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Melanocytotoxic effect of tyrosinase inhibitors and a general theory on cell viability and multiplication

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Biologia. — *Melanocytotoxic effect of tyrosinase inhibitors and a general theory on cell viability and multiplication.* Nota di GIORGIO MORPURGO, MARCELLA NAZZARO–PORRO e SIRO PASSI, presentata (*) dal Socio G. MONTALENTI.

RIASSUNTO. — Nel presente lavoro si presenta una ipotesi circa il sistema di controllo che regola la attività funzionale e la moltiplicazione dei cloni cellulari differenziati nei mammiferi.

Secondo la ipotesi la attività cellulare è regolata fondamentalmente dalla stato conformazionale della proteina che ne costituisce il suo prodotto principale, per esempio tirosinasi nel caso dei melanociti, immunoglobuline nei linfociti, globina nelle cellule eritroidi, ecc. Lo stato conformazionale corrispondente alla attività cellulare (per esempio enzima legato al substrato) stimola la sintesi di nuova proteina ed eventualmente la moltiplicazione cellulare. Una eccessiva stimolazione può portare alla morte della cellula per iperfunzione. Al contrario la rottura genetica del sistema di controllo può portare alla trasformazione tumorale. Si descrivono alcuni dati sperimentali da noi ottenuti sul controllo di malattie iperpigmentarie (ivi compreso il melanoma) e, sulla base della ipotesi, si interpretano i dati della letteratura su sistemi cellulari molto diversi come il sistema immunitario, il sistema eritropoietico, le cellule beta del pancreas.

INTRODUCTION

The growth, multiplication and cell activity of a pluricellular differentiated organism must be subject to a thorough control. The loss of the control mechanism would lead to a disorderly pattern of growth and eventually to cancer. In complex organisms, such as mammals, part of the control mechanism is outside of the cells themselves. For instance, multiplication of the erythropoietic tissues is controlled by erythropoietin; the metabolism and the multiplication of the thyroid cells are under the control of the thyreotropic hormone (TSH), etc.

This report aims at suggesting the hypothesis that a control mechanism of cell activity and cell multiplication might exist within the cell itself. According to the hypothesis cell activity is dependent on the conformational state of its main final product (when the product is a protein) or on the conformational state of the enzyme responsible for its synthesis (when the product is not a protein). We call " final product " the main product of a differentiated specialized cell line, i.e. hemoglobin for erythropoietic cells, thyroxin for thyroid cells, melanin for melanocytes, immunoglobulin for lymphocytes, etc.. It must be clear that the " final product " can be identified only in some tissues; for instance in the liver cell it is difficult to indicate the main product of the cell.

(*) Nella seduta del 15 giugno 1978.

In the following paragraphs we shall decribe some cases supporting the previous statements.

TYROSINASE INHIBITION AND MELANOCYTOTOXIC EFFECT

Pityriasis versicolor is a common disease of the human skin caused by a yeast, *Pityrosporum orbiculare*. Its colonization in the stratum corneum causes the appearence of cutaneous spots which often are initially hyperpigmented and then completely depigmented. In the affected areas the

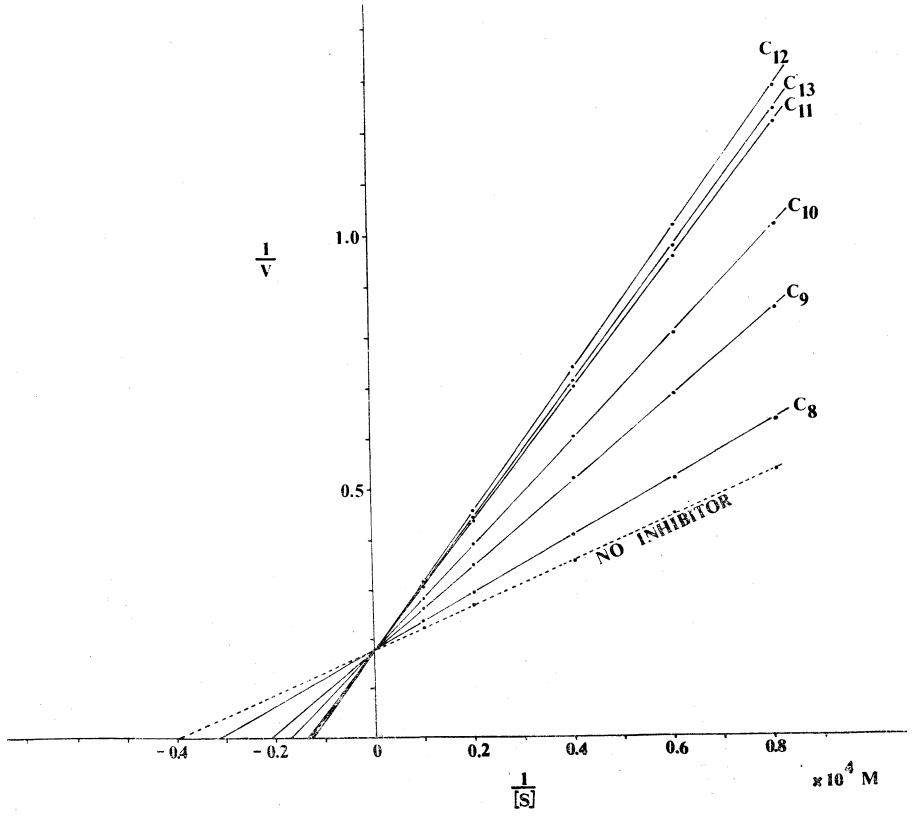

Fig. 1. – Lienwever-Burk plot showing competitive inhibition kinetics of dicarboxylic acids on tyrosinase. L-dopa was used as substrate. V = V max.; S substrate concentration.

melanocytes are clearly suffering or degenerated [1]. Our previous data [2] have shown that *Pityrosporum* is able to oxidize unsaturated fatty acids giving rise to the formation of dicarboxylic fatty acids; we have also shown that dicarboxylic fatty acids higher than C_8 are competitive inhibitors of tyrosinase activity *in vitro* (Fig. 1) and therefore of melanin formation. Dicarboxylic fatty acids from C_8 to $C_{1,}$ have proved to have a remarkable selective cytotoxic activity on both normal and pathological melanocytes. Our researches [2, 3] have shown that: 1) the application of a cream containing C_9 dicarboxylic

acid (azelaic acid) has a pronounced depigmenting effect on Chloasma, toxic melanoderma and Poikiloderma of Civatte; 2) The application of the same cream to the pretumoral condition Lentigo Maligna produces the apparently total regression of the lesion (Plate I, *a*, *b*); 3) Intraperitoneal, subcutaneous or oral administration of azelaic acid or dodecandioic acid to albino mice Balb's C with transplanted Harding-Passey melanoma resulted in significant retardation or inhibition of growth of the tumor (Table I).

TABLE I

Inhibitory effect of dicarboxylic acids on sprouting and growth of Hardy-Passey melanoma in mouse.

N° of animals	Drug (*)	Day of appearance	Results after 35 days		
			Survival	An. with tumors	size
15	—	7	0 All dead with tumors 30–50 mm diam.		
15	C_9	15	10	8	5–30
15	C_{12}	15	12	9	5–30

(*) Dicarboxylic acids were administered per os ad libitum as sodium salt in water 20 mg/ml.

These observations led us to postulate the existence of a relationship between the functional activity of the cell and its life and multiplication. The hypothesis was also supported by the fact that the other melanocytotoxic agents also inhibit melanin formation [4] by acting as alternative substrates for the enzyme. Catechols in particular [4] inhibit melanin formation in this way.

In the hydroxyanisoles series, meta, ortho and para, the melanocytotoxicity parallels the affinity of the drug for tyrosinase [5]. We have also evidence (Passi *et al.*, unpublished) that hydroquinone, another depigmenting agent, is a substrate for tyrosinase. Recently it has been shown that also the excess of natural substrates, tyrosine and dopa, produces cellular degeneration [6, 7]. Moreover phenylthiourea, a powerful non competitive inibitor of tyrosinase provides complete protection to the cells from the toxicity of the melanin precursor (6).

Lerner [8] has put forward the hypothesis that tyrosinase activity can result in the production of some compound toxic to the cell itself. He states that " a melanin precursor, one that is either a phenol or catechol derivative, or the phenol tyrosinase complex is lethal for the melanocytes ". Riley [10] has tried to explain the cytotoxic action of the substrates through the production of free radicals. This explanation is no longer valid with reference to dicarboxylic acids which are not metabolized.

Because undoubtedly there exists a relationship between the activity of tyrosinase and cell life a possible hypothesis is that this is regulated by the conformational condition of the enzyme responsible for the buildup of the final product. Each enzyme exists in at least two conformational conditions: 1) unbound enzyme (E); 2) bound to the substrate (ES). We suggest the hypothesis that the state E would inhibit the formation of new enzyme molecules and of cell multiplication, while the state ES would stimulate the formation of new enzyme and consequently cell multiplication (Fig. 2). The cell

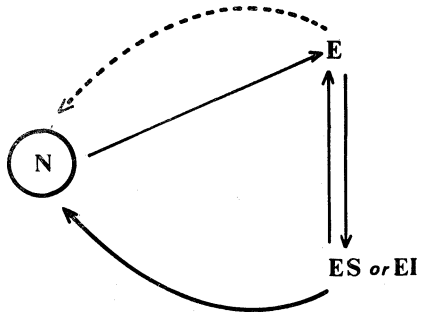

Fig. 2. – A theoretical scheme explaining the regulation of melanocyte activity. N = nucleus; the dotted arrow indicates inhibition, the thick solid arrow activation.

estimates the level of its activity and thereby of the necessity to form new enzyme and eventually to multiply by constantly measuring the ratio between the E and ES forms. In physiological conditions the enzyme should be totally in the E form or else both forms should be simultaneously present. We suggest that whenever the balance is altered in the direction of excess of bound enzyme there is an excessive stimulation of the cell which can lead to damage and its ultimate destruction.

If this hypothesis is well grounded we should be aware of the following consequences:

1) The competitive inhibitors (EI is considered equivalent to ES) must be cytotoxic. 2) Non competitive inhibitors are not necessarily cytotoxic. 3) The excess of substrate or the excessive stimulation of the cell must be cytotoxic. 4) The genetic deficiency of the enzyme and the physiological absence of its functioning must not damage the cell. 5) The cytotoxic phenomenon must be preceded by a transient hyperfunction.

We have already discussed point 1 and 3. Point 4 is confirmed by the fact that in genetically determined albinism the melanocytes are intact. Regarding point 2, reduced glutathione and ascorbic acid, simply by acting as antioxidants, inhibit tyrosinase without any cytotoxic effect [9, 10]. Our data on cultures of human melanocytes with C_9 or C_{12} dicarboxylic acids (Breatnach *et al.* unpublished) clearly show that cell damage is preceded by an abnormal accumulation of melanosomes.

Experimental diabetes and the viability of pancreas cells

Diabetes can be artificially induced in animals by various chemical treatments. In these cases it is caused by selective destruction of the β pancreas cells. The best known diabetogenic agents are alloxan, dehydroascorbic acid and streptozotocine. It is not known how the diabetogenic agents exert their selective cytotoxic action. One possibility is that they act by oxidizing the SH groups of glutathione [12, 13]. The oxidized glutathione could in turn cause the production of abnormal insulin through the formation of a disulfide bridge with one of the residues of the oxydized cysteine. The formation of altered insulin could then stimulate the cell to produce new insulin molecules and lead to its ultimate destruction by hyperfunctioning.

Whatever the mechanism of action of the diabetogenic agents, the first effect of alloxan administration is an enormous increase in the formation of β granules and in the release of insulin which occurs only five minutes after alloxan administration [12].

Thereafter the cytotoxic effect is manifested. These observations are again in keeping with the hypothesis of a regulatory action exerted by the final product and that hyperfunction leads to cell damage. Strongly in favour

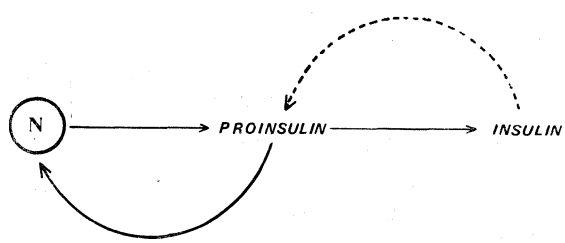

Fig. 3. – A theoretical scheme explaining the regulation of the pancreas cells. Symbols as in Fig. 2.

of the hypothesis is also the observation that in some animals (cat) the administration of an excess of glucose produces permanent diabetes through β cell destruction [12].

Also, in some cases of juvenile diabetes the first manifestation of the disease is not the lack of hormone but on the contrary its exalted secretion in relation to the glucose load.

A hypothetical scheme explaining the regulation of insulin production in the β cell is presented in Fig. 3.

Control of immunoglobulin production and of lymphocyte multiplication

According to the most accepted theory on antibody synthesis [14] at all stages the lymphocytes have the antibody present on the cell membrane. The antigen-antibody recognition would produce two different effects: 1) the stimulation of the synthesis of new molecules of the cellular clone which pro-

duces that particular antibody, 2) The multiplication of the clone. With the present theory the scheme can be further simplified: the antigen attaches itself to the antibody modifying its conformation. In the new conformation the antibody stimulates the synthesis of heavy chains (H) [15]; their synthesis causes cell multiplication. This theory is also supported by the fact that lymphocyte multiplication can be stimulated by using antibodies against those produced by the lymphocytes [14]. The quantity of free antibodies on the membrane should determine whether the cell should multiply or not. The scheme in Fig. 4 illustrates the above considerations.

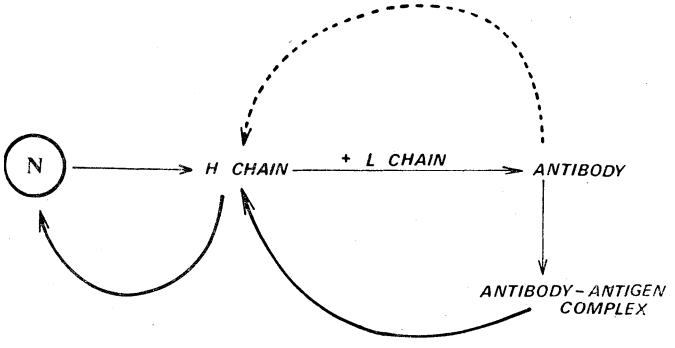

Fig. 4. – A theoretical scheme explaining the regulation of activity and multiplication of immune cells. Symbols as in Fig. 2.

Such scheme can also explain the phenomenon of high dose immunological paralysis (the low dose is a complicated event which needs the cooperation of B and T cells). Very likely the paralysis is caused by the selective destruction of the cell clones able to recognize the antigen and produce the specific antibody [16, 17]. According to the hypothesis the excess of antigen would bring all the molecules of the antibody into the bound condition (antigen + antibody) determining the excessive stimulation and consequently cell damage and death.

MULTIPLICATION AND DIFFERENTIATION OF ERYTHROID CELLS

Multiplication and differentiation of the erythroid cells are also under the control of the final products of globin and heme synthesis. The synthesis of globin and of the other proteins is dependent on the presence of hemin [18]. The hemin inhibits the synthesis of an inhibitor of the initiation factor IF 2 [18]. Because the differentiation of the cell is possibly controlled by hemoglobin, in the erythroid cells synthesis multiplication and differentiation would be under the control of the final products.

The theoretical scheme that best corresponds to the fact is the following: under a stimulus whose nature is not known the stem cells of the bone marrow are transformed into ERC (erythropoietin responsive cells) and then into proerythroblasts. These cells are irreversibly committed in the erythroid line. At this moment the cells normally begin to synthetize hemoglobin. With the rise in Hb concentration the cell is transformed into erythroblast and

then, losing its nucleus, passes to the stage of reticulocyte and mature red cell. We can therefore hypothesize that the heme concentration stimulates the synthesis of the globin and both stimulate the multiplication of the ERC. On the contrary, the concentration of Hb would be the factor determining the differentiation of the cell into mature red cell and consequently the end of the reproductive potentiality. The scheme is shown in Fig. 5.

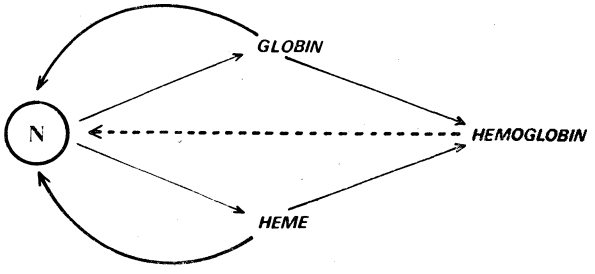

Fig. 5. – A theoretical scheme explaining the regulation of erythroid cell activity and multiplication. Symbols as in Fig. 2.

The hypothesis here outlined complies with the cell behaviour of some lines of a tumor of the mouse, the Friend Erythroleukemia. Erythroleukemic cells are considered to derive from the viral transformation of a proerythroblast. These cells, acquiring tumoral characteristics, lose at the same time the erythropoietin responsiveness. Normally only a very limited fraction of these cells produces Hb. Its formation nevertheless may be aroused in most cells by DMSO or by the presence of heme, but never by erythropoietin. The cells which synthetize Hb, no matter how stimulated, cease to multiply and through normal differentiation are transformed into erythrocytes [9].

Our hypothesis clearly explains this behaviour pattern: tumoral transformation can be based on the incapacity of the cells to respond to the erythropoietin stimulus and to synthetize Hb. Without it the cell does not differentiate and does not stop mitotic activity. The artificial stimulation of Hb synthesis converts the tumor cell into a normal one.

End product regulation and tumor

If the activity and the multiplication of the cell lines are regulated by the final product(s) it is conceivable that the rupture of the regulation mechanism could result in tumoral transformation. A cell which produces the final product without any control, if this is the regulator of the multiplication, and does not die as the result of hyperactivity (we shall discuss this possibility later) is potentially a tumor cell. In our scheme the tumoral transformation could originate in 1) the rupture of the mechanism which transmits the control from the final product to the nucleus; 2) the rupture of the mechanism of release of the final product (in endocrine or exocrine glands); 3) a genetic structural modification of the end product. The production of a modified protein which is not identified by the recognition mechanism would result in a tumor.

Some evidence supports this view: tumors of the endocrine glands generally release a high level of hormones in an uncontrolled way, i.e. insulinomas produce insulin independently of the concentration of glucose in the blood, thyroid tumors release thyroxine independently of the stimulus of TSH, erythroleukemic cells are independent of erythropoietin, etc. Independence from normal stimuli, by altering the synthesis of the final product is, in our hypothesis, the first cause of the tumor and not a peculiarity of it.

Particularly interesting in this respect is the case of a myeloma called Heavy Chain Disease (HCD). In the cases of HCD we know that the heavy chain produced to a great extent by the tumoral cells presents a deletion in the region between the constant and the variable part of the chain [9] which obviously hinders the formation of the complete antibody. Since the contemporaneous occurrence of a deletion in the H chain and a tumoral transformation is an extremely rare event the most plausible explanation is that the deletion and hence the impossibility of forming the antibody are the cause and not the result of the uncontrolled cell proliferation.

We must discuss now how the cell which has lost the regulation mechanism may become a tumoral cell instead of dying as the result of hyperfunction. We think that in most cases (HCD could represent an exception) in the beginning the failure in the control mechanism is only partial: it is sufficient to stimulate cell multiplication but not to damage the cell. In many experimental tumors—for instance in the experimental tumors of the thyroid—there is evidence that in the beginning the cells are only partially independent of the hormones of the hypophysis: following serial transplantetion the tumor becomes gradually totally independent probably because of successive mutational events and selection. Actually we consider that in most cases cell death caused by hyperactivity is the result of the sudden loss of the control mechanisms and we think that this constitutes an efficient defence of the organism against tumoral transformation. In fact we know that tumoral transformation is a complex event generally deriving from successive mutations which gradually cause the failure of the control mechanisms.

ACTION OF HORMONES

We have already stated that cell multiplication and activity is generally under hormonal control or other environmental factors like glucose concentration in the blood. In most cases the specific action of the hormone is to recognize the target cell. The action on the cell is generally aspecific, being in most cases, i.e. TSH, MSH epinephrine, glucagon, etc., mediated by cAMP. If the hormonal action is aspecific—and it really often stimulates the differentiated cell to do the only thing it can do—the importance of understanding what is going on inside the cell following the hormonal stimulation is patent. We want here to suggest the hypothesis that the hormonal action limits itself to the derepression of the synthesis of the enzyme responsible for the production

of the final product (or of the final product when it is a protein). Derepression of the synthesis would then consequently lead to the stimulation of cell reproduction. In effect TSH [21] stimulates in a few minutes the synthesis of thyroxine while cell multiplication follows after some days. There is also some evidence that erythropoietin directly stimulates Hb synthesis [22, 23, 24]; in sheep erythropoietin administration stimulates the synthesis of a foetal Hb normally absent in the adult. Also MSH stimulates the ex novo synthesis of tyrosinase in the melanocytes [26]. Evidence concerning this point is clearly insufficient but we think that the possibility of building up a unitarian theory of the phenomena of cell multiplication should be investigated.

Final remark

We finally want to point out that the logic governing cell multiplication and activity—if the scheme previously outlined is accepted—is not different from that governing the synthesis of enzymes in bacteria according to the scheme proposed by Jacob and Monod. In both cases regulation occurs through the monitoring of the cellular functioning. In prokaryotes that have no specialized cells each function is controlled independently in the same cell. In the complex eukaryotes it is often more convenient to regulate the number of cells than to regulate the concentration of protein in a constant number of cells. For instance, low oxygen pressure stimulates the increase in the total Hb content, not increasing the concentration of the Hb per cell but increasing the number of red cells per ml. of blood. This is clearly convenient because the Hb works with its maximum efficiency in a red blood cell as it is. To achieve this result the most logical system is monitoring the state of activity of the cell to stimulate the synthesis of the product when it is necessary and finally to induce cell reproduction when the concentration of the product has exceeded a certain threshold.

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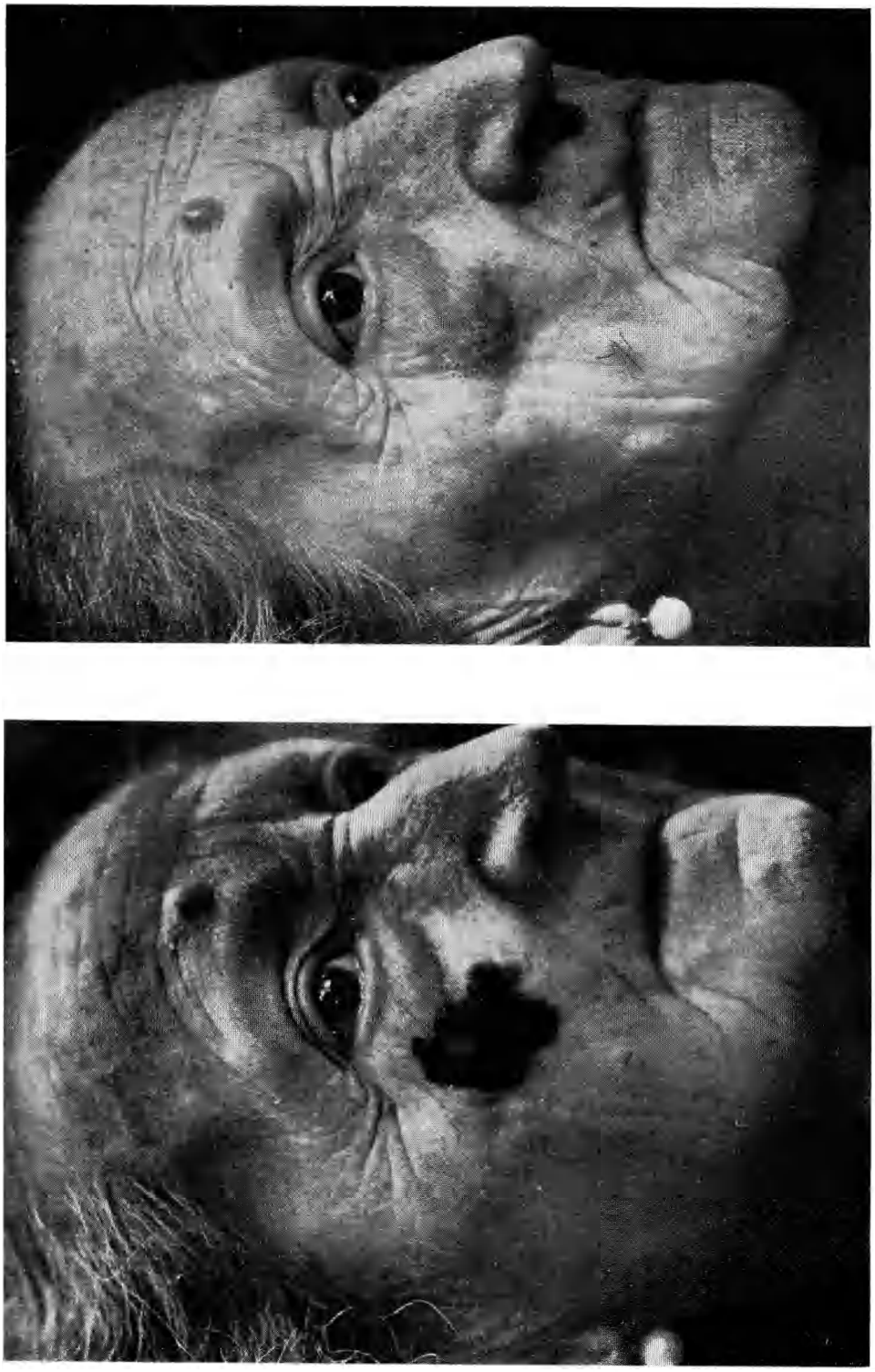

a *b*

A case of Lentigo Maligna treated with a cream containing azelaic (C_9) acid. *a*) before treatment; *b*) sixty days after the beginning of treatment.