thrombin activity is easily demonstrable and some clot forming activity is preserved.

1. Bar-Eli, A., Katchalski, E., *J. Biol. Chem.*, 1963, v238, 1690.

2. Cebra, J. J., Givol, D., Silman, H. I., Katchalski, E., *ibid.*, 1961, v236, 1720.

3. Rasmussen, P. S., *Biochem. et Biophys. Acta*, 1955, v16, 157.

4. Johnson, J. F., Seegers, W. H., in *Coagulation of Blood — Methods of Study*, L. M. Tocantins, ed., Grune & Stratton, New York & London, 1955, p. 120.

5. Ehrenpreis, C., Scheraga, H. A., *Arch. Biochem. Biophys.*, 1959, v79, 27.

6. Winzler, R. J., *Methods of Biochemical Analysis*, David Glick, ed., Interscience Publishers, New York, 1955, p303.

7. Dixon, M., *Biochem. J.*, 1953, v55, 170.

8. Shainoff, J. R., Page, I. H., *J. Exp. Med.*, 1962, v116, 687.

9. Sherry, S., Troll, W., *J. Biol. Chem.*, 1954, v208, 95.

10. Landaburu, R. H., Seegers, W. H., *Canad. J. Biochem. and Physiol.*, 1959, v37, 1361.

11. Fieser, L. F., Fieser, M., *Advanced Organic Chemistry*, Reinhold Publishing Corp., New York, 1961, p736.

Received June 20, 1963. P.S.E.B.M., 1964, v115.

Acid-Soluble Nucleotides and Peptides of Skin.* (28898)

MORTON URIVETZKY, SAM SEIFTER AND EDWARD MEILMAN
(Introduced by A. White)
Departments of Medicine and Laboratories, Long Island Jewish Hospital, New Hyde Park, N. Y., and Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York

Numerous reports have appeared concerning the presence of "peptide-nucleotides" in acidic and alcoholic extracts of mammalian liver(1-3) and microorganisms(4-7), and in one instance(8) the structure of such a compound has been determined. Although the function of these compounds has not been established, we decided to study perchloric acid-soluble preparations of neonatal animals to determine the extent and nature of the peptide-nucleotide pool, and whether proline and hydroxyproline were present in such a pool as possible intermediates in the metabolism of collagen.

Materials and methods. Animals and tissues. Rabbits less than one week of age were decapitated and their skins, less that of head and limbs, removed. Subsequent handling of tissues, including preparation and fractionation of extracts, was carried out at 0 to 5°C.

Extracts were prepared by homogenizing the skins in cold 0.25 M perchloric acid to yield a 20% suspension of the tissue; the mixture was centrifuged at 1500 × *g* for 10 minutes and the first extract was decanted. The residue was reextracted with a second equivalent volume of 0.25 M perchloric acid and centrifuged; the combined extracts were then filtered.

Extracts were examined at 260 mμ in a Beckman DU spectrophotometer using 1 cm cells. The total absorbancy at this wavelength, determined from the volume of extract and the optical density, was expressed as A_{260} units. In sub-fractions of the extract, prepared subsequently, measurements were also made at 275 mμ, and A_{275} units determined.

Adsorption of nucleotides on Norit and elution. Norit A was washed with 10^{-3} M Versene (pH 7), water, 4 N acetic acid and finally again with water until washings were neutral. The Norit was then dried at 110°, and a 10% suspension prepared in water.

The suspension was added to the perchloric acid extract (40 mg Norit for each 100 optical density units measured at 260 mμ in a 1 cm cell (A_{260} units)). The mixture was stirred for 2 hours and then filtered through a coarse sintered-glass funnel layered with

* Aided by grants from Nat. Inst. Health, USPHS, and from Am. Heart Assn.

Hyflo-supercel. The filtrate was treated with a second portion of Norit (one-third the previous amount used), stirred for one hour and refiltered.

The Norit residues were combined, washed with water and recovered by filtration. Elution of nucleotides from Norit was achieved by use of a solution containing triethylamine, ethanol and water (2:60:38 v/v). The washed Norit residue was transferred to a flask and 25 ml of this solution added for each 100 mg of Norit. The mixture was stirred overnight and filtered; the recovered Norit was treated for an additional 2 hours with half the volume of eluting agent used previously. The filtrates were combined, dried *in vacuo* at 30° in a rotary evaporator, and the residue was dissolved in water. A solution containing approximately 100 A_{260} units per ml was obtained.

Because Norit adsorbs some amino acids and uncombined peptides in addition to nucleotides, an effort was made to remove these before fractionation on Dowex-1. Two separate methods were used. In Experiment I, Norit residues, before treatment with the eluting solution of triethylamine, ethanol and water, were washed successively with 0.05 M tripotassium phosphate and 0.1 M potassium acetate. In Experiment II, phosphate and acetate washings were omitted; however, after the eluate was obtained and concentrated, it was treated further with Amberlite CG-50 resin before being placed on Dowex-1. For this purpose Amberlite CG-50 (Type I, H^+ form) was prepared according to the procedure of Davies and Harris(4). The concentrated eluate from Norit was stirred for 10 minutes with an equal volume of dry resin; the suspension was filtered and washed with water. Filtrate and washings were combined and used in this form for assays and for charging the Dowex-1 column.

Preparation of ion-exchange resins and fractionation of Norit eluate. Dowex-1-X4 resin (200-400 mesh, supplied as the chloride), from which the fines were removed, was washed on a Buchner funnel successively with 1 N NaOH, water, 1 N HCl and again water until free of chloride. A column, 20 × 1 cm, was prepared and the resin-bed was washed with 2 N HCl and water until the washings were free of chloride and had an A_{260} value of less than 0.05.

A portion of concentrated Norit eluate, adjusted to pH 7-7.2 and containing approximately 2,000 A_{260} units, was used to charge the column. In the second experiment reported below the charge solution was first treated with Amberlite CG-50 resin as described above. Columns were charged and washed at flow rates not exceeding 20 ml per hour; water was then used to remove unabsorbed substances until the eluate had an A_{260} value of less than 0.1. The unabsorbed fractions were combined and saved. A chloride elution gradient(9) was started after the column was mounted over a fraction collector. Elution was carried out at a rate approximately 10 ml per hour; 5 ml fractions were collected.

Identification and estimation of purine and pyrimidine bases and nucleotides. Each of the column fractions was read against a water blank at 260 and 275 mμ in a Beckman DU spectrophotometer using 1 cm cells. The 260 mμ readings were plotted to yield an elution pattern, and A_{275}:A_{260} ratios were used for preliminary identification of nucleotides in a given fraction(9). For more exact characterization, fractions in a given peak were combined and their spectra obtained in 0.1 N HCl and in 0.1 N NaOH with a Cary recording spectrophotometer. In some cases the contents of a single tube within a peak range was also studied in this manner. The molar extinction values used for estimating nitrogenous base concentrations were those employed by Hurlbert *et al*(9). Identification of the base was further confirmed, after hydrolysis of the nucleotides in 70% perchloric acid, by paper chromatography in isopropanol-HCl (10).

DPN and TPN were identified as their cyanide derivatives by spectral analysis.

Aliquots of nucleotide fractions were subjected to electrophoresis in a 0.05 M sodium acetate buffer, pH 4.0, on Whatman 3 MM paper strips. Electrophoresis was carried out at a constant current of 5 mA in a Spinco-Durrum apparatus for 16 hours in a cold room. Portions of some fractions were also

TABLE I. Gross Composition of Preparations from Rabbit Skins.

Fraction*	A_{260} units	Ninhydrin test, μM leucine equivalents	Peptides,† μg	Proline,† μM		Hydroxyproline, μM		Aminohexose,§ μM				
									Total		N-acetyl	
				H	U	H	U	H_1	H_2	U	H_1	H_2	U
Perchloric acid extract	4020												
Norit eluate	3140	740	2000	22.4		4.20							
Placed on Dowex-1	2200	397	1100	12.0		2.20							
Unadsorbed on Dowex-1	432	202	243	6.9		1.90							
Perchloric acid extract	12600												
Norit eluate	10350	2640	5250	112.2	19.1	9.16	.91	25.9	26.0	15.4	5.9	6.3	1.1
Placed on Amberlite	3750	1056	1750	37.4	7.8	3.05	.30	8.7	8.7	5.1	2.2	2.3	.5
Removed from Amberlite	3550	630	1040	24.7	2.2	1.63	.16	8.5	8.5	3.1	2.1	2.2	.4
Placed on Dowex-1	2130	365	614	17.6	1.6	1.17	.12	5.9	5.9	2.2	1.7	1.8	.3
Unadsorbed on Dowex-1	497	213	16	5.8	1.4	1.13	.11	1.2	1.2	1.2	.0	.0	.0

* Exp I is represented in upper portion of table; Exp II, in which Amberlite treatment was included, is in lower portion. Exp I was performed using 182 g of rabbit skin; Exp II was begun with 430 g of rabbit skin.

† Performed by method of Lowry *et al.* as mentioned in text; a serum albumin standard was used, and results are referred to this standard.

‡ H indicates that analysis was done after hydrolysis with 6N HCl as described in text; U represents analysis before hydrolysis.

§ H_1 = hydrolysis in 0.01 N HCl for 15 min; H_2 = hydrolysis in 0.1 N HCl for 15 min; U = unhydrolyzed.

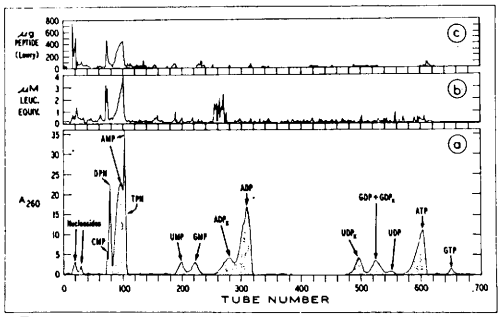
analyzed by 2-dimensional paper chromatography in the butanol-acetic acid water and *n*-propanol systems described for separation and detection of peptide-nucleotides(4).

Other chemical assays. Ribose and phosphate determinations were carried out according to procedures described by Hurlbert *et al*; ribose values of uridine containing fractions were low as expected. The ninhydrin method of Rosen(11) was employed for determination of amino acids and peptides. Total peptide content was estimated with the Folin-Ciocalteu reagent by the procedure of Lowry *et al*(12). Proline was determined by the method of Piez and co-workers(13), and hydroxyproline by that of Stegemann(14). Aminohexose content was measured by a modification of the Elson-Morgan reaction (15), and N-acetylaminohexose by the method of Reissig *et al*(16).

The presence of ester or anhydride groups was determined by a modification of the hydroxamate method of Lipmann and Tuttle (17). Ferric complexes formed after application of these procedures were characterized by their absorption spectra.

Results and discussion. Two separate fractionations of perchloric acid-soluble materials of rabbit skin were performed. As indicated previously the starting materials for these separations differed chiefly in the manner in which uncombined amino acids and peptides were removed preliminary to the charging of the Dowex-1 chloride columns. In Table I are presented analytical data which characterize the nature of the extracts obtained from the skins and of those ultimately placed on the Dowex-1 column. It is sufficient to note that in each experiment the starting solution for anion-exchange chromatography contained both peptides and nucleotides, and that the peptide materials contained both proline and hydroxyproline.

The elution patterns obtained using Dowex-1 chloride are presented in Fig. 1 and 2. Analytical data describing the nature of individual fractions in the patterns are presented in Tables II and III. In both figures and tables those fractions denoted by a subscript *x* contained derivatives or isomers of



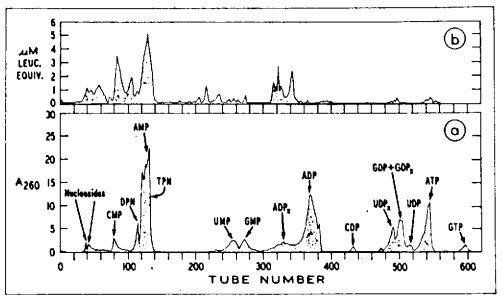


FIG. 1. Dowex-1 (chloride) elution patterns obtained in Exp. I. Constituents are expressed as total values per tube. (a) Absorbancy at 260 mμ; (b) Leucine equivalents in ninhydrin assay; and (c) Peptides (Lowry). The column was charged with an aliquot of concentrated Norit eluate containing 2200 A_{260} units. Elution gradients were: 0.002 N HCl → 0.01 N HCl (tubes 1-110) → 0.01 M NaCl in 0.01 N HCl (tubes 111-195) → 0.025 M NaCl in 0.01 N HCl (tubes 196-310) → 0.05 M NaCl in 0.01 N HCl (tubes 311-490) → 0.1 M NaCl in 0.01 N HCl (tubes 491-530) → 0.2 M NaCl in 0.01 N HCl (tubes 531-600) → 0.5 M NaCl in 0.01 N HCl (tubes 601-700).

FIG. 2. Dowex-1 (chloride) elution patterns obtained in Exp. II. Constituents are expressed as total values per tube. (a) Absorbancy at 260 mμ; and (b) Leucine equivalents in ninhydrin assay. The column was charged with an aliquot of concentrated Norit eluate (after treatment with Amberlite) containing 2130 A_{260} units. Elution gradients were identical with those given in the legend to Fig. 1 except that the indicated changes were made at tubes 140, 255, 450, 480 and 560.

the designated nucleotides as determined by chromatography or spectrophotometric assay. Simultaneous elution of nucleotides and peptides, as demonstrated by the elution patterns as well as by the ratio of ninhydrin values after and before hydrolysis (Tables II and III), occurred throughout the fractionation. Pre-treatment of the concentrated Norit eluate with Amberlite CG-50, as was done in Exp. II, primarily affected the composition of the fraction *not* retained on Dowex-1.

Because many eluted fractions contained both peptides and nucleotides, experiments were performed to determine the presence of groups (*e.g.*, esters or acyl anhydrides) which could react with hydroxylamine. Aliquots of Norit eluates, of Amberlite-treated concentrates of Norit eluates, and of concentrates of all column fractions were reacted with neutral or alkaline hydroxylamine and then either ferric chloride or ferric perchlorate. It was found that Norit eluates and the Amberlite-treated eluates, after treatment with alkaline hydroxylamine (pH 9) for 1 hour at 40°, yielded relatively small amounts of ferric complexes corresponding to approximately 0.25 micromole of "hydroxamate" for each 100 A_{260} units; reaction with neutral hydroxylamine failed to produce detectable amounts of hydroxamate. This value was obtained using the extinction coefficient for ferric acyl hydroxamates which have absorption maxima at 505-510 mμ; the materials analyzed here had maxima at 450-470 mμ. The possibility therefore exists that the derivatives formed with hydroxylamine were not solely acyl hydroxamates. In any event after eluates were fractionated on Dowex-1, none of the fractions obtained, when treated with hydroxylamine and ferric reagents, yielded detectable ferric complexes in the test tube. As mentioned below some of the fractions did produce ferric complexes on filter paper after spraying with hydroxylamine and ferric perchlorate reagents.

When eluant fractions were subjected to electrophoresis, only UDP_x and $GDP\text{-}GDP_x$ components were found to contain overlapping nucleotide and ninhydrin-positive materials which migrated toward the cathode. The areas on the filter paper which corresponded to these fractions did give positive color reactions when sprayed with hydroxylamine and ferric perchlorate. Attempts to elute the pigments from the papers for spectrophotometric analysis were unsuccessful because of the instability of the color.

One of our major objectives was to determine whether young skins contain peptide-nucleotides with constituent residues of proline and hydroxyproline as intermediates in

TABLE II. Exp I. Composition of Fractions Eluted from Dowex-1 Chloride.

Fraction	Nitrogenous base, μM total	Analysis—Results expressed as μM per μM of nitrogenous base: Ribose	Phosphate: Total	Phosphate: Labile	Proline	Ninhydrin test*: Before hydrolysis	Ninhydrin test*: Ratio after hydrolysis†
CMP	2.82	.13	1.09	.0	.0	1.68	3.8
DPN	6.56	1.62	1.54	.0	.0	.37	9.4
AMP	28.84	1.12	1.15	.0	.0	1.65	3.2
UMP	4.64	.10	1.04	.0	.17	.84	4.0
GMP	2.05	1.04	1.13	.0	.29	.90	3.7
ADP_x	7.18	1.73	1.72	.21	.16	2.30	2.7
ADP	19.30	1.08	1.97	.93	.09	.26	3.7
UDP_x	5.40	.40	2.10	.80	.15	.54	3.2
GDP + GDP_x	4.95	.80	2.02	.60	.12	.58	2.3
UDP	1.08	.10	2.10	.87	.09	.60	2.4
ATP	7.80	1.11	2.90	1.80	.0	.19	4.0
GTP	.94	1.20	3.10	1.87	.0	.0	2.6

* Results expressed as μM leucine equivalents.
† This is the ratio of leucine equivalents after hydrolysis with 6 N HCl to leucine equivalents before hydrolysis.

TABLE III. Exp II. Composition of Fractions Eluted from Dowex-1 Chloride.

Fraction	Nitrogenous base, μM total	Analysis—Results expressed as μM per μM of nitrogenous base: Ribose	Phosphate: Total	Phosphate: Labile	Proline	Aminohexose: Total	Aminohexose: N-acetyl	Ninhydrin test*: Before hydrolysis	Ninhydrin test*: Ratio†
CMP	3.42	.10	.94	.0	.0	.0	.0	4.35	4.0
DPN	4.40	1.60	1.80	.0	.0	.0	.0	1.12	8.4
AMP	21.88	1.15	1.02	.0	.0	.0	.0	2.66	3.4
UMP	5.75	.27	1.00	.0	.28	.0	.0	.83	4.5
GMP	2.20	1.14	.94	.0	.44	.0	.0	1.27	3.0
ADP_x	4.29	2.00	1.60	.13	.42	.0	.0	7.15	3.2
ADP	13.20	1.14	1.70	.98	.20	.0	.0	.24	2.6
CDP	.20	.05	1.86	1.00	.0	.20	.05	.50	1.4
UDP_x	3.80	.35	2.00	.90	.32	.81	.32	.72	3.5
GDP + GDP_x	4.80	1.02	2.10	.94	.16	.20	.15	.54	3.1
UDP	1.60	.10	1.87	.84	.26	.0	.0	.34	2.1
ATP	7.02	1.15	2.94	2.04	.0	.0	.0	.30	3.2
GTP	.72	.98	2.78	1.88	.0	.0	.0	.0	1.2

* Results expressed as μM leucine equivalents.
† This is the ratio of leucine equivalents after hydrolysis with 6 N HCl to leucine equivalents before hydrolysis.

the metabolism of collagen. While both of these amino acids in combined (presumably peptide) form were found in the Norit eluates (Table I), hydroxyproline in any form was not retained by the Dowex-1 column and appeared almost quantitatively in the first (unadsorbed) fraction. Proline, however, was present in combined form, presumably peptide, in several of the eluted fractions including UDP_x and GDP-GDP_x; the complete amino acid composition of these has not as yet been determined. Caution must be exercised in interpretation of these results because acid-soluble collagens could be extracted from young skins and provide the observed proline and hydroxyproline contents. On the other hand control experiments indicated that the peptides containing these amino acids were dialyzable, thus lessening the possibility that they were associated with undegraded collagen in the extracts.

The nucleotides and peptides of the remaining fractions migrated independently as anions under the electrophoretic conditions employed. The peptide in the AMP fraction was of particular interest because it was present in relatively large amount and migrated as though it were a strong anion. Following

electrophoretic separation it behaved as a single component in 3 chromatographic solvent systems, gave a 3-fold increase in ninhydrin reactivity after hydrolysis with 6 N HCl at 110° for 12 hours, and contained glutamic acid, glycine and cysteic acid. Hydrolysis of the DNP-derivative of this peptide yielded DNP-glutamic acid, glycine and cysteic acid. These observations suggest that the peptide is an oxidized derivative of glutathione. We are not certain to what extent the peptide was present in the native tissue, although control experiments in which glutathione was carried through treatment with perchloric acid, Norit and Dowex, did not yield significant amounts of cysteic acid. The same peptide was present in the Norit eluate before fractionation on Dowex-1, and therefore appears to be a *bona fide* component of untreated extracts of skin. In some respects this cysteic acid peptide resembles that found in association with nucleotides in liver(2), although the latter, containing taurine in addition to cysteic acid and β-alanine, as well as glycine and glutamic acid, would appear to arise from oxidation of a mixed disulfide of glutathione and CoA.

Peptides eluted from the anion-exchange columns subsequent to the AMP fraction migrated electrophoretically as slightly acidic, neutral or basic ampholytes. One would therefore have to speculate that these peptides were retarded by the resin due to conjugation with acidic moieties (*e.g.*, glycuronic acids, sulfates, phosphates or nucleotides). It is possible that in the process of their elution from the column some of the conjugates were hydrolyzed and appeared as free peptides and nucleotides in the fractions.

Summary. A cold perchloric acid extract was made of the skins of young rabbits, and treated with Norit to provide a concentrate which was then fractionated on Dowex-1 chloride. All fractions were investigated for the presence of peptide-nucleotide components by absorption spectrophotometry, ninhydrin analysis, paper electrophoresis and treatment with hydroxylamine. Small amounts of substances reacting with alkaline hydroxylamine were detected in preparations prior to column chromatography; eluted fractions, however, did not yield positive hydroxamate tests in the test tube. Two of the fractions when subjected to paper electrophoresis contained overlapping nucleotide and peptide components migrating toward the cathode, and indeed yielded positive color tests for hydroxamates after treatment with hydroxylamine and ferric perchlorate spray reagents. Proline was present in these fractions as well as in several others. While hydroxyproline was present in the Norit eluate, it was not retained on the Dowex-1 column and therefore did not appear in any of the eluted fractions either in free or combined forms. An acidic peptide containing glutamic acid (as N-terminal), glycine and cysteic acid was isolated from an eluted fraction which also contained adenylic acid.

1. Weinstein, C., Hammond, D., Berkman, J. I., Gallop, P. M., Seifter, S., *Fed. Proc.*, 1958, v17, 332.

2. Wilken, D. R., Hansen, R. C., *J. Biol. Chem.*, 1961, v236, 1051.

3. Ondarza, R. N., Aubaniel, M., *Biochim. Biophys. Acta*, 1960, v44, 381.

4. Davies, J. W., Harris, G., *Proc. Roy. Soc. (London) B*, 1960, v151, 537.

5. Harris, G., Neal, G. E., *Biochim. Biophys. Acta*, 1960, v43, 197.

6. Davies, J. W., Harris, G., *ibid.*, 1960, v45, 28.

7. Schuurs, A. H. W. M., Koningsberger, V. V., *ibid.*, 1960, v44, 167.

8. Bernlohr, R. W., Webster, G. C., *Nature*, 1958, v182, 531.

9. Hurlbert, R. B., Schmitz, H., Brumm, A. F., Potter, V. R., *J. Biol. Chem.*, 1954, v209, 23.

10. Hotchkiss, R. D., in Colowick, S. P. and Kaplan, N. O., *Methods in Enzymology*, Academic Press, Inc., New York, vIII, p716.

11. Rosen, H., *Arch. Biochem. Biophys.*, 1957, v67, 10.

12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.

13. Piez, K. A., Irreverre, F., Wolff, H. L., *ibid.*, 1956, v223, 687.

14. Stegemann, H., *Z. Physiol. Chem., Hoppe-Seyler's*, 1958, v311, 41.

15. Rondle, C. J. M., Morgan, W. T. J., *Biochem. J.*, 1955, v61, 586.

16. Reissig, J. L., Strominger, J. L., Leloir, L. F., *J. Biol. Chem.*, 1955, v217, 959.

17. Lipmann, F., Tuttle, L. C., *ibid.*, 1945, v159, 21.

Received June 20, 1963. P.S.E.B.M., 1964, v115.