BIOCHEMICAL STUDIES ON VITILIGO

BY

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INTRODUCTION

I. INTRODUCTION

The skin is sometimes said to be a window to human biology and pathology. Many processes of life whether normal or abnormal, are reflected on the body surface (Olwency, 1981).

Vitiligo is characterised by the development of depigmented patches on the skin (Goudie et al., 1979) which may be due to exhaustion of melanocytes, blackade of formation of melanin or disappearance of melancytes. Neurochemical factors and self destructive processes by the melahocytes may also have a possible role in the disease (Ajit Singh Kumar et al., 1980).

Vitiligo is one of the commonest pigmentary problems in India (Mathur et al., 1974). It is a very common disorder which affects atleast 1 per cent of the population and is usually gradually progressive over many years.

Vitiligo affects all races and both sexes seem equally vulnarable to the disease. Approximately 50 per cent of the patients develop some forms of the disease before age 10, but vitiligo may have it anytime from infancy to senscence (Domonkos et al., 1982).

True vitiligo must be differentiated from temporary secondary depigmentation which may be seen in tinea versicolor, pityriasi's rosea seborrheic dermatitis or following exposrure to anti oxidants. In these conditions pigmentation usually returns after subsidence of the mild to moderate inflammation or withdrawal of the chemical.

The etiology of vitiligo is unknown, but it must infrequently follows an insult of some kind to the system (Behl and Bhatia, 1973). The relative frequency of vitiligo has led to a discussion of the relationship between vitiligo and pernicious anemia, diabetes mellitus and an immunologically effective thyroid disturbances (Korting and Denk, 1976). Most of the Indian clinicians believe that gastrointestinal worms may be factors contributing atleast partially towards causation of vitiligo (Rahnbir Singh and Gurmohan Singh, 1980).

There is no satisfactory method of treatment for vitiligo. Administration of oxypsoralen topically, orally or both in conjunction with exposure to natural sunlight appears to be encouraging.

The black and brown pigment is of most animals are forms of melanin. The enzyme responsible for melanin formation is tyrosinase (Bender, 1975).

The activity of the enzyme tyrosinase depends on the availability of the trace element copper. It was suggested that zinc may play a part in the formation of melanin, Copper and zinc levels were estimated in serum and skin of the vitiligo patients (Molokia and Portnoy, 1973). The nature of association between zinc and melanin remains unexplored. It has been suggested that melanin may be acting as an potent chelating agent binding zinc (Ashok Ghorpade et al., 1982).

The interaction between copper and vitamin C, iron and vitamin C have been studied in other species (Milne and Tomayl, 1980).

The analysis of body tissues and tissue fluids for trace metal can often provide information that is useful for diagnosis and treatment of the disorders (Delver, 1976).

In the present investigation an attempt had been made to study

a) the tyrosinase activity in the skin and to evaluate the level of copper, zinc and iron in serum and skin,

b) ascorbic acid in blood and

c) melanin in urine, in order to understand their relationship to the development of vitiligo.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

The literature related to this investigation 'Biochemical studies on vitiligo' is presented under the following headings:

A. Introduction

B. Incidence of vitiligo

C. Effect of age and sex on vitiligo

D. Role of heredity on vitiligo

E. Mechanism of pigmentation

F. Histopathology

G. Classification of vitiligo

H. Dermatological studies on vitiligo

1. Digital pattern in vitiligo
2. Non-nervous vascular reactions in vitiligo
3. Sweat gland reaction in vitiligo

I. Biochemical changes in vitiligo

1. Enzymes of serum and skin
2. Role of trace elements in vitiligo
3. Vitamins and Tyrosine metabolism
4. Study of blood groups in vitiligo
5. Urinary phenolic acids
6. Influence of Hormones

7. Level of serum cholesterol in vitiligo patients
8. Differential count of leucocytes and hemoglobin in vitiligo patients
9. Effect of water
10. Serum and urine creatinine in vitiligo patients

J. Vitiligo and other associated diseases
1. Vitiligo and pernicious anemia
2. Vitiligo and intestinal parasites
3. Perkinson's disease and vitiligo
4. Vitiligo and grave's disease

K. Treatment
1. Allopathy
a) Steroids
b) Clofazimine
2. Photochemotherapy
a) Use of 8 methoxy psoralen
b) Topical use of Anacarcin Forte(r) oil

A. Introduction

Vitiligo is an acquired disfiguring pigmentary anomaly of the skin manifesting white patches, surrounded

by a normal or hyperpigmented border; the depigmented skin is normal in all other aspects. The hairs in the vilili-genous areas usually become white also, very rarely patches may have a red inflamma tory border.

B. Incidence of Vitiligo

Vitiligo is more prevalent in the tropics than in the cold regions. Dark skinned persons seem more frequent-ly and more severely effected than light skinned (Donmonkos et al., 1982).

The reported incidence of vitiligo in various dermatological clinics in India varies from 3.5 per cent to 4.3 per cent (Dutta, 1977). Trauma in predisposed individu-als may induce vitiligo as a Koebner isomorphic response (Coondoo et al., 1976).

Many disorders have been reported in which there is an associated higher incidence of vitiligo especially of the later onset types. Most of these are disorders of commonly speculated to be of autoimmune pathogenesis', for example hyperthyrodism, hypothyroidism, Addison's disease, pernicious anemia, hypoparathyrodisim and rheumatic disease.

Vitiligo like changes in the skin were detected in 27.2 per cent of the workers producing phenol containing additives. Dark haired men were found to be more subject to depigmentation (Telegina and Boiko, 1972).

C. Effect of Age and Sex on Vitiligo

Vitiligo affects all races and both sexes seem equally vulnarable to the disease. In about 50 per cent of the patients vitiligo starts before the 20th year of life and in about 20 to 30 per cent of cases there is a familial incidence (Korting and Denk, 1976).

D. Role of Heredity on Vitiligo

There is a pronounced dominantly inherited predisposition to the disease as indicated by the observation that about 25 per cent of affected persons have a family history of the same problem (Mochella et al., 1975).

E. Mechanism of Pigmentation

Melanogenesis involves the progressive oxidation of the aminoacid tyrosine and the product is subsequently

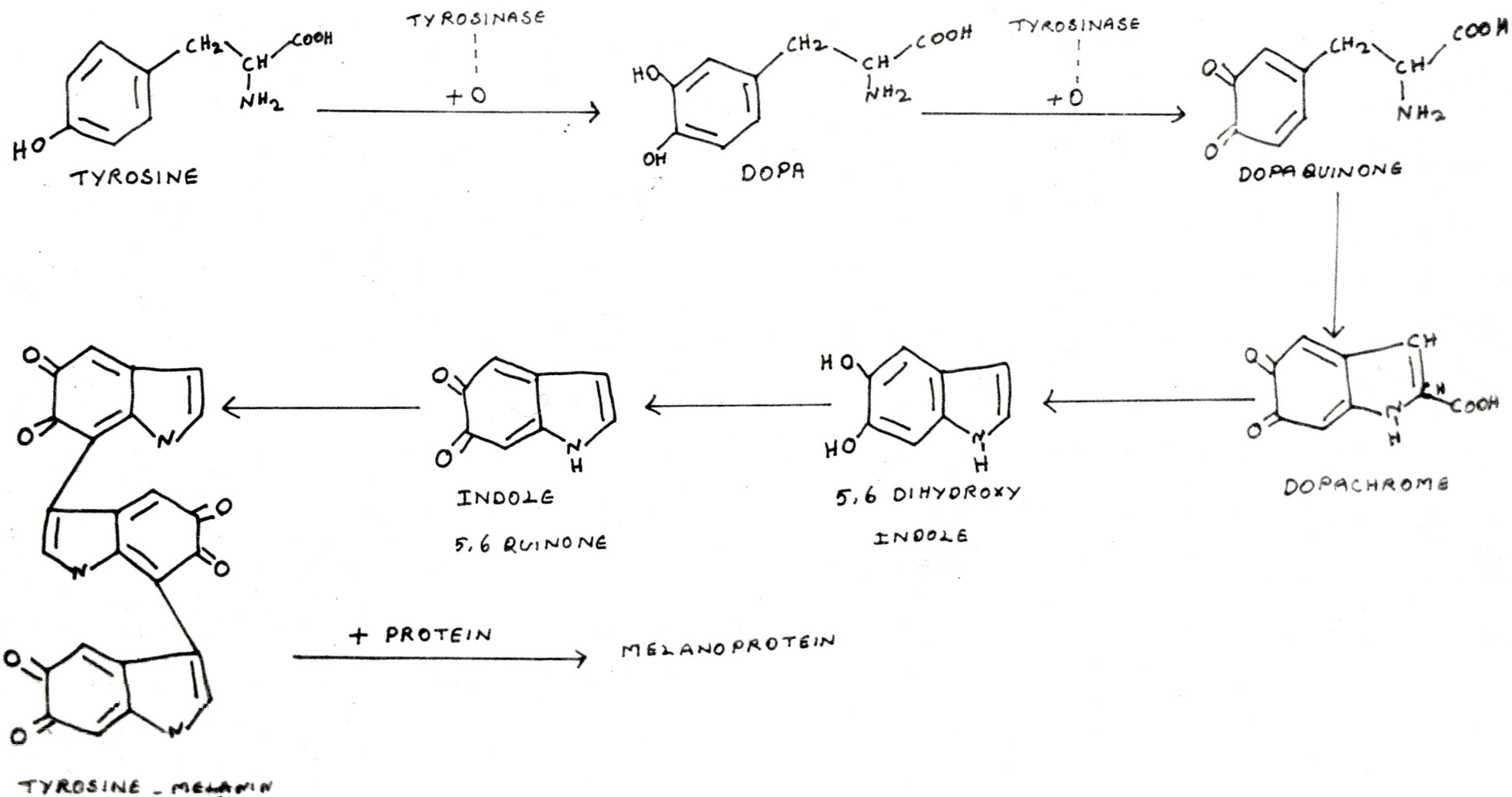
polymerised and linked to protein. Initially tyrosine is hydroxylated to dihydroxy phenylalanine (DOPA) which is then dehydrogenated to quinone. A single enzyme, a copper containing aerobic oxisase is now considered to be responsible for both tyrosinase and dopa oxidase activity. Dopa quinone rearranges to form an indole compound, leucodopachrone, which is subsequently dehydrogenated to dopachrome and then decarboxylated and rearranged to form 5, 6 dihydroxy indole. This is further oxidised to the corresponding quinone which becomes polymerised and linked to protein. The stages of melanogeneis are illustrated in figure 1.

Four biological processes are involved in melanin pigmentation.

1. Formation of melanosomes in melanocytes
2. Melanization of melanosomes
3. Secretion of melanosomes into keratinocytes
4. Transport of melanosomes in keratinocytes with and without degradation in lysosome like organells (Rook et al., 1979).

FIG: 1

STEPS OF MELANOGENESIS



F. Histopathology of Skin

There is marked absence of melanocytes and melanin in the epidermis. Histochemical studies shows lack of DOPA positive melanocyte in the basal layer of epidermis. Electron microscopic studies confirm the loss of melanocytes that appear to be replaced by langerhans cells. Degenerating melanocytes are found especially at the margins of vitiligo. There is also an increased cellularity of the dermis. Occassionally a raised erythematous border is seen and in this inflammatory vitiligo the skin is infiltered with lymphocytes and histocytes.

G. Classification of Vitiligo

Three types of vitiligo can be demonstrated histologically. In the absolute type no dopa positive melanocytes are present. Two relative forms exist. One is which very weakly Dopa positive melanocytes are present in normal number, the other in which the number of Dopa positive melanocyte is reduced but the remainder are enlarged and have elongated dendritic process. Melanocytes appear to be replaced by langerhans cells in some cases (Rook *et al.*, 1979).

The depigmentation may rarely become generalised or universal. Four types have been described according to the extent and distribution of the involved area;

a) localised, including a linear or segmented pattern
b) generalised
c) universal and
d) perinevia (halonerves)

H. Dermatological studies on vitiligo

1. Digital pattern in vitiligo

Significant differences were found in digital pattern of the two sexes in the controls as well as patients (Singh et al., 1983).

2. Non-nervous Vascular Reaction

There was a relatively increased blister resorption time, increased bleeding time and increased duration of blanch reaction in the vitiliginous skin. The results were interpreted as possibly showing an inhibited nerve tonus and altered conditioning, perhaps due to local sympathetic hypotonia (Dutta and Dermat, 1972).

3. Sweat gland Histology in Vitiligo

Resistance to the conductance of a minute direct electric current was used to asses the functional integrity of sweat glands in normal and affected skin. In 25 out of 71 cases of vitiligo increased resistance to the electric current was observed indicating sweat gland dysfunctions. Various degrees of inflammatory and fatty aerolar degeneration of sweat glands were observed (Gokhale et al., 1977).

I. Biochemical Studies on Vitiligo

1. Enzymes of Serum and Skin

a. Tyrosinase

The role of tyrosinase in controlling the melanin formation is seen in the gold fish (BENDER, 1975). Although the majority of the workers feel that the this single enzyme carry out both the conversion of tyrosine to DOPA and the conversion of DOPA to Dopaquinone (Fitzpatrick, 1971), studies showing that two distinct enzymes for the conversion of aerobic Dopa oxide and peroxide have been reported (Okun, 1973). Tyrosinase is currently felt to be the only enzyme required in the synethesis of melanin by the Raper-mason Scheme.

Dopa reaction was completely absent in vitiliginous areas in 24 cases and was present in low intensity in 6 cases.

In the hematoxylein and Eosin stained sections, lack of melanin pigment was apparant in the vitiliginous skin of all patients. In 5 cases the demarcation between normal pigmented and vitiliginous areas was fairly sharp (Sarin and Prabakar, 1980).

The activity of the enzyme involved tyrosinase depends on the availability of the trace element copper. Metal binding agents as well as metals competing with copper can inhibit such activity (Molokhia and Portnoy, 1973).

The incubation of skin with DOPA is used to detect the tyrosinase activity and thus for the demonstration of of melanocytes.

B. Peroxidase

Mammalian peroxidase can mediate enzymatic conversion of tyrosine to melanin in the presence of dopa as cofactor. Mammalian tyrorinase has strong doap oxidase activity but there is no conclusive evidence that it can

significantly oxidise tyrosine to melanin. Peroxidase may be the enzyme that initiate melain synthesis invivo ; tyrosinase may utilise dopa synthesised by the enzymatic activity of peroxidase. Tyrosinase may function primarily as a dopa oxidase (Malkinson and Pearson, 1971).

C. Ceruloplasmin

Giri et al., (1980) reported that when compared to normals the vitiligo patients had slightly higher levels of serum ceruloplasmin.

D. Alanine Transaminase

It was reported that serum alanine transminase were higher in vitiligo patients when compared to the normals (Giri et al., 1980).

2. Role of Trace Elements in Vitiligo

A deficiency of trace element can be wholly or partly responsible for a number of disorders (Martin Laker, 1982).

Multielement analysis of postmortam tissue specimens carried out by Tipton (1961) and Butt et al., (1964) showed the consistent presence of more than 20 trace metals in normal tissues.

a. Levels of Copper and Zinc

Copper serues as a prosthetic group of tyrosinase in the production of melanin pigements (Bradfield et al., 1980). Copper and zinc levels wefe estimated in serum and skin of nineteen vitiligo patients. In vitiligo there was a significant reduction in serum zinc while copper levels were increased in the hyperpigmented zone surrounding the lesions. Chemical analysis of melanosomes has revealed a high zinc content and it was suggested that zinc may play a part in the formation of melamin. Certain vitiligo had subnormal levels of copper (Molokhia and Portnoy, 1973).

The nature of association between zinc and melanin remains unexplored. It has been suggested that melanin may be acting as potent chelating agent binding zinc (Ashok Ghorpade and Reddy, 1982).

Various studies on serum copper levels in vitiligo patients have shown a tendency to low levels (Lal and Rajagopal, 1970). Behl et al., (1961) demonstrated significantly lower levels of serum copper in vitiligo cases compared to normal. Serum copper was determined in 21

normals and 24 vitiligo patients. The mean serum ionic copper was lower in this disease but the difference from the normals was not significant statistically (Rajagopal et al., 1971).

Serum concentration of zinc are certainly low in some patients with leg ulcers as well as in other chronic diseases. Skin levels on the other hand are not reduced (Dachowski et al., 1975) and serum levels do not correlate with whole body zinc (Hawkins et al., 1976).

Owing to the role of cuproprotein in melanin formation, Huziez et al., (1972) studied the two varieties of blood copper in 72 patients with vitiligo and 30 controls. The albumin bound copper in controls lay between 80-150 mg/100 ml. In 72 cases of vitiligo serum copper was less than 85 ug in 29 per cent and less than 50 ug in 10 per cent. In 50 per cent of cases the serum copper was normal. The few cases with a serum copper above 150 ug had some other diseases such as hepatitis, hyperthyrodism or cancer.

b. Level of Iron and Manganese

The mean serum iron values do not vary significantly in vitiligo patients when compared to normals in the corresponding age group.

The mean serum manganese values of patients do not differ significantly from the corresponding values of the normals. This shows that serum manganese levels are not changed in vitiligo (Giri et al., 1982).

c. Level of Sodium and Potassium

There is hardly any change in the levels of potassium and sodium between patients and controls of the same age groups (Giri et al., 1979).

3. Vitamins and Tyrosine Metabolism

The level of ascorbic acid in blood, which is a better indicator of vitamin C status in vitiligo patients ranges from 0.2 - 1.4 mg per cent with a mean of 0.5 mg per cent where as the normal value is 0.2 - 1.2 mg per cent with a mean of 0.73 mg per cent (Ve lov and Gopalan, 1964).

The mean ascorbic acid values are increased significantly in vitiligo patients when compared to normals upto 30 years. Above 30 years of age, the serum ascorbic acid values of patients do not show any significant variation from the normals of the same age group (Giri et al., 1982).

There is some evidence indicating that folic acid and vitamin B_{12} are also involved in tyrosine metabolism (Duncan, 1961).

4. Study of Blood Groups in Vitiligo

There was no significant difference in the distribution of various blood groups in the patients having the vitiligo and controls (Kuper *et al.*, 1981).

5. Urinary Phenolic Acids

Dopa besides being converted into melanin is also metabolished through other pathways accounting for urinary phenolic acids like 3-methoxy 4-hydorxy phenyl acetic acid and 3-methoxy 4-hydroxy phenylpyruvic acid. There was no significant difference in the excretion of the common urinary phenolic acids in the two groups. (Verma *et al.*, 1973).

6. Influence of Hormones

Melanogenesis is influenced by certain polypeptide hormones of the pituitary and also to some extent by the steroid hormones. Thus adrenocorticotrophic hormone like peptides affect its melanin content (Janet Marks, 1983).

7. Levels of Serum Cholesterol in Vitiligo Patients

There was statistically significant increase in serum cholesterol level of vitiligo patients when compared to the controls of the same age group (Giri et al., 1980).

8. Differe ntial Count of Leucocytes and Hemoglobin in Vitiligo Patients

When compared to controls the polymorph counts of the vitiligo patients were significantly different, the lymphocyte count was increased significantly (Giri et al., 1980).

Giri et al., (1979) reported the hemoglobin level to be between 11.1 to 12.8 per cent in vitiligo patients.

9. Effect of Water

An analysis of drinking water in and around Coimbatore, where prevalence of vitiligo is high, showed that copper and iron were present in very minute amounts., much below the standard limit suggested by the ICMR. Hence it was felt that water could be an aetiological factor in the development of vitiligo in and around the Coimbatore city. This was supported by the fact that the copper levels

in the serum of vitiligo patients were low (Sudha Naik, 1979).

10. Serum and Urine Creatinine in Vitiligo

Mean serum and urine creatine values were decreased significantly in vitiligo patients when compared to normals in corresponding age groups (Giri et al., 1982).

J. Vitiligo and Other Associated Diseases

1. Vitiligo and Pernicious Anemia

A relation has been found to exist between vitiligo and several presumbly autoimmune disorders, including pernicious anemia, thyroid disease and diabetes mellitus. Permicious anemia is about 30 times as frequent among the patients with vitiligo as among general population (Dawber, 1971).

2. Vitiligo and Intestinal Parasistes

Most of the Indian clinician believe that gastro intestinal disorders like chronic amoebiasis and intestinal worm may be factors contributing atleast towards the causation of vitiligo (Rahnbir Singh and Gurmohan Singh, 1980). Only 18 per cent of the cases were the intestinal parasites detected of course entomoeba toping the list (Behl and Bhatia, 1972).

3. Perkinson's Disease and Vitiligo

One case out of 28 with Perkinson's disease treated with L-Dopa had also vitiligo (Iarragirre, 1971).

4. Vitiligo and Grave's Disease

In addition to the characteristic features of Grave's disease, some patients have been noticed to have a typical vitiligo (Malkinson and Pearson, 1971).

K. Treatment

So far there is no satisfactory method of treatment for vitiligo.

1. Allopathy

a. Steroids

Harry Brostoff and Jonpthan Brasstoff (1978) reported that prolonged treatment with corticosteroids produced complete remmision of extensive vitiligo. There have been few reports of some repigmentation of each active lesions following intralesional or topical treatment with highly potent anti inflammatory corticosteroids (Kandil, 1970).

b. Clofazimine in Vitiligo

Vitiligo patients were given oral clofazimine and were exposed to sun rays for 30 minutes daily. Significantly

more patients in the treated group developed repigmentation. But the pigment disappeared soon after stopping the treatment (Shash et al., 1981).

2. Photochemotheraphy

The drugs used are phototoxmic compounds, psoralens which are known for decades for the treatment of vitiligo (Naik, 1983). The drug used was 8-methoxy psoralen.

Topical application of Anacarcin Forte (r) oil was tried in 10 cases of vitiligo. Seven cases showed excellent results. Side effects like intense itching vesication and pustulation was seen in two cases. One case showed urticarial rash (Panshi, 1980).

EXPERIMENTAL PROCEDURE

III. EXPERIMENTAL PROCEDURE

The experimental procedure for the present study 'Biochemical Studies on Vitiligo' is presented as follows:

1. Selection of patients
2. Collection of skin samples
3. Collection of blood and separation of serum
4. Collection of urine
5. Analysis of skin
6. Analysis of serum
7. Analysis of urine

1. Selection of patients:

Thirty vitiligo patients were selected at random from among those attending the Government General Hospital, Coimbatore, for treatment. They were of ages ranging from 5 to 70 years.

Thirty healthy normal individuals of the same age group were selected for comparison.

From five vitiligo patients, five skin biopsy samples were collected.

Skin biopsy samples were taken from five normal healthy individuals also for comparison.

2. Collection of skin samples:

The discs of skin which were punched out with local anesthesia have been used for clinical diagnosis. The punch was of various diameteres from 2 to 8mm.

3. Collection of blood and separation of serum:

The blood was collected as follows: (Oser, 1971). Tied a Tourniquet (of soft rubber tubing or a strip of bandage) tightly around the arm of the patient, a couple of inches above the elbow. Had the subject clench his fist firmly, washed the skin surface above the most prominent vein on the inner surface of the elbow (Usually themedian bassillic) with 70 per cent alcohol, allowed to dry, held the vein immobile by pressing on it with the thumb below the elbow and into the vein inserted a sharp sterile hypodermic needle (number 20) an inch and a half long which was attached to a dry, sterile syringe of suitable capacity. The needle should penetrate the vein from the side and at an angle of 50° with the surface of the arm, the level or opening of the needle being kept upward or the other-side. As soon as the blood was seen to enter the syringe retracted the plunger slowly until 5.5ml of blood had entered the syringe. Before removing the needle from the vein loosened the tourniquet, had the patient unclench his fist and on the skin, at the point of entrance of the needle, held in place a small pad of folded gauze, moistened with 70 per cent alcohol, withdrew the needle, detached it from the syringe

(not too vigorously which might cause helnolysis) and then transferred to a centifuge tube. Pressure on gauze pad will effectively prevent bleeding from the skin puncture. It is important that the pressure be maintained for minimum of five minutes, to prevent the formation of a painful hematoma at the site of puncture.

2.5ml of the blood was transferred to an oxalated bottle for the estimation of Ascorbic acid.

3.0ml of the blood, after being transferred to a centrifuge tube was allowed to clot. The clot was carefully removed and centrifuged, after which the supernatant was separated. The separated serum was frozen till used for analysis.

4. Collection of urine:

Morning urine samples were collected from both patients and normals in bottles containing small amount of xylene as preservative.

5. Analysis of skin:

a. Determination of tyrosinase activity in skin:

1. Preparation of the homogenate (Hsia, 1971):

To obtain cell free preparation for enzymic studies, homogenization of the skin has been a challenging problem.

Human skin was first cut into pieces with scissors in salt solution (0.9% w/v) and then subjected to homogenisation in Teflon Homogeniser. After homogenisation, the tissue debris was removed and the homogenate was then subjected to centrifugation and the supernatant was taken for the experiment.

2. Assay of Tyrosinase activity (Horowitz et al., 1970)

Principle:

The conversion of dopa to the red coloured oxidation product dopachrome, is followed photometrically. The initial rate of the reaction is proportional to the enzyme concentration.

Tyrosine $\xrightarrow{1/2\ O_2}$ dopa

2 Dopa $\xrightarrow{O_2}$ 2 dopaquinone + $2H_2O$

Dopaquinone $\longrightarrow$ leukodopachrome

Leukodopachrome + dopaquinone $\longrightarrow$ dopachrome + dopa

Tyrosine + 3/2 O_2 $\longrightarrow$ dopachrome + $2H_2O$

Reagents:

Sodium phosphate buffer, 0.1 M, PH 6.0

DL - Dopa 4 mg/ml in buffer.

Procedure:

1.0 ml of the supernatant was diluted to 4.0ml with buffer and equilibrated for 4 5 minutes in a water bath at 30°C. At time zero, 1.0 ml of the dopa solution was added rapidly with mixing and the absorbancy was read at 420 mu in a Klett-Summerson photo electric colorimeter. After incubation for 5 minutes at 30°C, the mixture was shaken again and a second reading was taken. The change in absorbancy is proportional to the enzyme concentration.

Similarly for standard 1 micromole of Dopa was added to the assay mixture and the change in absorbancy was measured.

Definition of unit:

The unit in the amount of tyrosinase, which catalyses the transformtion of 1.0 micromole of substrate per minute under the above conditions.

Calculation:

Initial reading of the standard	=	A
Final reading of the standard	=	B
Change in absorbancy of the standard	=	(B - A)
Initial reading of the sample	=	X

Final reading of the sample = Y

Change in absorbancy of the Sample = (Y - X)

Change in absorbancy of the standard i.e (B - A) represents 1 unit of the enzyme.

∴ Change in absorbancy of the sample i.e (Y - X) corresponds to $\frac{Y - X}{B - A}$ units.

2. Estimation of skin copper, zinc and iron:

Among the trace elements in skin copper, zinc and iron were analysed. For the estimation of the above trace elements Piper's (1966) method was followed.

Principle:

Skin on digesting with triple acid (Nitric acid, sulphuric acid and perchloric acid in the ratio of 9:2:1) liberates into solution the trace elements.

Procedure:

10.0 mg of the skin biopsy sample was taken in a microkjidhal flask which was previously washed with glass distilled water and dried and to this was added 2.0ml of the triple acid. The mixture was shaken well and

digested on a sand bath with occasional shaking. The digestion was continued till no more brown fumes evolved and the solution of the flask became colourless. The digested mixture was transferred to a graduated tube, the washings being done with double distilled water, and the solution was made upto the mark 5.0ml with double distilled water. This solution was used for analysing the trace elements, using the Atomic Absorption Spectrophotometer (AA 120 model) available in the Soil Science Department of Tamil Nadu Agricultural University.

6. Analysis of blood and serum:

a. Ascrobic acid in blood:

The method of (2, 4) Dinitrophenyl hydzazine was followed for the estimation of Ascorbic acid (Varley et al., 1980). The details of the method are given in Appendix I.

b. Estimation of serum copper, zinc and iron:

Among the trace elements in serum copper, zinc and iron were analysed. For the estimation of above elements Piper's method (1966) was followed.

Principle:

Serum on digestion with triple acid (Nitric acid, Sulphuric acid and Perchloric acid in the ratio of

9 : 2 : 1) liberates into solution the trace elements.

Procedure:

1.0ml of the serum sample was taken in a micro-kjeldhal digestion flask which was previously washed with glass distilled water and dried and to this was added 10.0 ml of triple acid. The mixture was shaken and digested on a sand bath with occasional shaking. The digestion was continued till no more brown fumes evolved and the solution in the flask become colourless. The digested mixture was transferred to a 25.0 ml standard flask, the washings being done with double distilled water. This solution was used for analysing the trace elements, using the Atomic Absorption Spectrophotometer (AA 120 model), available in Soil Science Department of Tamil Nadu Agricultural University.

7. Analysis of Urine:

Qualitative analysis of melanin in urine was done (Varley, 1981) (Vide Appendix II).

RESULTS AND DISCUSSION

IV RESULTS AND DISCUSSION

The results obtained in the present investigation "Biochemical Studies on Vitiligo" are discussed under the following headings:

1. Analysis of skin of vitiligo patients
   a. Tyrosinase activity
   b. Copper content
   c. Zinc content
   d. Iron content
2. Analysis of serum of vitiligo patients
   a. Ascorbic acid level
   b. Copper level
   c. Zinc level
   d. Iron level
3. Analysis of urine for in melanin content in vitiligo patients and normals.

1. Analysis of skin of vitiligo patients:

a. Tyrosinase activity:

The enzyme responsible melanin formation is tyrosinase. The tyrosinase activity in vitiligenous skin was compared with that of the controls. The comparison is presented in Table I. Figure 2 indicates diagrammatically the same.

The tyrosinase activity in vitiligenous skin was found to be decreased when compared to that of the controls. The decrease was significant at 1 per cent level.

b. Copper Content:

The amount of copper present in the skin of both patients and normals are shown in Table I. It is seen from the Table I that there was no significant difference in the skin copper values of vitiligo patients and the controls.

c. Zinc Content:

The levels of zinc in both patients and normals are presented in Table I.

It is seen from the Table I that there was no significant difference in the skin zinc values of the vitiligo patients and normals.

d. Iron Content:

The iron content of skin of both vitiligo patients and the normals are given in Table I.

It is clear from the values that there was no significant difference between the skin iron of the vitiligo patients and normals.

TABLE I

COMPARISON OF TYROSINASE ACTIVITY AND LEVELS OF TRACE ELEMENTS (COPPER, ZINC AND IRON) OF SKIN OF VITILIGO PATIENTS AND NORMALS

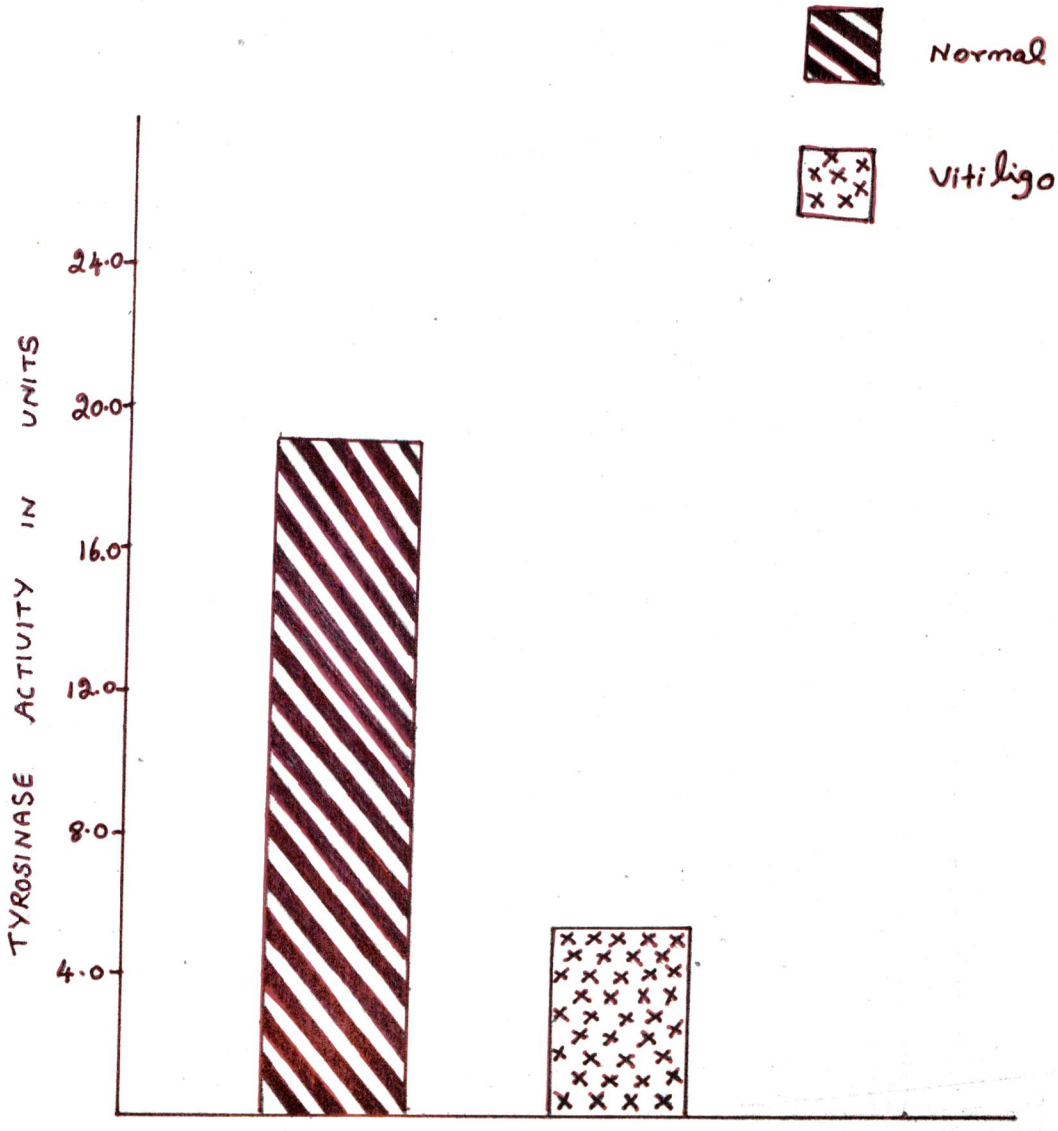
	Vitiligo Mean ± S.D.	Normal Mean ± S.D.	Groups compared	't' value
Tyrosinase activity in units	5.7 ± 1.320 (A)	19.0 ± 2.250 (a)	A Vs a	11.483**
Copper in mg/g	0.131 ± 0.022 (B)	0.157 ± 0.0147 (b)	B Vs b	1.238 NS
Zinc in mg/g	0.588 ± 0.035 (C)	0.937 ± 0.274 (c)	C Vs c	2.5289 NS
Iron in mg/g	0.89 ± 0.363 (D)	0.755 ± 0.2113 (d)	D Vs d	0.666 NS

** - Significant at 1 per cent level

N.S. - Not significant

FIG-2.

COMPARISON OF TYROSINASE ACTIVITY IN THE SKIN OF VITILIGO PATIENTS AND NORMALS



2. Analysis of Serum of Vitiligo Patients:

a. Ascorbic acid level:

The mean serum ascorbic acid levels of the vitiligo patients and the controls are presented in Table II. The same is diagrammatically indicated in figure 3.

There was no statistically significant differences in the serum ascorbic acid levels between different age groups among both the controls and vitiligo patients. So age is not considered for comparing the serum ascorbic acid levels.

From Table II it is evident that there was a marked increase in serum ascrobic acid in the vitiligo patients compared to normals. This increase is significant at 1 per cent level.

This is in agreement with the values reported by Giri, et al., (1979).

b. Copper level:

Copper is an important constituent of plasma and is present in two different fractions. The direct reacting copper fraction which reacts with sodium diethyldithio carbonate and ceruloplasmin which is the copper bound protein.

Serum copper levels estimated both in the patients and the controls are presented in Table II. Figure 4 diagrammatically represents the same.

Here the comparison was done irrespective of age. The mean serum copper level was found to be lowered, when compared to the control group. The difference between the two groups was found to be significant at 1 per cent level.

Giri et al., (1980) and Lal et al., (1970) also reported that there was decreased serum copper levels in the case of vitiligo patients, compared to the normals.

C. Zinc Levels:

There was no statistically significant difference in the serum zinc levels between different age groups among both the controls and vitiligo patients. So age is not considered for comparing the serum zinc level. The zinc levels of vitiligo patients and controls were estimated and values are presented in Table II. The same is indicated in Figure 4.

Table II shows that serum zinc levels of vitiligo patients were decreased compared to the normals. This decrease was significant at 1% level. This agrees with the earlier report of Giri et al., (1982).

d. Iron level:

The levels of serum iron of vitiligo patients and controls are shown in the Table II. The same is indicated digrammatically in Figure 4.

The comparison was done irrespective of the age group. It is evident from Table II that there was an increase in the serum iron level in the case of vitiligo patients compared to the normals and the increase was significant at one per cent level.

TABLE II

COMPARISON OF LEVELS OF ASCORBIC ACID AND TRACE ELEMENTS (COPPER, ZINC, IRON) IN THE SERUM OF VITILIGO PATIENTS AND NORMALS

	Vitiligo Mean ± S.D.	Normal Mean ± S.D.	Groups Compared	't' Test
Ascorbic Acid in g/100ml	0.8951 ± 0.302 A	0.6453 ± 0.200 A_1	A Vs A_1	3.768**
Copper in g/100ml	75.546 ± 6.986 C	92.136 ± 10.910 C_1	C Vs C_1	7.136**
Zinc in g/100ml	105.55 ± 15.785 B	159.67 ± 13.517 B_1	B Vs B_1	14.26**
Iron in g/100ml	85.283 ± 8.923 D	74.433 ± 4.793 D_1	D Vs D_1	5.867**

** - Significant at one per cent level.

FIG:3

COMPARISON OF SERUM ASCORBIC ACID LEVELS IN VITILIGO PATIENTS AND NORMALS

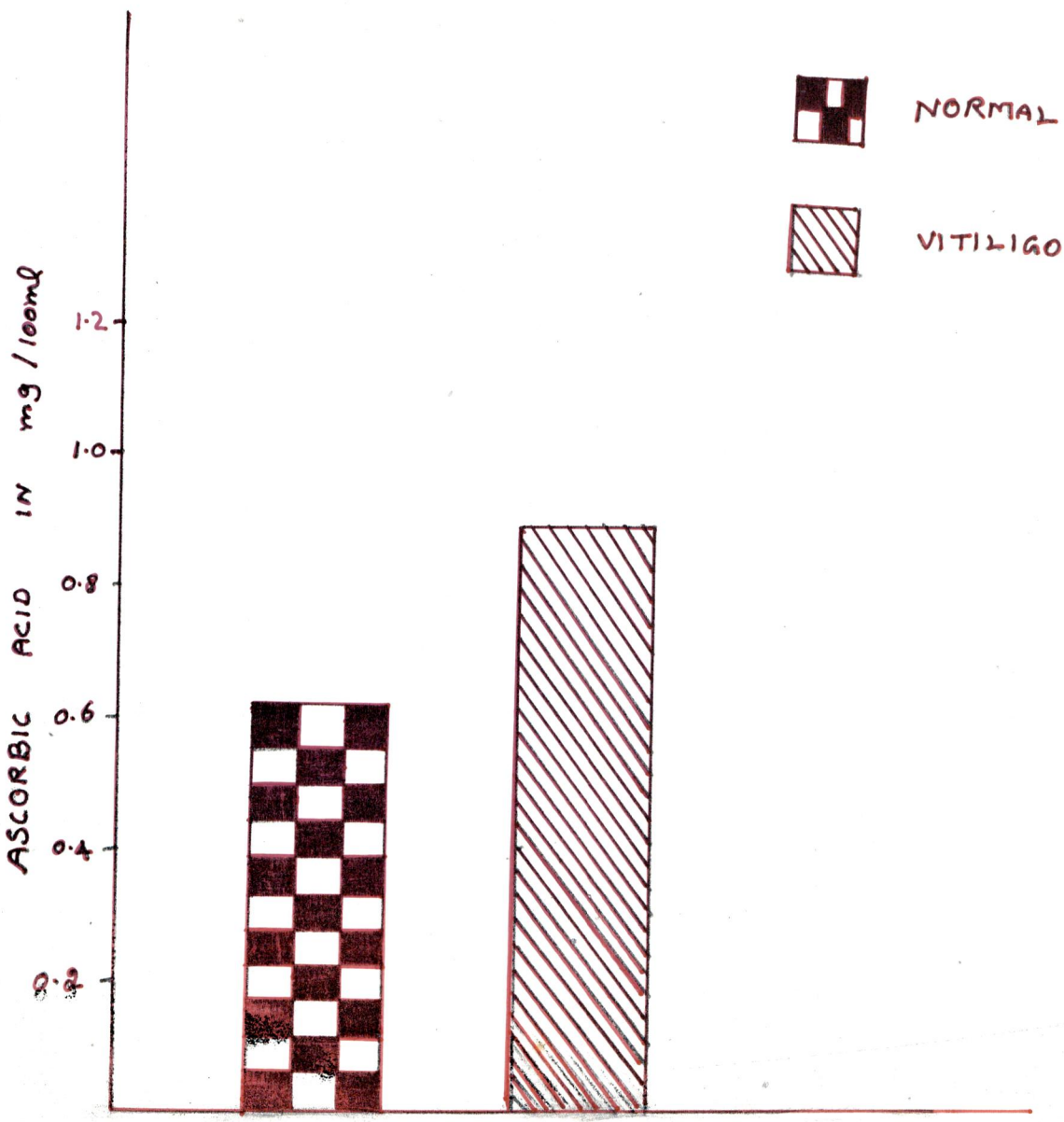
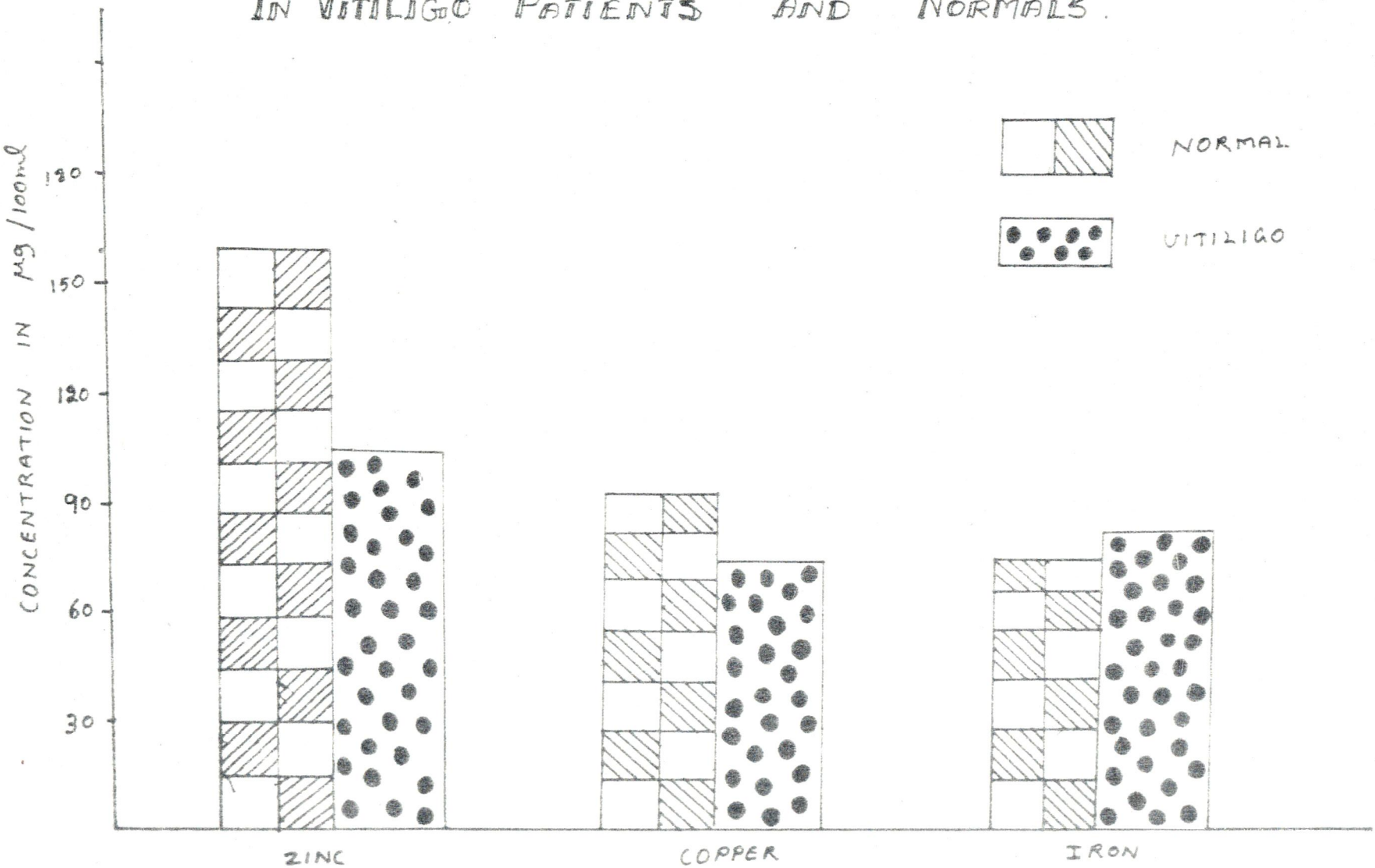


FIG: 4

COMPARISON OF SERUM ZINC COPPER AND IRON LEVELS IN VITILIGO PATIENTS AND NORMALS.



3. Analysis of urine for melanin content in vitiligo patients and normals:

Qualitative analysis of urine for melanin content was done both in normals and vitiligo patients. The tests showed negative results to both vitiligo patients and normals.

SUMMARY AND CONCLUSION

V. SUMMARY AND CONCLUSION

In the present investigation the biochemical changes occuring in skin, serum and urine of vitiligo patients were studied.

From five vitiligo patients among those attending the Government General Hospital, Coimbatore, five skin biopsy samples were taken to study the tyrosinase activity and the levels of zinc copper and iron. For comparison five skin biopsy samples were taken from normal healthy individuals.

Thirty vitiligo patients attending the Government General Hospital, Coimbatore were selected for collection of blood. Equal number of normal healthy people were also selected for comparison. With the blood samples collected from the patients and normals, levels of ascorbic acid, zinc, copper and iron were estimated. Patients selected for this study had moderate affliction of vitiligo.

Urine samples were collected to analyse whether melanin was excreted in the urine of the vitiligo patients. The urine samples from normal individuals were also subjected to similar tests.

Compared to the normals the mean tyrosinase activity of vitiligo patients was found to be lower. The difference

was significant at 1 per cent level. The tyrosinase activity of the vitiligo patients ranged from 4.38 to 7.02 units with a mean of 5.7 units. In normals, the value ranged from 16.75 to 21.25 units with a mean of 19.0 units.

A comparison of the levels of copper in the skin of normals and the patients suffering from vitiligo showed that there was no statistically significant difference between the two groups. The values of the normals varied from 0.1433 mg/g to 0.1717 mg/g with a mean of 0.157 mg/g. In vitiligo the value ranged from 0.153 mg/g to 0.109 mg/g with a mean value of 0.131 mg/g.

There was no significant difference between the vitiligo patients and the normals, when the skin zinc content of the vitiligo patients was compared with that of the normals. In normals the skin zinc content ranged from 0.663 mg/g to 1.211 mg/g with a mean value of 0.937 mg/g. In the case of vitiligo the value ranged from 0.553 mg/g to 0.663 mg/g with mean value of 0.588mg/g.

There was no statistically significant changes in the mean skin iron content ofvitiligo patients compared with normals. The value of the iron content of the normals ranged from 0.543 mg/g to 0.966 mg/g. with a mean value

of 0.755 mg/g. The iron content of the vitiligenous skin ranged from 0.527 mg/g to 1.253 mg/g with a mean value of 0.89 mg/g.

A comparison of the levels of serum ascorbic acid in controls and vitiligo patients indicated a higher serum ascorbic acid levels in vitiligo patients. This difference was statistically significant at 1 per cent level. The value of the serum ascorbic acid of normals ranged from 0.445 mg/100ml to 0.845 mg/100ml with a mean value of 0.645 mg per cent. The value of the serum ascorbic acid of vitiligo patients ranged from 0.572 mg/100ml to 1.1977 mg/100ml with a mean value of 0.895 mg/100ml.

The mean serum copper levels of the vitiligo patients was compared with that of the normals of corresponding age group and this showed a decrease in the level of the serum copper in vitiligo patients. The difference was significant at 1 per cent level. The values of the normals ranged from 81.226 µg/100ml. to 103.046 µg/100ml with a mean of 92.136 µg/100ml. But in the case of vitiligo patients the value ranged from 68.56 µg/100ml to 82.532 µg/100ml with a mean value of 75.546 µg/100ml.

There was a decrease in serum zinc levels of the vitiligo patients, when compared to the normals of the corresponding age groups. The decrease was significant at one per cent level. The serum zinc level of normals ranged from 146.153 µg/100ml to 176.187 µg/100ml with a mean value of 75.546 µg/100ml. In the case of vitiligo patients the value ranged from 68.532 µg/100ml to 82.532 µg/100ml with a mean value of 75.546 µg/100ml.

The mean serum iron value increased significantly at 1 per cent level in vitiligo patients when compared to the normals of the corresponding age group. There was an increase in the serum iron in the case of vitiligo patients. The normal values ranged from 69.64 µg/100ml to 79.226 µg/100ml with a mean value of 74.433 µg/100ml But in the case of vitiligo patients the values ranged from 76.36 µg/100ml to 93.206 µg/100ml with a mean value of 85.288 µg/100ml.

Melanin was found to be absent in the urine of both vitiligo patients and the normals.

Thus the investigation reveals that there was a decreased tyrosinase activity in vitiligenous skin, increased levels of serum ascorbic acid and iron, and lowered

level of serum copper and zinc in vitiligo patients when compared to the normals and the mealnin was absent in the urine of both vitiligo patients and normals.

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APPENDICES

APPENDIX I

DETERMINATION OF BLOOD ASCORBIC ACID BY THE (2, 4) DINITROPHENYL HYDRAZINE METHOD (Varley et al., 1980)

Principle:

The ascorbic acid isconverted into dehydroascorbic acid by shaking with norit and this is then coupled with (2, 4) dinitrophenylhydrazine in presence of thiorea as a mild reducing agent. Sulphuric acid then converts the dinitrophenyl hydrazone into a red compound which is assayed colorimetrically.

Reagents:

1. Trichloroacetic acid solution, 60g/litre.

2. (2, 4) dinitrophenyl hydrazine reagent.

Dissolve 2g of the solid in 100ml sulphuric acid (1 part of concentrated acid added to 3 parts of water). Add 4g of thiourea and shake to dissolve. Filter when necessary and keep in the refrigerator. To check whether enough thiourea is present add the reagent drop by drop to 2 ml nercuric chloride solution containing 10 g/litre. A copious precipitate of mercurous chloride should form after adding 2 to 5 drops.

3. Acid washed Norit. Place 200g Norit in a large flask, add a litre of hydrochloric acid (100ml concentrated

acid plus 900 ml water), heat to boiling and filter with suction. Transfer the cake of Norit to a beaker add a litre of water, stir thoroughly and filter. Repeat until the washings show a negative test for Fe^{+3} ions. Dry overnight at 110 - 120° C (not higher). Some batches of Norit do not need this washing. If a blank using unwashed Norit treated with trichloroacetic acid in the same as with trichloroacetic acid alone, then washing in unnecessary.

4. Sulphuric acid. Add 900 ml concentrated acid to 100ml water.

5. Standard solution of ascorbic acid:

Prepare a stock standard by dissolving 50 mg in 100ml trichloroacetic acid solution (40g/litre), or oxalic acid solution (5g/litre). For the working standard dilute 2ml of this to 100ml with the acid solution used. This solution contains 10mg/litre.

Technique:

To 6ml trichloroacetic acid in a centrifuge tube add 2 ml blood slowly, with constant stirring to produce a fine suspension. Stand 5 min., centrifuge then add 300mg. Norit

to the supernatant fluid, shake vigorously and filter. Place 2ml of the filterate into each of two test tubes. Keep one for the blank and to the other, the test, add 0.5 ml 2, 4 dinitrophenylhydrazine reagent. Stopper and place in a water bath at 37°C for exactly 3h. Remove and place both test and blank in ice cold water and slowly add 2.5 ml sulphuric acid, drop by drop taking about half a minute to do so, so that there is as appreciable rise in temperature. Finally add 0.5ml dinitrophenylhydrazine to the blank. Mix well the contents of both tubes which still in iced water. Remove, and after 30 minutes read at 540 nm, or using a yellow green filter against the blank. As standard treat 2ml of the working standard in the same way as the test.

Calculation:

Blood ascorbic acid (mg/100ml) =

$$\frac{\text{Reading of the unknown}}{\text{Reading of the standard}} \times 1.0$$

A Standard Curve can be Prepared as follows:

Blood ascorbic acid (mg/l)	0	2.5	5.0	7.5	10.0	12.5	15.0	20.0
Standard solution, 10mg/l/,1	0	0.5	1.0	1.5	2.0	2.5	3.0	4.0
Trichloroacetic acid, 40g/1(ml)	8.0	7.5	7.0	6.5	6.0	5.5	5.0	4.0

Add Norit, shake, stand, filter, take 2ml filterate and proceed as described for the test.

APPENDIX II

Qualitative Analysis of Melanin in Urine (Varley, 1980)

1. Ferric Chloride Test:

Add a few drops of ferric chloride solution to 10ml of urine in a test tube and note the formation of a grey colour upon further addition of the chloride, a dark precipitate forms consisting of phosphates and adhering melanin. An excess of ferric chloride causes the precipitation to dissolve. This is the most satisfactory method for the identification of the melanin in urine.

2. Nitroprusside Test of Thormahlen:

Reagents:

1. Sodium nitroprusside solution:

Prepare freshly before use by dissolving a few crystals in a few ml of water.

2. Thirty three per cent (33%) acetic acid.

3. Forty per cent (40%) sodium hydroxide.

Technique:

Add 3 or 4 drops of the nitroprusside solution to about 5ml of urine and make strongly alkaline with about 0.5 ml of the sodium hydroxide shake well to mix and make acid by adding a few ml of acetic acid. The presence of melanojen is shown by the development of a blue colour to blue-black colour. The actual colour seen depends on the colour of the original urine. If this was deep yellow the colour of the urine seen will be dark green. The less pigmented the urine, the bluer the colour produced.

APPENDIX III

RESULTS OF THE BIOCHEMICAL ANALYSIS OF SKIN OF VITILIGO PATIENTS

Patient No.	Tyrasinase activity in units	Copper (mg/g+)	Zinc (mg/g)	Iron (mg/g)
1	5.0	0.250	0.6	0.425
2	5.5	0.125	0.51	0.7
3	8.0	0.125	0.615	1.525
4	4.0	0.135	0.615	0.875
5	6.0	0.150	0.6	0.95

APPENDIX IV

RESULTS OF THE BIOCHEMICAL ANALYSIS OF SERUM OF VITILIGO PATIENTS

Patient No.	Ascorbic acid in mg/100ml	Copper (µg/100ml)	Zinc (µg/100ml)	Iron (µg/100ml)
1	1.0	78.5	78.0	87.5
2	1.0	65.0	85.0	80.0
3	0.33	75.0	120.0	84.5
4	0.59	65.0	118.75	101.0
5	1.50	80.0	92.0	72.0
6	1.0	75.0	98.0	92.0
7	1.5	67.6	112.5	84.0
8	0.9	65.0	112.5	77.5
9	0.75	83.5	102.0	105.0
10	1.0	73.0	117.5	85.0
11	1.75	70.0	110.0	85.0
12	0.85	80.0	90.0	100.0
13	0.90	65.0	68.0	70.0
14	0.75	70.0	100.0	75.5
15	0.90	75.0	80.0	85.0
16	0.7	80.0	125.0	85.0
17	0.75	76.0	115.0	85.0
18	1.5	79.5	135.0	84.0
19	0.85	85.0	104.5	90.0
20	0.70	88.5	115.0	88.0

Patient No.	Ascorbic Acid in mg/100ml	Copper (µg/100ml)	Zinc (µg/100ml)	Iron (µg/100ml
21	0.80	89.0	112.5	76.0
22	1.0	78.0	90.0	70.0
23	0.625	84.8	105.6	83.0
24	0.75	80.0	120.8	80.0
25	0.75	79.0	124.5	73.0
26	0.69	80.0	125.0	100.0
27	0.85	70.0	102.5	90.0
28	0.80	73.5	95.0	95.0
29	0.60	65.0	90.0	85.0
30	0.80	71.8	110.0	90.0