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Identification of a Natural Moisturizing Agent in Skin

KARL LADEN, Ph.D.,* and ROBERT SPITZER, Ph.D.†

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Synopsis—Sodium-2-pyrrolidone-5-carboxylate has been identified as a naturally occurring humectant in skin. A significant relationship was established between the moisture-binding ability and the PCA content of samples of stratum corneum.

Introduction

In 1952 Blank (1, 2) demonstrated that the lowered moisture content of the skin is probably the prime factor in causing the condition commonly called "dry skin." Contrary to older beliefs, the amount of oil in the stratum corneum is not the essential factor in determining the softness and flexibility of the skin. Thus, pieces of hardened stratum corneum immersed in various oils do not regain their flexibility, whereas immersion in water increases their flexibility.

The factors which influence the state of hydration of the stratum corneum may be classified into three general categories:

1. The rate at which water reaches the stratum corneum from layers beneath it.
2. The rate at which water leaves the skin surface by evaporation.
3. The ability of the stratum corneum to hold moisture.

The rate at which water reaches the skin surface is governed by the supply of water from the eccrine glands and moisture obtained by trans-

* Gillette Medical Research Institute, 6220 Kansas Ave., N. E., Washington D.C. 20011
† University of Illinois Medical School, Chicago, Ill.

epidermal transport. The latter source appears to be limited by the low water vapor permeability of the stratum corneum itself (3).

The removal of water from the skin by evaporation is governed by climatic factors such as temperature, humidity, and wind velocity and perhaps by the presence of sebum.

The factors affecting the ability of skin to hold moisture have been the object of many investigations (1, 2, 4–14). There is much evidence that the skin contains hydrophilic nitrogenous substances as well as other hydrophilic substances which enhance the ability of the skin to hold water. When these substances are extracted from the skin, its ability to hold moisture is greatly diminished. In addition, in various skin conditions associated with scaling the scales appear to have lesser amounts of these substances as well as a low capacity to bind moisture (15–19).

It was the purpose of this study to investigate the chemical nature of these natural moisturing agents present in skin. A second study to be reported at a future date will establish on a more quantitative basis the role played by water and these moisturizing agents on the physical properties of skin.

Analysis of the Water Soluble Components of skin

Whereas many differences are known to exist between stratum corneum and callus, both of these forms of keratin show the presence of natural moisturizing agents, and both show the same general physical properties with regard to softening with water. In these studies therefore, both callus and stratum corneum have been used as substrates for investigation. In the initial studies, the effect of removing the water-soluble components from callus on its ability to bind moisture was evaluated.

Sixty grams of callus was extracted with 750 ml of ether in a soxhlet extractor for seven hours. The ether-extracted callus was air dried and then placed in an Erlenmeyer flask with 500 ml of water and shaken for three and one-half hours. The mixture was suction filtered, an additional 300 ml of water was added to the callus, and the mixture shaken overnight. The latter process was repeated one more time. The filtrates were combined and freeze-dried to yield 9 g of water-extractable material. The water-extracted callus was air-dried.

The humectancy of samples was determined by drying the samples to constant weight in a vacuum desiccator and then equilibrating the

samples in small desiccator chambers, each containing an appropriate solution to maintain the desired relative humidity.

Data obtained in these experiments (Table I) closely paralleled results obtained by other investigators (2, 20). Thus, removing the water solubles from callus greatly diminishes the ability of the callus to hold moisture, and the water extractables can be shown to be hygroscopic.

In order to study further the nature of the water-soluble materials responsible for this humectancy, the water solubles from callus were fractionated on ion exchange resin columns, and the fractions obtained were evaluated for humectancy.

Columns containing C-G 400 Type I Amberlite resin were converted to the acetate form. Next, the water-soluble extract, dissolved in a small volume of water, was placed on the column. After washing the column thoroughly with water, the column was eluted with 11% formic acid, and fractions of the acidic eluant were taken to dryness and evaluated for humectancy.

The humectancy of these fractions was far below that expected based on the humectancy of the total extract. One possible reason was that the fractionation procedure had converted one or more of the moisturizing agents to a nonhygroscopic form.

These fractions were then subjected to paper chromatography.

From 20λ to 50λ of sample were spotted on No. 1 Whatman chromatographic grade filter paper, and one dimensional descending chromatography was run using a butanol-acetic acid-water mixture (12:3:5). After drying in a vacuum oven for several hours, the chromatograms were developed by spraying with a solution of 0.2% ninhydrin in bu-

Table I
Moisture-Binding Ability of Callus

Material	Per Cent Moisture Uptake = Wt. of Water × 100 / Wt. of Dry Sample	
	37% R.H.	70% R.H.
Unextracted callus	9.2	16.8
Extracted callus	5.8	12.3
Callus extractables	...	45.0

tanol to detect amino acids or by dipping into a solution of potassium iodide, potassium iodate, and starch. (A solution of 2% potassium iodide and 4% potassium iodate is mixed with an equal volume of 2% starch. A trace of NH_4OH is added to reduce background color.) Intense blue colors appear using this test where relatively large amounts of acidic material are present on the chromatogram.

The chromatograms indicated that, in addition to ninhydrin-positive amino acids, there was also present a ninhydrin-negative, potassium iodide-potassium iodate-starch positive acid. Since Pascher (9) had reported the existence of an acid, 2-pyrrolidone-5-carboxylic acid, in extracts of skin, a known sample of this material was chromatogrammed along with the unknown acidic material. Upon developing the chromatograms, both the known sample of 2-pyrrolidone-5-carboxylic acid (PCA) and the unknown sample had the same Rf value. To confirm that the unknown acid material was PCA, a known sample and the unknown acid were hydrolyzed with HCl. The hydrolyzed samples were rechromatogrammed, and upon developing it was found that in both instances the PCA spot had disappeared and a new ninhydrin-positive spot which corresponded in Rf value to glutamic acid had appeared. Since PCA on acid hydrolysis is converted to glutamic acid, it was concluded that the unknown acid in the water-soluble fraction was PCA.

Initial studies on the humectant properties of PCA indicated that this material was nonhygroscopic. There was a possibility, however, that the fractionating procedure had converted some of the moisturizing agents into a nonhygroscopic form. This possibility was investigated further with respect to PCA. Since PCA can arise as a result of cyclization of glutamic acid, the humectancy of glutamic acid was evaluated but was also found to be very low.

The pH of skin and of water extracts of skin are usually between pH 5–6. At this pH, amino acids are present in their zwitter-ion form. However, PCA, which has no free amino group, is present largely in the ionic form at this pH. Indeed, a titration curve of PCA *vs.* pH indicates that at pH 5.3 99% of the acid is in the sodium salt form. During the fractionation procedure, the amino acids are converted to the formic acid salts, which upon drying are converted back to the free amino acids. Any salt of PCA in the aqueous extract, however, is converted to the free acid during isolation. Thus, the amino acids undergo essentially no change during isolation, while PCA is converted from the salt to the acid form.

The sodium salt of PCA was, therefore, prepared and its humectancy evaluated. This compound was found to be highly hygroscopic and, at

Table II
Moisture-Binding Ability of 2-Pyrrolidone-5-Carboxylic Acid—
Its Sodium Salt and Glycerol

Compound	Per Cent Moisture Uptake at	
	31% R.H.	58% R.H.
Pyrrolidone carboxylic acid	<1	<1
Sodium pyrrolidone carboxylate	20	61
Glycerol	13	35

higher humidities, even dissolved in its own water of hydration. Table II compares the humectancy of PCA with that of its sodium salt and with the commonly used humectant, glycerol. It thus appeared that the ionic form of PCA could certainly partially explain the high humectancy of the callus water extractables. Experiments were, therefore, initiated to explore in a more definite manner the role of PCA as a naturally occurring moisturizing agent in skin.

Analysis of Skin Samples for PCA

Samples of stratum corneum and callus were analyzed for pyrrolidone carboxylic acid using paper chromatographic techniques.

Samples of stratum corneum were obtained by first clipping the hair from the back of the hand using an electric razor. Then, using a Spencer microtome knifeholder containing a razor blade, the skin could be carefully scraped from the backs of the hands, yielding up to 20 mg of stratum corneum.

The skin scrapings were submitted to two one-hour extractions at room temperature with ethyl ether. Five-tenths milliliter of water was then added to the dry scrapings, and an overnight extraction was performed. After centrifugation, 50λ to 200λ of aqueous extract was used for two dimensional descending paper chromatography. The solvent systems used were butanol-acetic acid-water (12:3:5) and phenol-water-ammonia (prepared by adding 1000 g phenol to 250 ml water; 2 ml of concentrated ammonium hydroxide is then added to 200 ml of the aforementioned solution). Generally, the first solvent was employed overnight and the second solvent for seven hours. The amino acids were identified with a 0.5% ninhydrin in butanol spray. In these studies the PCA was identified with a more sensitive and specific test. The chromatogram was placed in a chlorine atmosphere for twenty minutes, then blown free of residual chlorine and sprayed with a starch-iodine reagent (21). PCA appears as a brown spot. For comparison, water extracts of callus were also run.

The paper chromatograms from stratum corneum and callus were quite similar. PCA was found in both chromatograms. Identification of amino acids and PCA were accomplished by establishing reference chromatograms. Thus, in agreement with Pascher (6), PCA was demonstrated to be a normal component of stratum corneum.

While PCA can be shown to be a naturally occurring moisturizing agent in skin, its actual role in maintaining the skin in a hydrated state remained to be determined. In order to establish whether a relationship exists between PCA content of the skin and the skin's moisture binding ability, an analytical method to determine the amount of PCA in skin scrapings was developed.

Aliquots of the water extract of skin scrapings were submitted to ion-exchange chromatography using a 95 cm × 1.5 cm column of Dowex 50—X4, 100–200 mesh resin in an 0.1*N* citrate buffer at pH 3.0, using the general method of Moore and Stein (22). On this basis, the first 125 ml of effluent contained all of the PCA and, possibly, a small amount of aspartic acid. Aliquots of this effluent were analyzed with ninhydrin reagent (23) before and after hydrolysis with HCl. Since PCA produces no color with ninhydrin until after it is hydrolyzed with HCl, the difference between the ninhydrin value before *vs.* that after HCl hydrolysis yields the amount of PCA. Typical recoveries of standard PCA samples were in the range of 93–95%.

A fourteen-member panel was formed, and skin scrapings from the backs of the hands were obtained from each subject. The moisture-binding abilities of the scrapings were determined at 95% relative humidity. Water extracts of the samples were then prepared, and total ninhydrin-positive material was determined on an aliquot. The remainder of the extract was submitted to ion-exchange chromatography and PCA assay. The results are presented in Table III.

Statistical analysis of the data indicated that a reliable linear relationship is exhibited between moisture-binding ability and the PCA content of the skin scrapings. The product moment correlation coefficient, calculated from the data, gives $r = 0.652$. This value differs significantly from zero at the 0.98 level and thus points to the existence of a relationship between the variables studied. Some idea of the predictive ability of this relationship is gained by noting that 42.5% ($= r^2$) of the observed variation in the data is attributable to linear regression (24). The fourteen pairs of values are presented together with the two regressions in Fig. 1.

Table III
Analysis of Skin Scrapings

Subject	Per Cent Moisture Binding at 95% R.H.	PCA-Na (%)	μ-Moles Amino Acid (Mg Keratin)
1	110	3.36	3.00
2	113	2.51	1.90
3	132	7.30	3.71
4	94	2.32	2.07
5	123	5.09	2.24
6	86	2.43	2.01
7	78	1.94	1.29
8	93	4.26	2.11
9	78	2.43	1.77
10	102	3.72	1.96
11	89	4.04	1.76
12	89	5.60	2.38
13	96	3.16	1.40
14	85	1.63	1.65

Discussion and Summary

The earliest humans with whom we are acquainted, the cave men and their apish forebears, possessed hair which covered the entire surface of the body. It protected them from cold, heat, sun, rain and wind and also acted as a protective padding against injury. With the evolution and civilization of man, he shed his natural hairy covering to become increasingly hairless except for the scalp, axilla, and pubis. As a result of this diminished protection, the exposed skin has become more susceptible to the environmental conditions to which it is exposed. Even to the most casual observer, it is apparent that the onset of dry cold weather is rapidly followed by the appearance of dry and chapped skin. Indeed, correlations between environmental conditions and the onset of dermatitis hiemalis have been reported (25). The rapidity with which these changes occur suggests that the physical properties of the stratum corneum are to a large extent controlled by the environment to which it is exposed.

The importance of moisture in controlling the flexibility of stratum corneum is now well accepted. When exposed to conditions of low temperature and humidity, the stratum corneum can rapidly become more brittle because of loss of moisture from the underlying layers. This increase in skin stiffness results in fissures and the general conditions seen in dry skin.

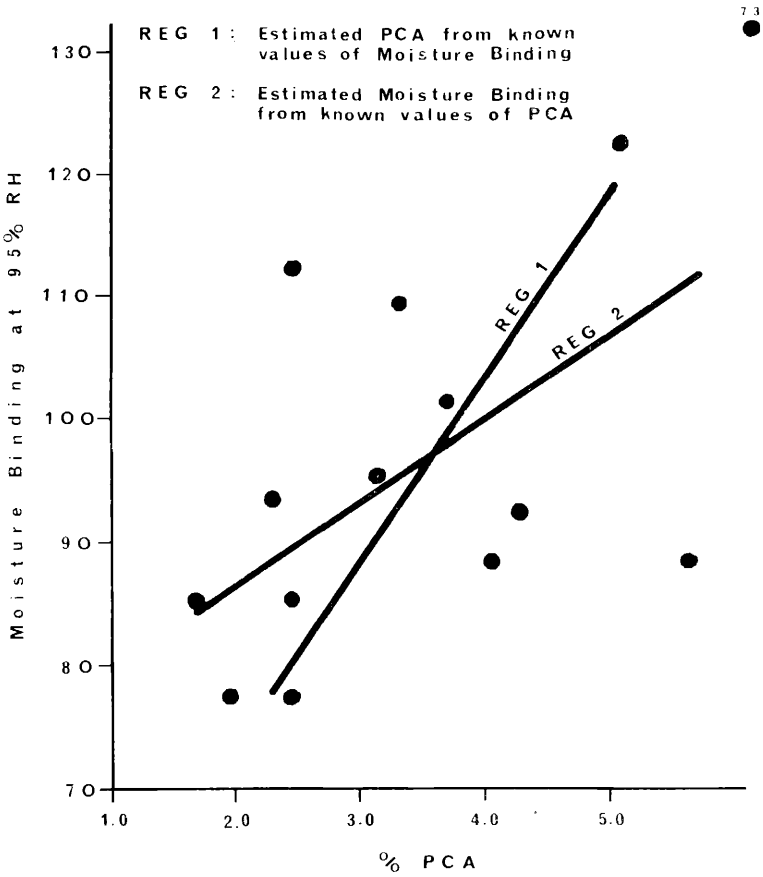

Figure 1. Moisture binding vs PCA content for fourteen member panel

Counteracting this tendency of the skin to dry out is the presence in the skin of naturally occurring humectants. It is the role of these humectants to help preserve the moisture content of the skin and thereby maintain it in a soft and supple condition.

The detailed chemical composition of all of the natural humectants has not been determined. Simple amino acids make up a large proportion of the water-soluble constituents of skin in which these humectants are found. However, it can be shown that at the pH at which these amino acids are found in skin they are relatively nonhygroscopic.

The occurrence of PCA in the human body appears to be largely limited to the stratum corneum. It is found only in trace amounts in other tissues and organs. In this respect the amount found naturally

occurring in the skin is surprisingly high (2% by weight). Speaking only from a teleological viewpoint, it would seem that the production of large amounts of the material almost exclusively in the skin must serve some function.

At the pH of skin, it can be shown that PCA exists almost entirely in the salt form. In this form, it is found to be highly hygroscopic and at higher relative humidities dissolves in its own water of hydration. Analytical studies have established a relationship between the amount of this material found in the stratum corneum and the moisture-binding ability of the stratum corneum.

The pathway by which this material is synthesized in the skin is unknown. The aqueous extracts of the skin are relatively rich in free amino acids. It has been proposed that these arise from the catabolic breakdown of proteins during keratinization. Since PCA can be formed *via* the cyclization of glutamic acid with heat, it is possible that some enzymatic cyclization of glutamic acid occurs in the skin.

It has been shown (26, 27) that in the enzymatic synthesis of glutamine from glutamic acid there is formed an intermediate γ-activated glutamate. In the presence of NH_4+ ions this is converted to glutamine. In the absence of NH_4+ ions, however, the intermediate is converted into PCA.

Attempts were made to determine whether glutamic acid in skin could act as a direct precursor of PCA, perhaps *via* formation of an γ-activated glutamate. Rat skin homogenates were used in the experiments after the presence of PCA was established in both guinea pig skin and rat skin. Under the conditions used in these experiments, PCA was not formed from glutamic acid. Obviously, this does not preclude the possibility that it could form under different conditions.

An alternative pathway by which PCA may be formed may involve the catabolism of proteins occurring during keratinization. If an activated glutamate is formed during protein catabolism, this may be converted directly into PCA. Thus, protein-bound glutamate rather than free glutamate may represent the precursor of PCA. Further experimentation in this area is needed.

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