THE EFFECT OF SODIUM DILANTIN ADMINISTRATION UPON THE CHEMISTY OF THE SKIN *

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The gingival hyperplasia associated with Dilantin therapy has been reviewed by Ziskin, Stowe and Zegarelli (1). Although a similar pathologic change could be induced in the ferret (2), it has been impossible to produce this condition in rats (3). However, Shafer, Beatty and Davis (4) have demonstrated that Dilantin-treated rats show a marked increase in the tensile strength of their healing wounds. These workers attributed this elevation in the tensile strength of wounds with parenteral Dilantin therapy to an increase in collagen synthesis, the same mechanism underlying the gingival hyperplasia occurring in young epileptics under Dilantin therapy. Forscher and Cecil (5) were unable to find any significant differences in the hydroxyproline content of healing palate tissue between control and Dilantin-treated rats.

This paper describes the effects of massive daily intraperitoneal injections of Dilantin Sodium upon the distribution of hydroxyproline, nitrogen and hexosamine within the connective tissue (skin) of the rat, as determined by using a sequential extraction technique to effect chemical dissection of the tissue.

MATERIAL AND METHODS

Thirty male Sprague-Dawley rats (210 to 230 g) were subjected to daily intraperitoneal injection of 1 ml of isotonic saline containing a suspension of 25 mg per ml of Dilantin Sodium.[1] At 2, 5, 7, 11 and 16 days after initiation of the experiment, groups of 6 rats each were sacrificed by either anesthesia or exsanguination. Six g samples of abdominal rat skin were collected from each animal, carefully shaven and dissected clean of adhering fat, fascia and muscle and minced with scissors. The tissues were then stored frozen until the completion of the experiment. Samples of each skin (300 mg) were then hydrolyzed for 8 hours at 100° C with 10 ml of 4 N HCl per g of skin. The sera were diluted 1:1 with 8 N HCl and hydrolyzed in similar fashion. The acid hydrolysate was analyzed for total hexosamine (6), hydroxyproline (7) and nitrogen, as has been described previously (8).

Hydroxyproline, as a signal amino acid of collagen, is an excellent indicator of this protein (9). Hexosamine may be found in both ground substance mucopolysaccharides and serum glycoproteins contaminating the matrix of the skin (10). Nitrogen is a standard measure of total protein.

Duplicate 5 g samples of rat skin were extracted with 50 ml of 0.15 M NaCl in the cold (3° C) using a Lourdes high-speed tissue blender for 45 minutes. After extraction, the mixtures were stored for 18 hours at 4° C.

After centrifugation at 25,000 × G and 4° C, the clear supernatant was removed. The insoluble residue was then extracted similarly with 0.5 M NaCl and after standing for 18 hours in the cold, the residue after centrifugation was in turn re-extracted with 100 ml of 0.5 M citrate buffer (pH 3.6). These conditions for extraction are such as to solubilize about 75 to 95 per cent of all the material exhaustively extracted by each solvent.

These fractions were mixed 2:1 with 12 N HCl and hydrolyzed as described above for hexosamine, hydroxyproline and nitrogen analysis.

Since cortisone administration does not increase the dermal content of either hexosamine or collagen (11), and since we were unable to measure any increase of these components in the skin of rats stressed by intraperitoneal injection (unpublished data), untreated animals of weights similar to those of the experimental animals were used as a control.

Statistical evaluation of the data presented below indicated that means which differ by more than 15 per cent are significantly different ($p = 0.05$). This level of significance, i.e., differences of more than 15 per cent are significant, obtains for all the results described in all of the tables.

EXPERIMENTAL AND RESULTS

The results of the analysis of rat skin for hydroxyproline, hexosamine and nitrogen after various numbers of daily intraperitoneal injections of 25 mg of Dilantin Sodium were expressed per gram of stored, cleaned and shaven tissue and are presented in Table I.

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[1] Parke, Davis (sodium 5,5-diphenylhydantoinate).

After four doses of Dilantin there is a significant increase in dermal hydroxyproline. This increase becomes maximal after 11 injections of drug with the treated rat skin containing about 150 μmoles more of hydroxyproline per g of skin than does the untreated animal. Hexosamine increased by about 4 μmoles per g during this period, while the nitrogen content increased by 6 mmoles per g.

The chemical analyses described above were based upon the weight of cleaned and shaven rat skin which had been stored in the frozen state from 4 to 21 days. During the first four days all tissues lost about 30 per cent of their weight through sublimation of dermal water. With further storage in the deep freeze less than a few milligrams of water were lost per gram per day. For this reason the values reported in Table I and below must be corrected for the loss of 300 mg of water per g if the results are to be expressed per gram of *fresh* cleaned and shaven skin. Because of the extraordinary constancy of this 30 per cent loss of weight via water sublimation, we prefer to report most of our data per gram of *stored* cleaned and shaven skin.

The absolute dermal content of water from normal rats and animals subjected to 7 and 11 doses of Dilantin was determined by lyophilization. The concentration of dermal collagen may be calculated from the finding that pure collagen contains about 13.6 per cent hydroxyproline, or 1 μmole per g (12). The amount of noncollagenous protein may be calculated from the fact that collagen contains about 18.5 per cent nitrogen or 13.3 μmoles per mg (12) and most other proteins contain about 16 per cent or 11.4 μmoles of nitrogen per mg. Multiplication of the micromoles of hydroxyproline, or milligrams of collagen, per gram of skin times 13.3 results in the micromoles of collagen nitrogen, and the difference between this value and the value for the total dermal nitrogen divided by 11.4 results in the milligrams of noncollagenous protein. The difference between the sum of the weight of water, collagen, and noncollagenous proteins, in milligrams per gram of tissue, and 1,000 represents the milligrams of fat in the skin. The results of these calculations are shown in terms of *fresh* shaven and cleaned skin in Table II. Correction of the values per gram of *stored* tissue reported in Table I for water loss results in essentially identical results.

TABLE I

The analysis of whole rat skin after Dilantin administration *

No., daily inject.	Hypro	Hex	N
	μmoles/g	*μmoles/g*	*mmoles/g*
0	300 ± 27	11.0 ± 0.8	6.1 ± 0.5
2	330 ± 31 †	14.7 ± 0.9 †	9.0 ± 0.6 †
4	349 ± 30	14.6 ± 1.0	10.7 ± 0.6
7	366 ± 28	15.2 ± 1.1	10.5 ± 0.7
11	468 ± 35	15.7 ± 1.0	12.4 ± 0.8
16	447 ± 35	15.1 ± 1.1	12.5 ± 0.7

* Mean values of duplicate extracts analyzed in triplicate Hypro = hydroxyproline, hex = hexosamine, N = nitrogen.

† All values below the line are significantly increased above normal ($p < 0.05$).

TABLE II

The chemical anatomy of fresh rat skin before and after various doses of Dilantin

Component	Normal	After 7 doses	After 11 doses
	mg/g	*mg/g*	*mg/g*
Water*	550	300	300
Collagen	200	245	300
Noncollagenous protein	122	395	382
Fat†	128	60	18

* By lyophilization.
† By difference.

The fat content of normal skin as calculated by difference was 128 mg per g. Kao, Schwartz, Treadwell and McGavach (13) recently reported the fat content of the skin of rats of equivalent weight to be about 125 mg of lipid per g of fresh skin.

The effects of Dilantin administration upon the hydroxyproline, hexosamine and nitrogen content per milliliter of rat sera after various numbers of Dilantin injections are shown in Table III.

These data indicate that there are statistically significant decreases in serum hydroxyproline and hexosamine and significant increases in serum nitrogen. None of these changes is as marked as those found in the skin.

In an attempt to determine the nature of the changes in dermal chemistry with Dilantin administration, the skin samples were extracted sequentially with 0.15 and 0.5 M sodium chloride as

TABLE III

The analysis of rat serum after Dilantin administration *

No., daily inject.	Hypro	Hex	N
	μmoles/g	*μmoles/g*	*mmoles/g*
0	0.70 ± 0.05	3.3 ± 0.2	0.68 ± 0.05
	†	†	†
2	0.45 ± 0.03	1.7 ± 0.1	0.80 ± 0.06
4	0.33 ± 0.02	1.1 ± 0.1	0.81 ± 0.06
7	0.40 ± 0.03	1.3 ± 0.1	0.86 ± 0.06
11	0.52 ± 0.03	1.2 ± 0.1	0.88 ± 0.06
16	0.40 ± 0.04	1.1 ± 0.1	0.88 ± 0.07

* Mean values of triplicate analyses of sera from six animals.

† All values below the line are significantly different from normal ($p < 0.05$).

described above. Microscopically, the cellular material was intact only after isotonic saline extraction, and the electron microscope indicated that the collagen was morphologically intact after both saline extractions. The amount of hydroxyproline, hexosamine and nitrogen extracted per gram of skin by these solvents is shown in Table IV.

The quantity of material soluble in isotonic saline, although occasionally differing significantly from the untreated normal, did not show any real pattern of response to Dilantin administration. The amount of hydroxyproline soluble in 0.5 M saline decreased significantly from normal with the second dose of Dilantin, while the hexosamine soluble in this fraction increased significantly with drug administration. The decreases in 0.5 M saline-soluble nitrogen with Dilantin were only occasionally statistically significant.

More meaningful changes in the quantity of material extractable from the skin of Dilantin-treated rats were found in the analysis of the citrate buffer extracts shown in Table V.

There was an immediate increase of 18 μmoles per g of hydroxyproline with Dilantin administration. The increase of 150 per cent with respect to hydroxyproline was paralleled by a 100 per cent increase in soluble nitrogen. Hexosamine was increased maximally (700 per cent) after four doses of Dilantin, suggesting that the syntheses of hexosamine and collagen are independent.

There was a slight increase in the ratio of hydroxyproline to nitrogen in this extract which may indicate a greater purity of the acid-soluble collagen produced in response to Dilantin administration.

By summation of all three soluble fractions, the total soluble material of the skin may be calculated. Subtraction of this value from the total skin content, described in Table I, permits the calculation of the insoluble dermal components, as shown in Table VI.

Initially, there was an increase in soluble dermal hydroxyproline, or collagen, with Dilantin administration. The amount of soluble nitrogen was significantly increased under these conditions. The soluble hexosamine increased about 30 per cent with Dilantin. The ratio of soluble hydroxyproline and hexosamine to nitrogen remained essentially constant following the daily administration of this drug. These ratios did not differ from those obtained from normal untreated rat skin.

The insoluble hydroxyproline increased significantly above normal after the fourth dosage of Dilantin. After the eleventh dose of the drug, there was a 60 per cent increase in insoluble hy-

TABLE IV

Analysis of the saline-soluble component of rat skin after Dilantin administration

No., daily inject.	Extracts					
	0.15 M NaCl			0.50 M NaCl		
	Hypro	Hex	N	Hypro	Hex	N
0	7.7	5.1	1.30	29	2.4	1.20
2	11.1*	5.7	1.39	21*	3.0*	0.95*
4	8.9*	6.5*	1.33	17*	3.4*	1.10
7	6.1	6.3*	1.43	23*	2.9*	1.10
11	9.0*	6.4*	1.17	20*	3.5*	0.93*
16	6.5	5.9*	1.11*	15*	3.4*	0.95*

* Significantly different from normal ($p < 0.05$).

TABLE V

Analysis of the citrate-soluble components of rat skin after Dilantin administration

No., daily inject.	Hypro	Hex	N
	μmoles/g	*μmoles/g*	*mmoles/g*
0	12.0 ± 0.8	0.10 ± 0.1	0.60 ± 0.05
	*	*	*
2	30.7 ± 2.0	0.45 ± 0.3	1.27 ± 0.10
4	30.1 ± 2.0	0.70 ± 0.7	1.30 ± 0.10
7	30.0 ± 2.0	0.70 ± 0.6	1.28 ± 0.10
11	28.5 ± 2.0	0.65 ± 0.5	1.14 ± 0.13
16	30.0 ± 2.0	0.76 ± 0.5	1.28 ± 0.10

* All means below the line are significantly elevated above normal ($p < 0.05$).

TABLE VI

The total soluble and insoluble component of rat skin after Dilantin administration

No. daily inject.	Total soluble			Insoluble		
	Hypro	Hex	N	Hypro	Hex	N
0	48.7	7.6	3.1	251	3.4	3.0
2	62.6*	9.2*	3.6	267	5.5*	5.4*
4	56.0	10.6*	3.7*	293*	4.0	7.0*
7	59.1*	9.8*	3.8*	307*	5.4*	6.7*
11	57.5*	10.5*	3.2	410*	5.2*	9.2*
16	50.0	10.1*	3.1	397*	5.0*	9.4*

* Significantly elevated above normal ($p < 0.05$).

droxyproline. This profound increment in collagen was not paralleled with a similar increase in insoluble hexosamine; this material increased maximally (50 per cent) after only two injections of Dilantin. The concentration of insoluble nitrogen doubled after the fourth Dilantin injection, and tripled after the eleventh injection. The increase in the total dermal nitrogen of 6 mmoles per g of skin with Dilantin, as shown in Table I, seemed to be associated almost entirely with the insoluble components of the skin. Similarly, most of the increase of 150 μmoles of hydroxyproline per g of tissue was also found in the insoluble fraction. The ratio of hydroxyproline to nitrogen of very pure tendon collagen and soluble collagen is about 84 (μmoles per mmole), while a similar ratio of the insoluble residue from normal skin was about 83. With Dilantin administration, the ratio of hydroxyproline to nitrogen decreased to about 42, evidence of a disproportionate increase in insoluble noncollagenous protein.

DISCUSSION

From the data reported above, it may be concluded that Dilantin produces four major alterations in dermal chemistry: *1*) a marked decrease in tissue water from 55 to 30 per cent and in dermal fat from 12.5 to 0.2 per cent; *2*) a marked increase in collagen (50 per cent); *3*) a marked increase in hexosamine (37 per cent); *4*) a marked increase in a noncollagenous protein.

That these changes are not due to contamination of the tissues with blood elements is indicated by the minor changes in the hexosamine, hydroxyproline and nitrogen content of the sera of these animals. The profound increase of these components in the skin is associated with a quantitatively minor decrease of hydroxyproline and hexosamine in the sera.

The isotonic saline-soluble components of the skin, mostly mucopolysaccharides, serum proteins, glycoproteins and so forth, are not meaningfully altered with Dilantin administration. The most important change in the 0.5 M saline-soluble materials is a significant decrease in the hydroxyproline content of this fraction, which is believed to extract the precursor of collagen (14) as well as glycoproteins and other proteins which are strongly bound to the insoluble components of the skin.

Citrate buffer (pH 3.6) soluble material, an extractant which removes reticulin and/or *"procollagen"* (14), shows a marked increase (150 per cent) in soluble collagen with Dilantin administration. The parallel increase in acid-soluble nitrogen (100 per cent) associated with this hydroxyproline increment serves to maintain a constant ratio of hydroxyproline to nitrogen (micromoles per millimoles) of .24. The constancy of this ratio indicates that the increases in acid-soluble nitrogen are approximately proportional to the increases in acid-soluble collagen with Dilantin administration.

The essential independence of the collagen and hexosamine synthesizing processes is suggested by the fact that the increase in acid-soluble hexosamine does not parallel the increments in nitrogen or hydroxyproline with respect to time. This hexosamine-containing moiety produced in response to Dilantin is not a glycoprotein, since the contribution of nitrogen from this compound would have altered the hydroxyproline-nitrogen ratios considerably. It cannot be a *free* mucopolysaccharide, since it is not soluble in either saline extract. This material is most probably a mucopolysaccharide, tightly bound to the acid-soluble proteins by bonds of higher energy than those that can be destroyed by high ionic strength solvents.

The calculation of the concentration of insoluble material in the skin of rats treated with Dilantin indicates that there is a profound increase in insoluble collagen (58 per cent), insoluble hexosamine and nitrogen. Most of the 150 mg increase in dermal collagen with Dilantin administration described in Table I was found in the insoluble residue. Similarly, most of the 6 mmole increase in dermal nitrogen with drug dosage is insoluble. The increase in dermal hexosamine (4 μmoles) is

evenly divided between the total soluble and insoluble fractions. This latter component in the insoluble fraction is maximally elevated after two doses of Dilantin, while the hydroxyproline and nitrogen require about 11 doses of the drug to effect maximal increment.

The nature of this insoluble hexosamine-containing component may well be a mucopolysaccharide strongly bound to either the insoluble collagen or the other insoluble proteins described below, and as such might bear a relationship to the mucopolysaccharide soluble only in citrate buffer.

The ratio of hydroxyproline to nitrogen (micromoles per millimoles) of the insoluble residue from the untreated animals was similar to that of pure collagen (i.e., 83). With Dilantin administration, this ratio decreased to about 45, indicating that the increase in insoluble nitrogen was far greater than the increase in insoluble dermal collagen. One of the major alterations in the chemistry of the skin in response to Dilantin is therefore the appearance of a unique insoluble noncollagenous protein. That this material is probably not a glycoprotein is indicated by the variation of the hexosamine to nitrogen ratios with increasing Dilantin dosage as calculated from Table VI.

SUMMARY

Dilantin administration results in the following changes in the chemistry of rat skin:

1. A marked decrease in dermal water and fat.
2. A marked increase in acid-soluble collagen and acid-soluble mucopolysaccharide.
3. A marked increase in insoluble collagen and hexosamine.
4. The appearance of a unique insoluble noncollagenous protein.

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