present studies seem to indicate that GERL-CV is also involved with γGTP maturation and activation. In addition, we have found that other regulatory factors in melanogenesis, namely, dopachrome conversion factor (DCF), indole blocking factor (IBF), and indole conversion factor (ICF), first described by Pawelek and co-workers [9,10], are present in high specific activities in the CV system. The distribution of these various activities in various subcellular fractions from pigmented melanomas allows us to postulate mechanisms for the restriction of melanin formation to melanosomes, and to offer an explanation for the absence of melanin in CV, despite the presence of high tyrosinase activity [5].

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Abbreviations:

CV: coated Vesicle.
DCF: dopachrome conversion factor
DDC: diethyl dithiocarbamate
DHICA: 5,6-dihydroxy indole 2-carboxylic acid
DOPA: 3,4-dihydroxy phenyl alanine
GERL: golgi-associated endoplasmic reticulum of lysosome.
IBF: indole blocking factor
ICF: indole conversion factor
IQ: indole-5,6 quinone
MS: melanosome
PMS: premelanosome
γGTP: γ-glutamyl transpeptidase

MATERIALS AND METHODS

Subcellular Fractionation Coated vesicles were isolated from BL-6 murine melanoma grown subcutaneously in nude mice (BALB/C, nu/nu) and from Greene's malignant melanoma maintained in the laboratory by serial transplantation into Syrian (Golden) hamsters, by the method of Usami et al [11]. Briefly, the tissues were homogenized in 1 V of MES buffer (pH 6.5) containing 0.1 M MES (2-N-morpholino ethane sulfonic acid), 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.02% sodium azide. The homogenate was centrifuged at 20,000 g for 30 min and the supernatent was again centrifuged at 55,000 g for 60 min. The pellet was suspended in MES buffer containing 10% sucrose. Coated vesicles were precipitated by centrifugation for 60 min at 100,000 g. The pellet was homogenized in a small volume of the buffer and underwent continuous 30%-50% sucrose density gradient ultracentrifugation for 16 h at 50,000 g. A turbid band at about 35%-40% sucrose was collected and diluted fourfold with the buffer and centrifuged at 100,000 g for 60 min. The pellet was resuspended and again ultracentrifuged in a continuous 5%-30% sucrose gradient for 60 min at 100,000 g. A band at the density of about 14%-20% sucrose was collected, pooled, and precipitated by centrifugation. The purified CV were resuspended in a Mg^{+2} free MES buffer.

Premelanosome (PMS) and melanosome (MS) subfractions were separated from the 20,000 g pellet (large granule fraction) by sucrose density gradient ultracentrifugation as described earlier [12]. The so-called Golgi apparatus (G_2) and soluble fractions from

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Greene's melanotic melanoma were isolated as described previously [3,12].

Electron Microscopic Study Isolated fractions were observed under an electron microscope by the technique of negative staining using a 1% phosphotungstic acid solution.

Tyrosinase Assay Tyrosinase was measured spectrophotometrically by following production of dopachrome (DC,orange) from a solution of 1.0 mM L-DOPA in a 3-ml reaction mixture containing 0.2 M phosphate buffer (pH 6.8). Activity was expressed as ΔE/min/mg protein as per Hamada and Mishima [13].

γ-GTP Assay γGTP was measured according to Tate and Meister [14]. The reaction mixture contains 0.1 ml cell homogenate, 0.9 ml substrate solution of 2.5 mM L-γ-glutamyl-p-nitroanilide, 20 mM glycyl glycine, 75 mM NaCl, and 50 mM Tris-HCl buffer (pH 8.0) and was incubated for 10 min at 37°C with constant stirring. The reaction was terminated by the addition of 1.0 ml of 3 N acetic acid, and the absorbance of the p-nitro-anilide released was measured spectrophotometrically at 410 nm. The enzyme activity was expressed as ΔE/10 min/mg protein.

Protein Determination Protein was determined by Lowry's method using bovine serum albumin as standard [15].

Synthesis of Dopachrome (DC) Dopachrome was synthesized using the method of Korner and Pawelek [9] modified from the method of Mason [16]. Briefly, ice-cold dopa (0.5 mg per ml of 0.1 M sodium phosphate buffer, pH 6.8) was mixed with Ag_20 (30 mg Ag_20: 1 mg dopa) for about 1 min and filtered through a 0.22-μm millipore filter.

Dopachrome conversion factor (DCF) activity was measured spectrophotometrically by the method of Korner and Pawelek [9]. The standard assay consisted of 0.5 ml DC solution and the extract of the fractions (0.2 mg protein) and 0.05 M sodium phosphate buffer (pH 6.8) in a total volume of 3 ml. The decrease in absorbance at 475 nm was noted. Diethyl dithio carbamate (DDC) was used to block the tyrosinase interference in DCF assay.

Indole Conversion Factor (ICF) and Indole Blocking Factor (IBF) Activity [10,17] The extract of CV and other organelles (0.2 mg protein) were mixed with 0.5 ml DC solution in a total volume of 3 ml containing 0.05 M phosphate buffer, pH 6.8. Increases in absorbance at 540 nm with time due to melanin synthesis were observed that could be readily detected visually.

RESULTS

Electron Microscopic Findings CV fractions consist almost exclusively of coated vesicles that appear as small spheroid bodies having a coated surface (Fig 1). Figures 2 and 3 show PMS and MS fractions, respectively. These fractions contain many ellipsoid bodies, which frequently reveal an oriented parallel array of membranes in the PMS fractions (Fig 2).

Tyrosinase and γGTP of CV Coated vesicle fractions are not only rich in tyrosinase but also contain substantial amounts of γGTP (Figs 4 and 5). The high γGTP activity in CV fractions is also presumably due to the presence of the ectozyme portion of this enzyme.

Dopachrome Conversion Factor (DCF) Activity Coated vesicle fractions have higher DCF activity than PMS (Fig 6). Their different DCF activity is clearly seen in the presence of tyrosinase inhibitors like DDC (1mg), which indicates that, unlike tyrosinase, DCF is unaffected by DDC and should be assayed in the absence of tyrosinase interference when the substrate still contains dopa.

Indole Conversion Factor (ICF) and Indole Blocking Factor (IBF) Activity Melanin formation from dopachrome is inhibited in the presence of CV, whereas an increase in indole conversion ability to melanin is observed in MS. PMS show some blocking factor activity but much less than that of CV (Fig 7). After 40-min incubation, a rise of only 25% in absorbance was observed at 540 nm in the presence of CV whereas rises of 90%, 160%, and 210% were noted in the presence of PMS, buffer (control), and MS, respectively. It appears, therefore, that during differentiation of melanosomes from CV through PMS, a gradual increase in indole conversion ability to melanin appears at the expense of indole blocking factor activity. Studies with Greene's melanotic melanoma also show similar results in the activity of these factors within subcellular fractions (Table I).

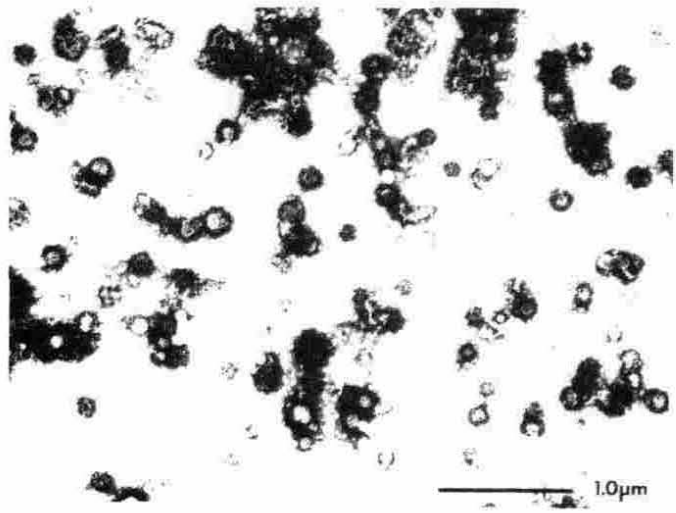

Figure 1. Electron micrograph of coated vesicle fractions. It contains numerous spheroid bodies that exhibit coated surface structure. Negative stain with 1% phosphotungstic acid (×24,150).

DISCUSSION

In the recent concept of mixed melanogenesis, while tyrosinase is involved in both eumelanin and pheomelanin formation, another enzyme, γGTP, glycoprotein in nature [18], plays the essential role in pheomelanogenesis [7,19]. Previously, it was reported that premelanosomes are not only rich in tyrosinase but also contain sub-

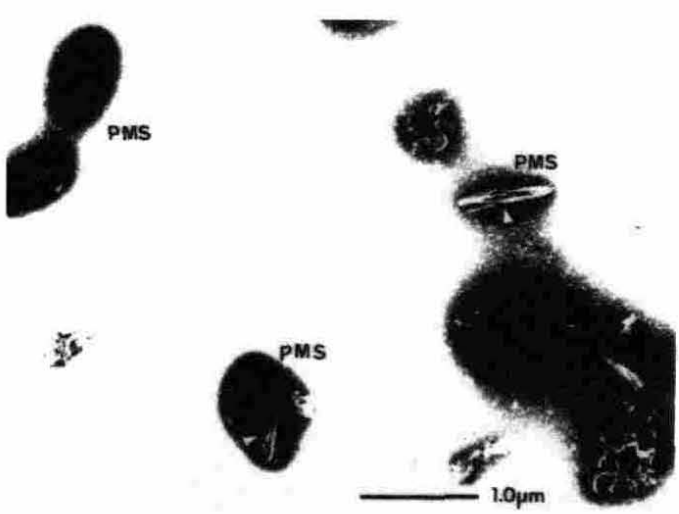

Figure 2. Electron micrograph of premelanosome fraction. It contains an oriented parallel array of membranes. Negative stain with 1% phosphotungstic acid (×21,000).

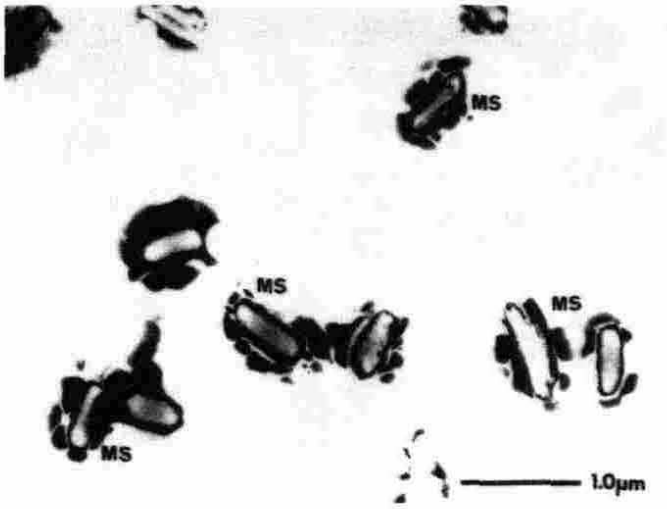

Figure 3. Electron micrograph of melanosome rich fractions. It contains numerous ellipsoid bodies which appear uniformly dense. Negative stain with 1% phosphotungstic acid (×21,000).

stantial amounts of γGTP [8]. In the present study we have shown that CV also contain high tyrosinase as well as γGTP activity (Fig 4). Compared with the other subcellular fractions, it becomes obvious that tyrosinase-rich CV fractions contain the highest γGTP activity among the so-called golgi fractions, premelanosomes, and soluble fractions (Fig 5). These findings seem to indicate that, similar to the tyrosinase maturation process, γGTP synthesized by ribosomes is glycosylated and concentrated in GERL and then transferred to PMS presumably by CV. As far as we know, this is the first report of a possible function of coated vesicles in the initiation of pheomelanogenesis as well as eumelanogenesis in vivo.

Recent studies on melanin biosynthesis as a polymerization have shown that the process distal to the tyrosinase action sites is not spontaneous but under some regulatory control: "dopachrome conversion factor (DCF)," which promotes the conversion of dopachrome (DC) to 5,6-dihydroxy indole 2-carboxylic acid (DHICA) [9]; "indole blocking factor (IBF)," which inhibits the conversion of DC to indole quinone (IQ); and "indole conversion factor (ICF)," which promotes the conversion of DC to IQ through colorless indole derivatives [9,10]. The activity of the indole conversion factor can be partially accounted for as melanosomal tyrosinase [17,20]. King et al reported that DCF and indole blocking factor activity is due to a new enzyme, Mr. of 34,000, which is heat labile, protease sensitive, and not inhibited by tyrosinase inhibitor, DDC [21]. They have also shown that subcellular distribution of this new enzyme is similar to that of tyrosinase among microsomal, melanosomal, and soluble fractions in B-16 melanoma. Hearing et al, on the other hand, reported the association of indole blocking factor activity with non-membrane bound tyrosinases, while the membrane-bound tyrosinase is associated with the activity that accelerates melanin formation from DC [22].

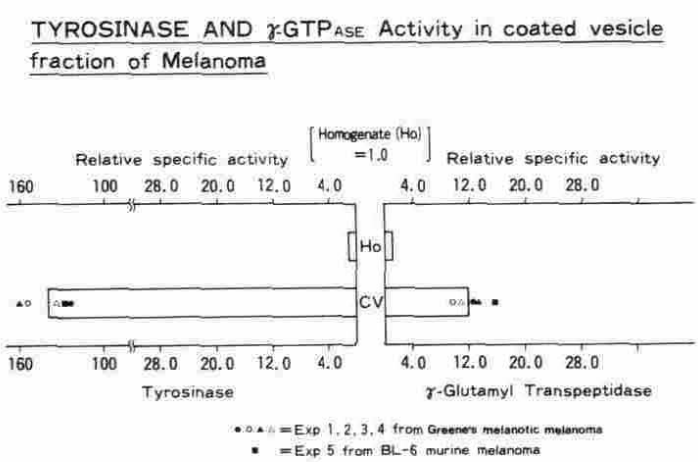

Figure 4. Tyrosinase and γGTP activity in CV fractions of melanoma. Their specific activities relative to homogenate (=1.0) have been presented. Experiments 1, 2, 3, and 4, respectively from Greene's melanotic melanoma, and experiment 5 from BL-6 murine melanoma.

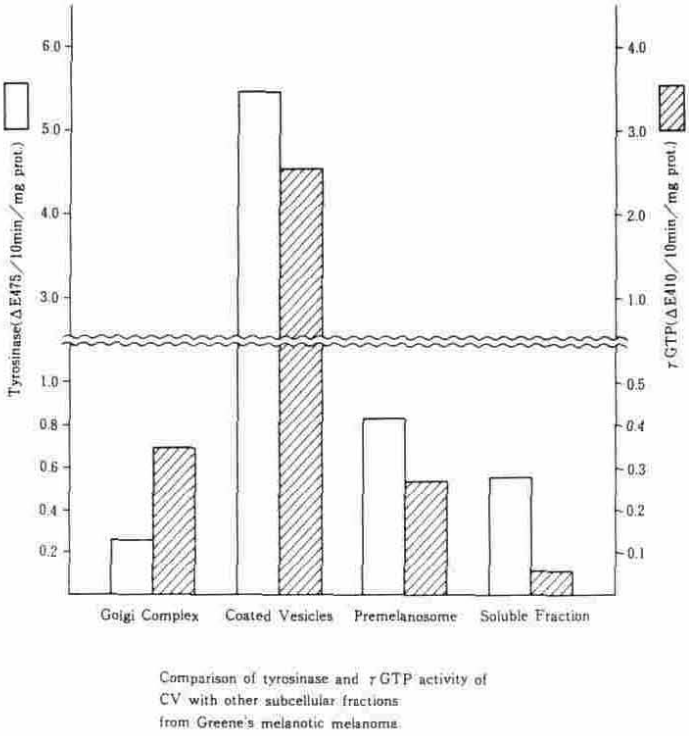

Figure 5. Comparison of tyrosinase and γGTP activity of CV with other subcellular fractions from Greene's melanotic melanoma.

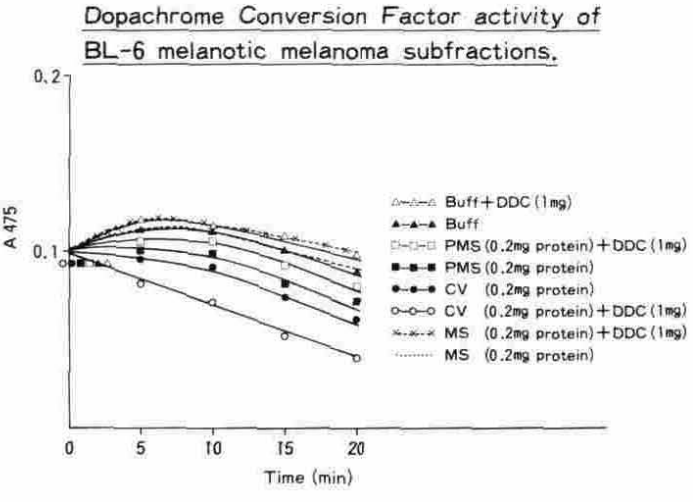

Figure 6. Dopachrome conversion factor activity of coated vesicle, premelanosome, and melanosome fractions from BL-6 melanoma. Freshly prepared dopachrome solution (0.5ml) was incubated with the extract, as indicated in the figure in a total volume of 3 ml at pH 6.8 and 37°C. The decrease in absorbance due to conversion of dopachrome to colorless indole derivative was noted. The experiments were repeated several times with similar results each time.

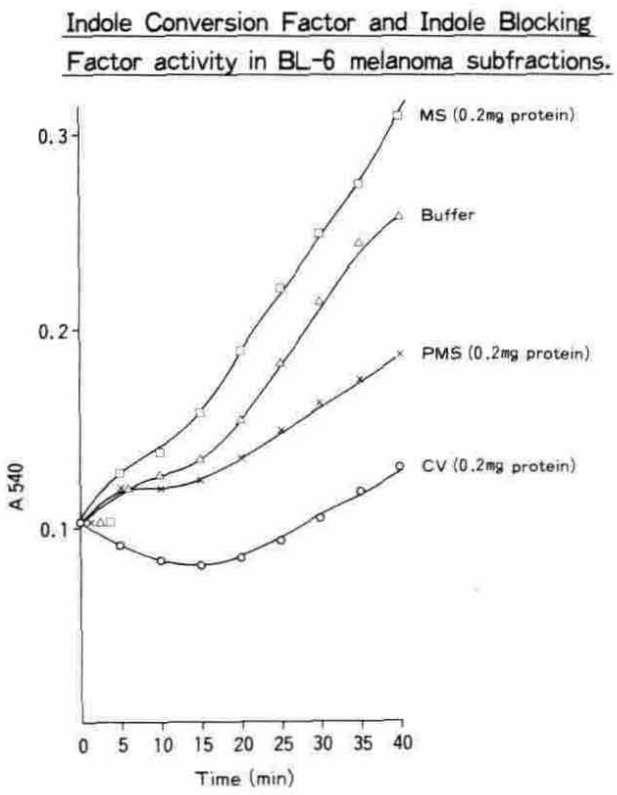

Figure 7. Indole conversion factor and indole blocking factor activity in BL-6 melanoma subfractions. Dopachrome solution (fresh), 0.5 ml was incubated with the extracts at pH 6.8 and absorbance was noted at 540 nm with time. The increase in absorbance at 540 nm was due to melanin synthesis which could be detected visually. The initial decrease was due to DCF activity because dopachrome shows some of its absorbance at 540 nm. The higher value over buffer (control) represents ICF activity and the reverse represents the activity of IBF. The experiments were repeated several times with similar results each time.

However, intracellular localization of these factors (DCF, ICF, and IBF), in reference to various functionally differentiated melanogenic compartments, has not been clarified yet.

The present study shows that DCF activity in CV fractions is significantly higher than PMS when tyrosinase activity is blocked by DDC during DCF assay (Fig 6). MS fractions have no DCF activity. These results are in agreement with the observation of King et al [21] that DCF is different from tyrosinase and seem to indicate that in CV, dopa conversion to dopachrome and thereafter to colorless indole compound takes place more quickly than in PMS because of higher tyrosinase and DCF activity in CV. Moreover, CV show indole blocking factor activity, while MS exhibit indole conversion factor (Fig 7). Premelanosomes are observed to exhibit only moderate IBF activity. Therefore, it is rational to assume that during the process of differentiation of melanosomes that occurs through sequential stages of development of PMS (stage I, stage II) [23], the blocking factor may lose its activity, and thus indole conversion ability gradually appears.

We have previously shown that the CV isolated from melanoma contain primarily the membrane bound tyrosinase, T_3, in addition to T_1, whereas the melanosomal fraction of these pigment cells contains T_3 only [24,25]. Hearing et al [22] have recently reported differential functions of multiple forms of tyrosinase in the melanogenesis pathway. It was shown that soluble tyrosinase (T_1) contains indole blocking factor activity, while membrane-bound tyrosinase (T_3) accelerates melanin formation from dopachrome.

Our present findings combined with previous reports indicate that during the process of maturation of tyrosinase, which seems to proceed from T_2 to T_1 and then to T_3 with sequential changes in carbohydrate moieties as tyrosinase is transferred from ribosome to PMS through such melanogenic compartments as GERL and CV [2], the blocking factor may either become inactivated or transformed to ICF.

Hence, we can conclude from our present results that 1) DCF is different from tyrosinase as well as from indole blocking factor, 2) CV have high DCF that converts DC to colorless indole compound, 3) MS have only ICF activity, whereas in moderately tyrosinase-rich PMS, levels of DCF and IBF activity are lower than in CV or MS, and 4) changes of T_1 form to T_3 may be associated with changes of indole blocking activity to indole conversion activity.

Last, the presence of high DCF and indole blocking factor activity in CV clearly indicates why melanization does not occur there in vivo, even though CV contain high tyrosinase activity.

Table I. Dopachrome Conversion Factor (DCF), Indole Conversion Factor, (ICF) and Indole Blocking Factor (IBF) Activity in Subcellular Fractions from Greene's Melanotic Melanoma[a]

Fractions	Percentage of activity		
	DCF	ICF	IBF
CV (0.2 mg protein)	87.5%	8.4%	91.6%
PMS (0.2 mg protein)	25.0%	57.7%	42.3%
MS (0.2 mg protein)	N.D[b]	137.8%	—
		Buffer control = 100%	

[a] Dopachrome conversion in presence of buffer after 20 min. of incubation with dopachrome (as described in *Materials and Methods*) is considered 100% (control). DCF activity of the subcellular fractions was calculated as the difference of percentage of dopachrome conversion in 20 min. from buffer (control). Indole blocking factor activity = Indole conversion in presence of buffer (control) − Indole conversion in presence of samples after 40 min. incubation with dopachrome as described in *Materials and Methods*.

[b] N.D: Not detectable.

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SYMPOSIUM ON METABOLIC DISORDERS AND NUTRITION RELATED TO THE SKIN

A clinical oriented symposium on "Metabolic Disorders and Nutrition related to the skin" organized jointly by the European Society for Dermatological Research and the Netherlands Society of Dermatology and Venereology will be held in Utrecht, the Netherlands, March 8–10, 1990. For further information please contact Willem A. van Vloten, MD, Department of Dermatology, University Hospital Utrecht, Postbox 85500, 3508 GA Utrecht, the Netherlands.