WATER SOLUBLE VITAMINS IN NORMAL HUMAN SKIN*

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Extensive vitamin studies in animal and plant tissues have been carried out by numerous workers. However, the information concerning vitamins in human skin is scant and incomplete (1). It is recognized that among the water soluble vitamins are integral parts of the enzyme systems essential to all forms of life, and ascorbic acid is of functional importance to higher animals and plants. Information regarding the content and distribution of vitamins in human skin is indispensable for studying normal as well as abnormal metabolic processes in skin.

Following are the results of a preliminary study on the concentration of water soluble vitamins in normal human skin. Further studies on the concentration of these vitamins in the epidermis and the dermis will be reported later.

EXPERIMENTAL

For assay of each of the water soluble vitamins, biological, microbiological, and chemical methods are well established. In the case of skin, the problem was to find a method of preparing an extract suitable for assay purposes without appreciable destruction of vitamins.

Various methods have been tried for this purpose. Autoclaving in dilute acid for extraction of the vitamins' folic acid, B_{12}, riboflavin, niacin, and biotin, autoclaving in dilute acid followed by enzymatic digestion at the proper pH for pantothenic acid and thiamine, and grinding in dilute acid with addition of an abrasive for ascorbic acid have been found satisfactory.

Fifteen normal skin samples obtained from patients undergoing surgical amputations were frozen and stored in a deep freeze (−20°C). The frozen skin was ground and lyophilized into dry powder according to a procedure previously described (2).

I. *Riboflavin, Niacin, Biotin and Pantothenic Acid*

Extraction procedure

Riboflavin and niacin: Twenty ml. of 1 N H_2SO_4 was added to 0.1 g. skin powder in a 50 ml. erlenmeyer flask. The flask was mechanically shaken for 30 min. and autoclaved 30 min. at a steam pressure of 15 lbs. per sq. in. After cooling to room temperature, the pH was adjusted to 4.5–5.5 with 1 N NaOH. The mixture was diluted to 50 ml. with water and filtered through No. 2 Whatman filter paper. The filtrate was adjusted pH 6.8 with 20% NaOH and

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stored under toluene in the refrigerator. For riboflavin the whole extraction process must be performed under dim light and the extract stored in brown bottles.

Biotin: Twenty ml. 2 N H_2SO_4 was added to 0.2 g. skin powder in a 50 ml. erlenmeyer flask. The flask was mechanically shaken for 30 min. and autoclaved 1–2 hours at a steam pressure of 15 lbs. per sq. in. The pH was adjusted to 3.0 with 1 N NaOH. The mixture was extracted once with an equal amount of petroleum ether. The ether layer was washed once with a small amount of water to insure full recovery of the sample. The aqueous layers were combined, adjusted to pH 6.8 with 1 N NaOH, diluted to 100 ml., and filtered. The extract was stored under toluene in the refrigerator.

Pantothenic acid: Four-tenths g. Mylase P (Wallerstein Laboratory), 10 ml. water, 0.2 ml. glacial acetic acid, and 1 ml. 1 N NaOH were added to 0.4 g. skin powder. The final pH of the mixture was about 4.2–4.5. The flask was mechanically shaken for 30 min. and autoclaved 30 min. at a steam pressure of 15 lbs. per sq. in. After cooling to room temperature, it was incubated at 37°C overnight. The mixture was adjusted to pH 6.8, diluted to 250 ml., and filtered through No. 2 Whatman filter paper. The extract was stored under toluene in the refrigerator. An enzyme blank was run simultaneously to correct the final value on the sample.

Assay procedure

For the assay of riboflavin, niacin, biotin, and pantothenic acid the microbiological method, using *Lactobacillus casei* E. (3–6) was used.

The assay was run in 25 x 150 mm. pyrex test tubes. For each sample, 5 tubes containing from 2–10 ml. of skin extract, at 2 ml. increments, were required. Ten ml. of the double concentration basal medium, with the particular vitamin omitted, was run into each tube to which the appropriate amount of skin extract was added. The total volume was made up to 20 ml. with distilled water. The tubes were plugged and autoclaved 7 min. at a steam pressure of 15 lbs. per sq. in. After cooling to room temperature, one drop of inoculum suspension was added aseptically to each tube. It was shaken to insure uniform distribution of cells. The tubes were incubated 15–20 hours at 37°C. The growth response was determined by measuring the optical density of the culture medium with a Coleman Junior Spectrophotometer at a wavelength of 660 mu. Analyses of a series of 6 tubes containing pure vitamin solution of proper concentration per tube were carried out simultaneously for each assay run. The calibration curve was obtained by plotting optical density against the amount of vitamin per tube.

The following concentration ranges in mcg. per tube were used for obtaining calibration curves:

Riboflavin	0–0.10
Calcium pantothenate	0–0.15
Nicotinic acid	0–0.30
Biotin	0–0.001

II. *Thiamine*

Extraction procedure

A considerable portion of thiamine in animal tissues is combined in the form of cocarboxylase, (7, 8) which may be released as free thiamine by various enzymes. The general opinion is that complete extraction of thiamine from tissues offers a rather difficult problem (9). The following procedure has been found fairly satisfactory for skin.

Forty ml. 0.1 N H_2SO_4 was added to 1 g. skin powder in a 125 ml. erlenmeyer flask. The flask was mechanically shaken for 30 min. and autoclaved 30 min. at a steam pressure of 15 lbs. per sq. in. After cooling to room temperature, 0.25 g. pepsin was added. The mixture was incubated 12–15 hours at 42–45°C., then heated on a waterbath 10 min. to inactivate the enzymes. After cooling to room temperature, 15 ml. acetate buffer, pH 4.5

(10), 0.25 g. takadiastase, and a few drops of benzene were added. Benzene was preferred to toluene because it was easier to eliminate on the water bath. The mixture was incubated 12–15 hours at 40–42°C. and heated on the waterbath 10 min. to eliminate benzene and inactivate enzymes. It was filtered through No. 1 Whatman filter paper. The filtrate was stored in the refrigerator.

Assay procedure

For the assay of thiamine several chemical methods, besides biological and microbiological methods, have been introduced. Prebluda and McCollum (11–13) proposed a colorimetric method which has not been used extensively because of its poor sensitivity. Jansen suggested a thiochrome reaction which was developed into an assay method by Hennessy (14)

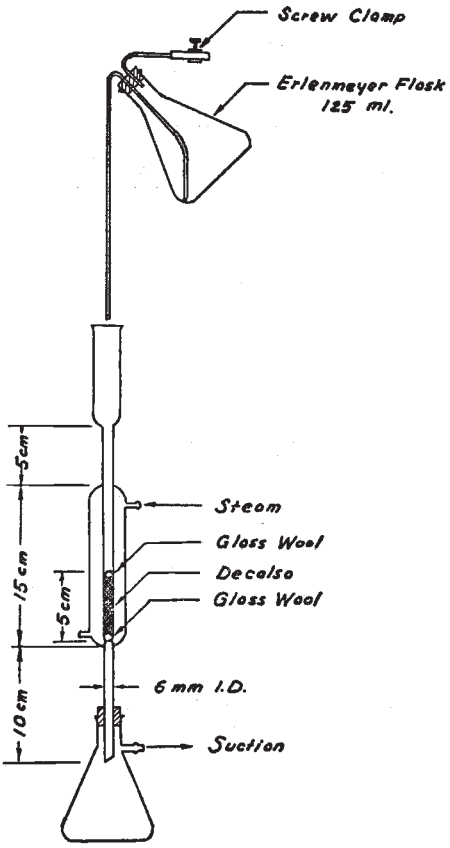

FIG. 1. Apparatus for extraction of thiamine

and Conner (10). Harris and Wang (15, 16) later modified this to simplify the procedure. However, Harris' modification gives inconsistent results in the case of skin. The procedure proposed by Conner and Staub (10) was modified to suit our purpose.

A battery of 8 Decalso adsorption apparatus was set up (Fig. 1). The Decalso column has an I.D. of 6 mm. and a constriction 10 cm. from the bottom end. The steam jacket has an effective length of 15 cm. The column was filled with activated 60–80 mesh Decalso (Permutit Co., New York, N. Y.) to a length of 5 cm. The Decalso was changed for each assay. The Decalso was activated according to the following procedure: 100–200 g. 60–80 mesh Decalso

was placed in a 1000 ml. beaker provided with a mechanical stirrer. Three hundred ml. 3% acetic acid was added. The mixture was boiled 15 min. while stirring. The solution was removed by decantation. The process was repeated with 300 ml. of 25% neutral KCl solution. In total, 4 alternate washings were carried out. The Decalso was then repeatedly washed by boiling 15 min. with 800 ml. distilled water until the washings were free of chloride ion. Usually 6 washings were sufficient. The washed Decalso was dried and stored in a stoppered bottle.

For adsorption of thiamine, the skin extract was placed in the 125 ml. erlenmeyer flask (Fig. 1) and allowed to drip onto the Decalso column by means of the attached siphon system which was started by a rubber bulb. The rate of flow was maintained at 1–2 ml. per min. by adjusting the screw clamp. After the extract had passed through, the column was washed 4 times with 20 ml. boiling distilled water and finally dried by applying suction for 1 min. Steam was then admitted to the steam jacket. The adsorbed thiamine was through the column at nearly boiling temperature. The steam jacket served to heat the KCl for elution. The elute was collected in a 25 ml. volumetric flask and made up to volume with 25% KCl solution.

TABLE I

Thiamine content of Beef and Bread

SAMPLE	VALUE FOUND MCG. PER GM. AS RECEIVED	PUBLISHED VALUE (21, 22)
Enriched white bread,		
No. 1	2.8	2.7
No. 2	2.9	
Beef, chuck roast,		
No. 1	0.5	0.49, 0.63
No. 2	0.7	

The thiamine in the elute was oxidized to thiochrome and the amount of thiochrome formed was determined by measuring fluorescence produced under ultraviolet radiation with a Coleman Electronic Photofluorometer. Filters B-1 and PC-1 were used. Quinine sulfate reference solution (0.3 ml. per 1000 ml. 0.1 N H_2SO_4) was used to set the meter. A complete blank with all the reagents and enzymes should be carried out for each assay run to correct the final result. Since it is believed that a small amount of thiamine may be lost during the extraction process and that Decalso usually gives 80–90% adsorption, an inside standard is used to obtain the calibration curve.

In order to verify the dependability and accuracy of the extraction and assay method used for thiamine, a series of food samples with published thiamine content was assayed. The results tabulated in Table I show that the values obtained agree with those of other investigators.

III. *Folic Acid and Vitamin B_{12}*

Folic acid and B_{12} were determined microbiologically in the aqueous extracts obtained from skin powder after the skin, in acetate buffer at pH 4.5, was autoclaved at 15 lbs/ sq. in. pressure for 15 min. *S. fecalis* was used for the folic acid assay and *Lactobacillus leichmannii* for the vitamin B_{12} assay using the procedure described by Swendseid et al. (17).

IV. *Ascorbic Acid*

Extraction procedure

Because ascorbic acid is very unstable in solution, autoclaving and enzymatic digestion cannot be used for its extraction. Grinding in dilute acid solution with the aid of an abrasive has been studied and found to be satisfactory.

One g. of skin powder was mixed with 1 g. of alundum (Norton Co.) and ground to a fine pulp in 10 ml. 5% trichloroacetic acid with a mortar and pestle. The pulp was transferred to a 50 ml. centrifuge tube with the aid of 15 ml. 5% trichloroacetic acid. The tube was stoppered, mechanically shaken for 10 min., and centrifuged at 2500 r.p.m. for 5 min. The supernatant liquid was collected in a 50 ml. volumetric flask. The residue was resuspended in 5 ml. 5% trichloroacetic acid and ground up again. The same process was repeated except 10 ml. of 5% acid was used for washing. The residue from the second grinding was again suspended in 10 ml. 5% acid and allowed to stand at room temperature 12–15 hours and centrifuged 5 min. The supernatant liquid was combined with previous portions and made up to volume with 5% trichloroacetic acid.

TABLE II

Water soluble vitamin content of normal whole human skin mcg. per gm. dry skin

SKIN SAMPLE	B_{12}	FOLIC ACID	RIBOFLAVIN	NIACIN AS NICOTINIC ACID	PANTOTHENIC ACID AS CALCIUM SALT	BIOTIN	THIAMINE AS HYDROCHLORIDE	ASCORBIC ACID
1	0.016	0.15	3.0	19	6.5	0.050	0.3	73
3	0.018	0.095	1.2	13	7.1	0.047		
4	0.022	0.11	1.8	12	3.2	0.044		42
5	0.024	0.10	1.8	13	2.5	0.036		25
6	0.020	0.13	1.2	9.6	1.3	0.055		39
7	0.026	0.12	2.5	19	4.6	0.044	0.3	64
8	0.016	0.09		19	2.2	0.039		
9	0.024	0.095		14	2.1	0.050	0.3	40
10							0.3	19
11							0.4	28
12							0.3	
13							0.2	
14							0.2	
15							0.2	
Average....	0.021	0.11	1.9	15	3.7	0.046	0.3	41

Assay procedure

Lowry (18) and Mills (19, 20) proposed an assay procedure which depends upon the oxidation of ascorbic acid to diketo-*l*-gulonic acid by a very mild oxidant. The diketo-*l*-gulonic acid is coupled to 2,4-dinitrophenylhydrazine to form a red dye which is determined colorimetrically. This procedure gives the total content of ascorbic acid. For the assay of ascorbic acid in skin extracts, the procedure was followed except that 20 ml. of skin extract and 0.5 g. of acid washed Norit were used. The resultant red dye was measured with a Coleman Junior Spectrophotometer, using 19 x 105 mm. round Coleman cuvettes. The meter was set at a wavelength of 520 mu. A calibration curve was obtained by running a series of standard samples containing 0, 0.4, 0.8, 1.2, and 2.0 mcg. crystalline ascorbic acid per ml., following the same procedure.

RESULTS

The concentration of vitamin B_{12}, folic acid, riboflavin, niacin, pantothenic acid, biotin, thiamine, and ascorbic acid, derived from the dermis and epidermis of fifteen normal human beings, is tabulated in Table II. Each skin sample reported was assayed in duplicates agreeing to within 10 per cent. The accuracy of

the assay methods was checked by recovery tests which ranged from 90 to 110 per cent.

The published values of the concentration in human tissues of folic acid, riboflavin, niacin, pantothenic acid, biotin, thiamine, pyridoxine, and inositol are tabulated in Table III.

No attempt has been made to explain the disagreement on vitamin contents of folic acid and thiamine in skin. It is possible that the condition of the individuals from whom the samples were obtained and the methods of assay used were the main factors responsible for the different values.

TABLE III

*Published Values on Water Soluble Vitamin Contents of Normal Human Tissues (γ/gm. dry tissue)**

TISSUE	FOLIC ACID	RIBOFLAVIN	NICOTINIC ACID	PANTOTHENIC ACID	BIOTIN	THIAMINE	PYRIDOXINE	INOSITOL
Liver	27–85	48–64	197–206	113–153	2.3–2.6	7.3–12.8	6.9–12.8	2060–3030
Brain-Cerebrum	3.1–7.4	8.8–14	84–94	55–79	.13–.42	5.9–8.9	2.5–3.4	6720–7030
Lung	6.1–12	7.5–11	84–123	16–40	.07–.26	3.5–7.7	0.4–2.0	1740–3180
Kidney-Renal cortex	8.9–12	61–123	163–172	79–116	2.9–4.2	12–18	3.6–7.9	4220–6410
Spleen	11–17	13–32	98–132	21–31	.18–.31	4.8–5.8	.27–1.7	1540–5310
Muscle	2.9–4.4	7.7–12	122–193	28–69	.08–.26	3.2–6.1	2.4–4.2	810–2650
Stomach-Cardiac mucosa	4.8–7.2	24–25	91–143	27–33	.51–1.2	2.0–4.8	.87–2.0	2230–5780
Ileum	5.8–6.9	16–22	73–164	24–30	.28–.49	1.9–6.2	.74–1.9	2300–3920
Colon Mucosa	6.1–16	7.3–12	40–137	12–63	.28–.75	4.0–5.7	.64–2.1	2380–6860
Mammary gland	0.53–	2.9	12	4.7	0.052	0.52	0.52	330
Ovary	5.1	20	84	18	0.11	2.8	0.70	2700
Testes	5.4–14	11–14	53–156	19–44	.29–.81	3.4–6.9	0.6–2.3	4420–15,630
Seminal ducts	4.4	5.1	46	10	0.76	3.5	0.19	500
Skin	2.3–2.7	2.1–3.4	18–24	6.1–9.3	.023–.085	.95–1.7	.18–.66	230–790

* Converted from γ/gm wet tissue to γ/gm dry tissue.

DISCUSSION

It has been postulated that skin, like muscle, derives its energy mainly from carbohydrate metabolism. The latter is carried out by glycolysis of carbohydrate and subsequent oxidation of pyruvic acid and acetic acid, which are also reaction products of fat and protein metabolism, to the final end-products, carbon dioxide and water, via Kreb's citric acid cycle. Decarboxylation of pyruvic acid to acetic acid requires a co-enzyme which is made up partly of thiamine. Furthermore, nicotinic acid is present in enzymes which catalyze several reactions in carbohydrate metabolism. Riboflavin, pantothenic acid, and biotin also play important roles in the metabolism of either carbohydrate or of protein and fat. The presence of these as well as other vitamins in considerable concentration in human skin is in support of this postulation.

It is interesting to note that in whole skin the content of water soluble vitamins is less than in all other tissues. These results may be explained by the relatively large amount of inert dermis in skin. Giroud (23) pointed out that ascorbic acid content of horse skin was concentrated in the prickle cell layer of epidermis. The concentration of other vitamins in the skin may be greater in the metabolically active epidermis than in the inert dermis.

SUMMARY

The concentration of vitamin B_{12}, folic acid, riboflavin, niacin, pantothenic acid, biotin, thiamine, and ascorbic acid in the skin of fifteen normal human beings, and the methods of extracting these vitamins from skin have been reported.

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