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THE RESORPTION OF SODIUM DILANTIN-PRODUCED DERMAL COLLAGEN *

By J. C. HOUCK

(From the Biochemistry Research Laboratory, Children's Hospital Research Foundation, and the Department of Biochemistry, Georgetown University Medical School, Washington, D. C.)

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Dilantin has long been known to result in hyperplasia of the gingiva (1) and to increase the tensile strength of rat skin (2). We have shown that daily intraperitoneal injections of 25 mg of Dilantin resulted in marked increases in the insoluble collagen, hexosamine, and insoluble noncollagenous protein (scleroprotein) content of rat skin (3). Four daily doses of the drug were sufficient to significantly increase both the total and the insoluble collagen and nitrogen concentrations in the skin. Maximal increases in these components were obtained after ten doses of Dilantin. This paper describes the changes in the dermal concentrations of these materials at various periods of time after the conclusion of either four or ten daily doses of this drug in rats.

MATERIALS AND METHODS

Two groups of 36 male Sprague-Dawley rats (300 to 330 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] One group of animals was given 4 daily injections of drug, while the other group was injected daily for 10 days. At 0, 1, 3, 6, 10, and 14 days after the conclusion of drug administration to both groups, 6 rats from each treated group were sacrificed, and 2-g samples of abdominal skin were removed, shaven, and dissected clean of adhering fat, fascia, and muscle. These tissues were then minced and 0.5-g aliquots removed for analysis of total dermal hydroxyproline, nitrogen, and hexosamine. These tissues were not permitted to stand in the frozen state long enough to lose dermal water via sublimation, as was done previously (3). The remaining tissues from each group of 6 rats were pooled, and duplicate 4-g samples of pooled tissue were extracted sequentially in the cold with 0.5 M NaCl, 0.5 M NaCl, and 0.5 M citrate buffer (pH 3.6) as has been described previously (3, 4). These extractions remove quantitatively ground substance, neutral-soluble, and acid-soluble collagen, respectively (4). Neutral-soluble collagen presumably represents the extrafibrillar precursor of fibrous collagen, while acid-soluble collagen is related to reticulin and the surface of the collagen bundle (5).

Both the extracts and the whole skin samples were hydrolyzed in 4 N HCl at 100° C for 8 hours. These hydrolysates were analyzed in triplicate for their nitrogen (6), hexosamine (7), and hydroxyproline (8) content as a measure of protein, mucopolysaccharides, and glycoproteins (9), and collagen (10). The results of the analyses of the whole skin were expressed as the mean and standard deviation of the replicate analyses of 6 animals in micromoles of hexosamine or hydroxyproline or in millimoles of nitrogen per gram of fresh, cleaned rat skin. The hydroxyproline, hexosamine, and nitrogen contents of the extracts were similarly expressed as the mean of the triplicate analyses of extracts prepared from duplicate pools of fresh, cleaned skin. The standard deviations of all means were never in excess of 6 per cent of the mean, and all means differing by more than 2 SD were, statistically, significantly different ($p < 0.05$). The difference between the total dermal concentration of these materials and the sum of their soluble concentrations was calculated. The insoluble dermal concentration of each material was therefore obtained.

The amount of nitrogen involved in proteins other than collagen may be calculated from the difference between the total nitrogen and the nitrogen content of the dermal collagen, as revealed by multiplying the micromoles of hydroxyproline (1 μmole of hydroxyproline = 1 mg of collagen) by 13.3 as described previously (3–5). This calculated value may then be translated into total noncollagenous dermal protein.

As described previously (3), control animals receiving daily intraperitoneal injections of isotonic saline demonstrated no increase in dermal collagen.

EXPERIMENTAL RESULTS

The results of the analyses of whole rat skin at 0, 1, 3, 6, 10, and 14 days after either 4 or 10 doses of Dilantin are presented in Table I. These figures are compared statistically both with Day 0 and with the analyses of normal rat skin from animals of an equivalent weight. This table shows that the elevated dermal collagen concentration resulting from 4 doses of Dilantin was reduced to normal 6 days after the conclusion of drug ad-

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

TABLE I

Analysis of whole rat skin after the administration of 4 or 10 daily doses of Dilantin *

After dose	No. of doses:	Hydroxyproline 4	Hydroxyproline 10	Hexosamine 4	Hexosamine 10	Nitrogen 4	Nitrogen 10
days		*μmoles/g*		*μmoles/g*		*mmoles/g*	
0		*257*	*310*	10.1	10.4	*7.5*	*8.1*
1		*280*	*310*	**16.3*	**15.0*	*8.2*	**7.1*
3		*230	*270*	**15.1*	**12.0*	**6.6*	**6.3*
6		*218	*330*	**13.5*	**14.7*	**5.7*	*7.3*
10		*214	*320*	**13.9*	**12.5*	**5.6*	**7.0*
14		*212	*330*	**15.0*	**11.6*	**6.1*	**6.6*
Normal		220 ± 12		10.0 ± 0.5		4.8 ± 0.2	

* Starred values are significantly different from Day 0 ($p < 0.05$); italicized means are significantly different from normal ($p < 0.05$).

ministration, while no resorption of Dilantin-produced collagen had taken place up to 2 weeks after the tenth and final dose of the drug had been administered. With collagen resorption (4 doses) there was an increase in dermal hexosamine concentration, while a similar, if less profound, increase in this material was found after 10 doses of drug despite the continued elevated dermal collagen concentration after this amount of Dilantin. The total dermal nitrogen content decreased similarly after the conclusion of the administration of both levels of drug.

Lyophilization studies indicated that at Day 0 both sets of tissues contained 300 mg per ml of water, and that by 14 days after the final drug injection both sets of skin contained about 400 mg of water per g. The normal dermal water content for this weight of animal was 550 mg per g (3).

Initially, both doses of Dilantin resulted in about 350 mg per g of noncollagenous protein on Day 0. Two weeks later the animals which had received 4 doses contained 300 mg per g, while those rats which had been given 10 doses contained 200 mg of noncollagenous protein per g fresh skin. Normal skin from rats of a similar weight contains 160 mg of this material per g.

The hydroxyproline, hexosamine, and nitrogen contents of the isotonic saline extracts of skin are shown in Table II. These results indicate that the hexosamine and nitrogen soluble in this solvent decreased below normal 2 weeks after conclusion of the administration of either 4 or 10 doses of drug. The hydroxyproline content of this fraction returned to normal during this time. Assumed that the ratio of hexosamine to nitrogen (micromoles per millimoles) in this fraction is a measure of the ground substance (4), then the isotonic saline extracts of skin from rats receiving 4 doses of drug contained 4.9 μmoles of hexosamine per mmole of nitrogen at Day 0 and 3.5 at Day 14. Similar values for animals receiving 10 doses were 5.4 and 5.9. Isotonic saline extracts from normal rats contained 5.9 μmoles of hexosamine per mmole of nitrogen.

TABLE II

Analysis of the 0.15 M saline-soluble components of rat skin after the administration of 4 or 10 daily doses of Dilantin *

After dose	No. of doses:	Hydroxyproline 4	Hydroxyproline 10	Hexosamine 4	Hexosamine 10	Nitrogen 4	Nitrogen 10
days		*μmoles/g*		*μmoles/g*		*mmoles/g*	
0		*6.2*	*6.0*	4.5	*4.3*	*0.94*	0.80
1		*6.3*	*6.5*	**3.7*	*5.3	*0.99*	**1.04*
3		**9.0*	**7.2*	**6.9*	*5.3	1.04	*0.93
6		*5.7*	*6.0*	*4.7*	**3.4*	*0.78	**1.07*
10		*6.1*	*5.1	*5.8	4.8	0.81	*0.99
14		*5.1	*4.2	**2.4*	*3.8*	**0.69*	**0.65*
Normal		4.7 ± 0.23		5.0 ± 0.3		0.85 ± 0.05	

* See Table I.

TABLE III

Analysis of the 0.50 M saline-soluble components of rat skin after the administration of 4 or 10 daily doses of Dilantin *

After dose	No. of doses:	Hydroxyproline 4	Hydroxyproline 10	Hexosamine 4	Hexosamine 10	Nitrogen 4	Nitrogen 10
days		*μmoles/g*		*μmoles/g*		*mmoles/g*	
0		*11.8*	*13.4*	2.5	*2.3*	0.77	*0.63*
1		**15.5*	*19.8	**3.8*	**3.5*	0.84	**1.00*
3		**17.7*	**17.1*	**4.7*	*3.2	**0.93*	**1.02*
6		**16.8*	**16.5*	**3.8*	*3.2	0.69	**0.99*
10		*12.8*	**15.9*	*3.2	2.7	0.78	**0.90*
14		* *9.0*	*14.7*	*3.3	*3.2	**0.63*	**0.93*
Normal		20.4 ± 1		2.9 ± 0.17		0.78 ± 0.04	

* See Table I.

Table III shows that the neutral-soluble collagen content of skin remained low for at least 2 weeks after concluding the administration of either 4 or 10 daily doses of Dilantin. These results also suggest that the depression of neutral-soluble collagen was less profound in those animals which had received 10 than in those receiving 4 doses. The hexosamine content of this fraction increased to normal with time after the administration of either dose of the drug. Despite the decrease in neutral soluble collagen, the nitrogen content of this fraction obtained from animals after 10 doses of drug was elevated above normal. Similar transient increases in 0.5 M NaCl-soluble nitrogen were found after 4 doses of Dilantin, but the nitrogen content of this fraction returned to normal or subnormal value 6 days after the last dose of drug.

The citrate-soluble collagen content of rat skin from animals 1 day after the fourth dose of drug demonstrated a profound increase of 1,500 per cent above normal, and 600 per cent above Day 0, as shown in Table IV. This degree of elevation in citrate-soluble collagen was maintained over the whole course of 2 weeks subsequent to Dilantin administration. The citrate-soluble collagen content of skin from animals after 10 doses of drug was decreased to normal.

Citrate-soluble hexosamine was found for 2 weeks after administration of 4 doses of Dilantin, and disappeared 1 day after the tenth dose of this drug. The nitrogen content of this fraction was elevated above normal in animals receiving 4 doses, and decreased to normal in those rats which had been given 10 doses of drug.

Table V shows the results of the analysis of the insoluble components of rat skin at various days after 4 or 10 doses of drug. This table indicates that, with the disappearance of insoluble collagen from the skin of rats which had received 4 doses of drug, there was an increase in insoluble hexosamine, while with the constant concentration of insoluble collagen found in the skin of animals receiving 10 doses, this increase in insoluble hex-

TABLE IV

Analysis of the 0.50 M citrate-soluble components of rat skin after administration of 4 or 10 daily doses of Dilantin

After dose	No. of doses:	Hydroxyproline 4	Hydroxyproline 10	Hexosamine 4	Hexosamine 10	Nitrogen 4	Nitrogen 10
days		*μmoles/g*		*μmoles/g*		*mmoles/g*	
0		21	*19*	*0.50*	*0.43*	*0.91*	*0.98*
1		**130*	*23*	**0.98*	*0.00	**1.10*	**0.79*
3		* *53*	*9	**0.91*	*0.00	**1.10*	**0.63*
6		* *59*	*7	**0.75*	*0.00	**1.20*	*0.46
9		* *50*	*5	**0.68*	*0.00	**1.10*	*0.42
14		**106*	*9	**0.70*	*0.00	**1.80*	*0.53
Normal		8 ± 0.4		0.00		0.48 ± 0.03	

* See Table I.

TABLE V

Analysis of the insoluble components of rat skin after administration of 4 or 10 daily doses of Dilantin *

After dose	No. of doses:	Hydroxyproline 4	Hydroxyproline 10	Hexosamine 4	Hexosamine 10	Nitrogen 4	Nitrogen 10
days		*μmoles/g*		*μmoles/g*		*mmoles/g*	
0		217	*274*	*2.7*	*3.5*	*4.9*	*6.1*
1		**128*	*261*	**7.8*	**6.2*	**5.7*	**4.3*
3		**150*	**236*	*2.6*	3.5	**3.5*	**3.7*
6		**136*	**301*	**4.2*	**7.8*	*3.0	**4.8*
10		**145*	*293*	**4.7*	**5.0*	*3.0	**4.7*
14		* *92*	**302*	**8.6*	**4.5*	*3.0	**4.6*
Normal		187 ± 15		2.1 ± 0.2		2.7 ± 0.2	

* See Table I.

osamine was much less profound. The insoluble dermal nitrogen concentration decreased more markedly after only 4 doses than after 10 doses of drug.

The insoluble noncollagenous protein (scleroprotein) content of rat skin was calculated as described above from the amount of insoluble noncollagenous nitrogen. Table VI describes the changes in scleroprotein with time after 4 or 10 doses of Dilantin, and relates the concentration of insoluble hydroxyproline and hexosamine to the amount of scleroprotein in the tissue. These results show that scleroprotein produced by 4 doses of drug was stable for at least 2 weeks after the final injection. It is interesting to note that on Days 1 and 14 both the scleroprotein content and the citrate-soluble collagen concentration of these tissues were maximally elevated. The ratio of insoluble hexosamine to scleroprotein increased with time after 4 doses of drug. One day after 10 doses, this ratio was lower than at 14 days after 4 doses. Further time after 10 doses did not markedly alter the ratio of insoluble hexosamine to scleroprotein. Essentially little change in the ratio of insoluble hydroxyproline to scleroprotein was recorded with time after 4 doses of drug, while this ratio in animals after 10 doses increased with time to about 50 per cent of normal.

DISCUSSION

In view of the significant decrease in dermal water with Dilantin, the concentration of total solids in the tissue increased from about 450 to 700 mg per g. This increase in solids was numerically greater than the increase in total dermal collagen. Therefore, the collagen concentration, while increasing from 220 to 257, or 310 mg per g wet weight of skin, decreased from 500 to 368, or 440 mg per g of dermal solids, with 0, 4, and 10 daily doses of drug. Despite this proportionate decrease in the concentration of collagen on a basis of solid weights, the collagen content of the

TABLE VI

Scleroprotein and insoluble hexosamine and hydroxyproline in rat skin after the administration of 4 or 10 daily doses of Dilantin *

After dose	No. of doses:	Scleroprotein 4	Scleroprotein 10	Hexosamine/mg scleroprotein 4	Hexosamine/mg scleroprotein 10	Hydroxyproline/mg scleroprotein 4	Hydroxyproline/mg scleroprotein 10
days		*mg/g*		*μmoles*		*μmoles*	
0		*175*	*210*	*0.154*	*0.166*	*1.28*	*1.31*
1		**350*	* *70*	**0.245*	**0.089*	**0.37*	**3.75*
3		**132*	* *43*	*0.196*	**0.082*	*1.14*	**5.50*
6		**106*	* *70*	**0.396*	**0.113*	*1.28*	**4.31*
10		* *96*	* *70*	**0.490*	**0.072*	*1.50*	**4.23*
14		*158*	* *53*	**0.542*	**0.085*	**0.58*	**5.61*
Normal		18		0.118		10.4	

* See Table I.

skin itself was increased in that the whole tissue contained increased amounts of both collagen and other proteins. That this increase was not due merely to tissue dehydration is shown by the fact that the ratios of total hydroxyproline to nitrogen content of the skin were altered significantly—a consequence of more than simple water loss. As a per cent of the weight of the entire fresh, wet, cleaned skin of each rat, collagen increased from 22 ± 1 to 31 ± 2 per cent with 10 daily doses of Dilantin.

The increased total dermal collagen concentration resulting from 4 doses of drug was gone from the skin 3 days after the last dose had been administered. The total collagen concentration produced by 10 doses was still elevated in the skin 2 weeks after drug administration had been concluded. Resorption of Dilantin-produced collagen within 2 weeks was then possible only when small doses of drug had been used. This resorption of total dermal collagen was associated with large increases in citrate-soluble collagen and large losses in insoluble collagen. The elevated total collagen produced with large doses of the drug was not resorbed, and the citrate-soluble collagen content of the skins of these animals decreased toward normal, while the insoluble collagen concentration remained constant for at least 2 weeks after the administration of 10 doses of drug.

Dermal neutral-soluble collagen concentration was decreased similarly in the face of either no change or a loss of insoluble collagen from the skin. Neutral-soluble collagen was also decreased in the face of an increase in insoluble collagen, as described previously (3). Citrate-soluble collagen was increased slightly (from 8 to 20 mg per g) with increasing insoluble collagen concentration (3), remained normal with no change in insoluble collagen, and was increased profoundly (from 8 to over 100 mg per g) with a decrease in insoluble collagen (Table IV). Citrate-soluble collagen therefore seems to be a somewhat more reliable indicator of changes in insoluble dermal collagen concentration than is neutral-soluble collagen.

If resorption of dermal collagen involves a conversion of insoluble collagen *into* the acid-soluble form, it appears unlikely that such a reaction involves collagenolysis via peptide-bond hydrolysis, since the products of this proteolysis would most probably be found in the isotonic or even the 0.5 M NaCl extracts. Since no increase in the hydroxyproline concentration of these fractions was found, collagen resorption could occur either by a physicochemical solubilization of insoluble collagen fibers, as suggested by Grant and Alburn (11), or by enzymatic hydrolysis of collagen during which the products of collagen proteolysis were immediately disposed of via the lymphatic system and the circulation. This latter possibility, although not supported by serum hydroxyproline findings (there were no significant elevations in the serum hydroxyproline concentration after Dilantin administration), has been suggested to explain the specificity of the loss in dermal collagen with local inflammation (6). This persistently normal serum hydroxyproline concentration in the face of major losses of collagen also makes it difficult to explain what happened to the physicochemically solubilized collagen in the citrate extracts, since this material must also leave the skin.

Associated with both collagen resorption and Dilantin-induced synthesis of collagen was the appearance of unique acid-soluble hexosamine-containing component and relatively constant and large amounts of scleroprotein. Associated with stable drug-induced collagen was the disappearance of acid-soluble hexosamine and a marked loss in scleroprotein. That the scleroprotein was not a glycoprotein is suggested by the variation in the ratio of insoluble hexosamine to scleroprotein. Presumably the scleroprotein is related to that previously described (3) as produced during Dilantin administration. The insoluble hexosamine may well be a mucopolysaccharide associated intimately with insoluble collagen or scleroprotein (3, 4).

It appears that collagen resorption produced large amounts of scleroprotein and acid-soluble hexosamine, since both of these materials were largely gone when no losses in insoluble Dilantin-produced collagen were observed. Both acid-soluble hexosamine and scleroprotein were apparently involved in either the synthesis or the degradation of the insoluble dermal collagen produced by Dilantin administration to rats.

Finally, the amount of ground substance (as estimated by the ratio of the isotonic saline-soluble hexosamine to nitrogen) was apparently decreased

with collagen resorption while remaining normal when no decreases in dermal collagen were found, as shown in Table II.

SUMMARY AND CONCLUSIONS

The dermal collagen produced by 4 doses of Dilantin was resorbed within a week after the conclusion of drug administration. After 10 doses, however, dermal collagen was stable for at least 2 weeks. Collagen resorption was associated with a profound decrease in insoluble collagen and ground-substance concentrations and a marked increase in the citrate-soluble collagen hexosamine content of the skin. The increased scleroprotein content of rat skin during administration of the drug was essentially maintained for at least 2 weeks despite the loss in insoluble collagen that occurred during this period after 4 doses of drug. After 10 doses, the scleroprotein content of rat skin decreased within 3 days to about one-fifth of that found during drug administration, despite the continued high levels of insoluble dermal collagen. The ground-substance and citrate-soluble hexosamine and collagen content of skin from animals which demonstrated no loss in Dilantin-produced (10 doses) collagen returned to normal shortly after termination of drug administration.

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REFERENCES

1. Ziskin, D. E., Stowe, L. R., and Zegarelli, E. V. Dilantin hyperplastic gingivitis: Its cause and treatment; differential appraisal. Amer. J. Orthodont. 1941, **27**, 350.
2. Shafer, W. G., Beatty, R. E., and Davis, W. B. Effect of Dilantin sodium on tensile strength of healing wounds. Proc. Soc. exp. Biol. (N. Y.) 1958, **98**, 348.
3. Houck, J. C., Jacob, R. A., and Maengwyn-Davies, G. D. The effect of sodium Dilantin administration upon the chemistry of the skin. J. clin. Invest. 1960, **39**, 1758.
4. Houck, J. C., and Jacob, R. A. Chemical dissection of rat skin. Proc. Soc. exp. Biol. (N. Y.) 1960, **105**, 324.
5. Jackson, D. S. Some biochemical aspects of fibrogenesis and wound healing. New Engl. J. Med. 1958, **259**, 814.
6. Houck, J. C., and Jacob, R. A. The chemistry of local dermal inflammation. J. invest. Derm. 1961, **36**, 451.
7. Blix, G. The determination of hexosamines according to Elson and Morgan. Acta chem. scand. 1948, **2**, 467.
8. Martin, C. J., and Axelrod, A. E. A modified method for determination of hydroxyproline. Proc. Soc. exp. Biol. (N. Y.) 1953, **83**, 461.
9. Boas, N. F. Distribution of hexosamine in electrophoretically separated extracts of rat connective tissue. Arch. Biochem. 1955, **57**, 367.
10. Neuman, R. E., and Logan, M. A. The determination of collagen and elastin in tissues. J. biol. Chem. 1950, **186**, 549.
11. Grant, N. H., and Alburn, H. E. Collagen solubilization by mammalian proteinases. Arch. Biochem. 1960, **89**, 262.