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Non-Protein Nitrogen Changes in Serum and Plasma of Rats Following Thermal Injury.* (20275)

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Disturbances in nitrogen metabolism are commonly observed after severe burns. Azotemia is one of the principal features of the metabolic changes. In general, the distribution of the elevated blood non-protein nitrogen is normal, but, occasionally, in patients(1,2) and rats(3,4) with very extensive deep burns, a large part (as much as 80%) of the azotemia is due to abnormal amounts of "residual" nitrogen whose nature has not been determined. Duval *et al.*(5) and Lambret *et al.*(6) postulated that severe burns result in a polypeptidemia. They based their conclusions on an observed increase of that amino nitrogen which is soluble in trichloracetic acid, but precipitated by phosphotungstic acid. Christensen(7) has presented evidence which casts doubt on the reliability of such determinations and interpretations.

In the present investigation, ultrafiltrates of plasma and serum from normal and thermally injured rats were utilized in a study of the non-protein nitrogen changes. In addition to the routine chemical measurements, absorption spectra in the ultraviolet were obtained, and changes of individual amino compounds were followed by ion exchange chromatography.

Methods. A. *Thermal technic.* Male albino rats of the Wistar strain, weighing between 225 and 275 g were anesthetized with ether and thermally injured deeply over about 30% of the body surface by dipping their backs in hot water (90°C) for 35 seconds(8). Rosenthal and McCarthy(4) have found that the increase in plasma residual nitrogen in similarly injured rats is at a maximum 10 to 12 hours after trauma. Accordingly, in the present study 11 to 12 hours after injury, the surviving rats (80% of those injured) were sacrificed, and their blood collected by heart punctures. Control rats, anesthetized but not burned, were studied simultaneously. Purina chow and water were offered *ad libitum* to all rats. B. *Chemical and spectrophotometric measurements.* Bloods from 16 injured rats

* The experimental work was done at the Medical College of Virginia.

and from 10 control rats were separately pooled and allowed to clot in vessels immersed in ice water. The bloods were then spun in a refrigerated centrifuge, and the sera filtered at 3°C through "Visking" sausage casing under 40 lb per square inch of nitrogen in an ultrafiltration apparatus made of Monel. All subsequent manipulations were performed within 24 hours. Serum ultrafiltrates were used since anticoagulants and protein precipitants generally absorb light in the region 220-290 mμ, seriously interfering with spectrophotometric measurements carried out in this wavelength range. The ultrafiltrates were analyzed for total filterable nitrogen, urea nitrogen, and amino nitrogen before and after acid hydrolysis with 6 N HCl. The hydrolyses were carried out in sealed tubes heated at 110° for 12 hours. The analyses were performed by the methods of Sobel *et al.*(9,10). Since many naturally occurring nitrogenous substances show characteristic spectral changes when irradiated with ultraviolet light, absorption spectra of the ultrafiltrates of the sera from normal and injured rats were obtained before and after ultraviolet irradiation in sealed silica cells. An Argon-Mercury lamp, 80% of whose output was at 254 mμ, was used. The absorption spectra of the ultrafiltrates were obtained with a Beckman DU spectrophotometer. C. *Chromatographic measurements.* The bloods from 20 injured rats and 20 control rats were separately pooled in vessels immersed in ice water. Sodium citrate (2.5%) in a 1:5 dilution with the blood was used as an anticoagulant. The bloods were centrifuged within a few minutes after collections. The plasma was separated from the cells and immediately frozen in 5 ml vials. For analysis, the frozen plasma was thawed at 3°C, and ultrafiltration immediately carried out. Changes in individual amino compounds were followed by an ion exchange chromatographic technic employing Dowex-50 and a colorimetric ninhydrin method, both developed by Stein and Moore(11,12). All hydrolyses were carried out in sealed vials with 6 N HCl at 110°C for 20 hours. The resultant hydrolysates were evaporated by heating under a stream of air. The dry residue was then made up to about 3 ml with H_2O, and the pH adjusted to approximately 2.5 with 2 N NaOH.

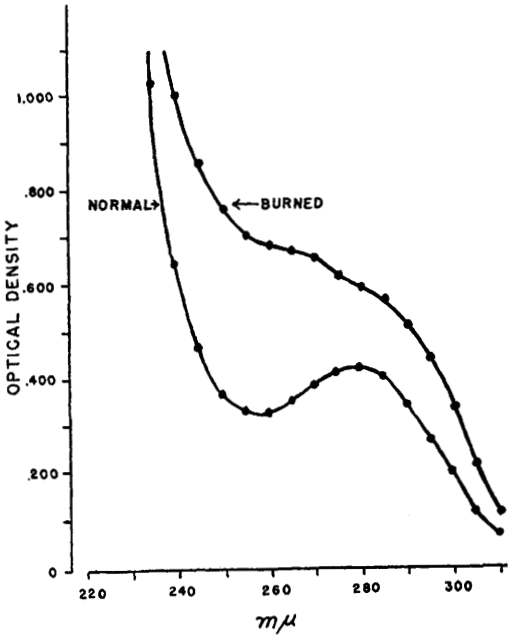

FIG. 1. Absorption spectra of sera ultrafiltrates of normal and thermally injured rats.

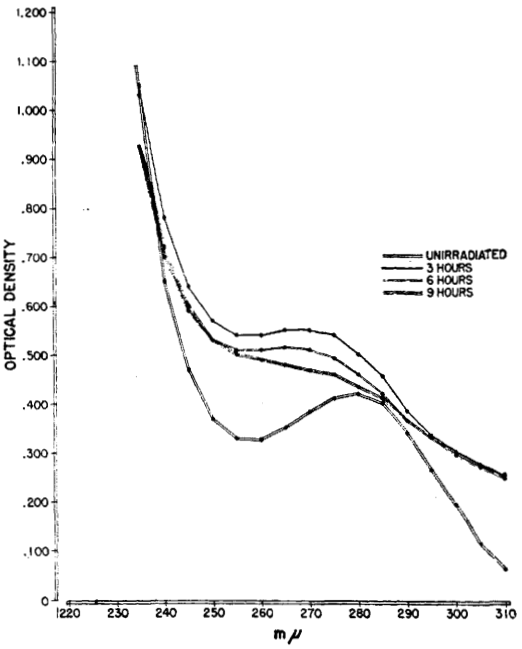

FIG. 2. Effect of ultraviolet irradiation on absorption spectrum of serum ultrafiltrate of normal rats.

Results. I. *Spectrophotometric measure-*

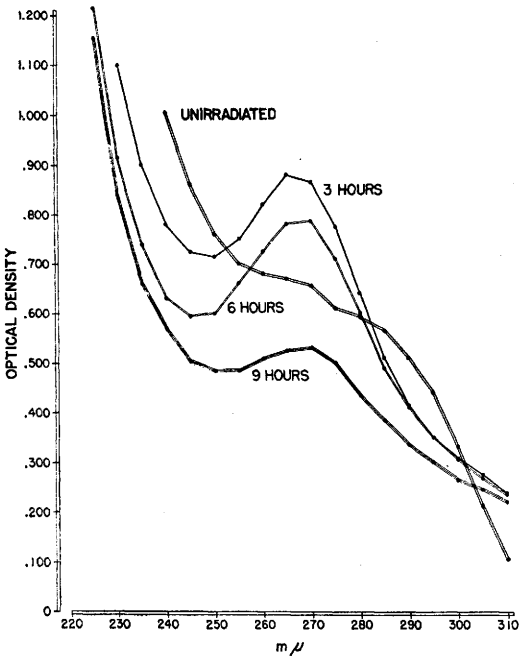

FIG. 3. Effect of ultraviolet irradiation on absorption spectrum of serum ultrafiltrate of thermally injured rats.

ments. The absorption spectra of the sera ultrafiltrates are shown in Fig. 1. The ultrafiltrate of the sera from burned rats shows a markedly altered spectrum. There is an increase in absorption over the entire range of wavelength, but especially in the region of the normal minimum at 255 mμ. The changes in the spectrum of the filtrate of normal rat sera after 3, 6, and 9 hours of irradiation are shown in Fig. 2. There is an initial rise of the entire spectrum, particularly in the region of minimum absorption (255 mμ). With further irradiation, there occurs a slight drop in the spectral pattern. When the serum ultrafiltrate of injured rats is irradiated, there is initially a decrease in the region 240-250 mμ, and a marked increase in the region 255-275 mμ (Fig. 3). With further irradiation, there is a uniform fall, so that after 9 hours of irradiation, the entire spectral pattern is lower than that of the unirradiated sera ultrafiltrate. II. *Chemical measurements.* The results of the chemical measurements are shown in Table I. There are concomitant rises in concentrations of plasma urea, and free and combined amino nitrogen. III. *Chromatographic measurements.* A chromatogram of normal pooled rat plasma ultrafiltrate is shown in Fig. 4. The separations are, in general, clear cut. The following features of the chromatographic patterns should be noted: 1) The first component, or forepeak stable to acid hydrolysis, was found in all plasma ultrafiltrate specimens. It has been tentatively identified as taurine. 2) The second and sixth peaks (Fig. 4) are unstable to acid hydrolysis. The first of these peaks is an amino conjugate. Its composition will be discussed later. The sixth peak was studied by isolating it from single runs of ultrafiltrate, hydrolyzing, and rechromatographing (Fig. 5). It is composed of asparagine, serine, and glutamine. 3) There was no measurable cystine in any specimen. 4) In some runs, the histidine peak showed resolution into two components. It is probable, therefore, that the "histidine" figures are high, since they may include another component. 5) Tryptophan is destroyed by the acid buffers employed. 6) The basic substance immediately preceding lysine was found in all samples. It does not yield the typical amino-ninhydrin purple color, but rather a blue-green one. Its identity has not been established. 7) Arginine could not be separated from other basic, ninhydrin reactive material. Chromatograms were therefore stopped after lysine.

Table II shows the similarity of the results of the chromatographic separations and colorimetric ninhydrin determinations of some amino acids of normal rat plasma as compared with measurements made by a microbiological method(14). The results obtained by

TABLE I. Effect of Severe Thermal Injury on Nitrogen Partition of Rat Serum Ultrafiltrate. (mg N/100 ml of serum ultrafiltrate.)

	Total filterable N	Urea N	Amino N	Amino N after hydrolysis	Combined amino N	Combined amino N as % of total filterable N
Normal	28.3	14.0	6.3	11.8	5.5	19
Burned	130	76.0	18.9	47.3	28.4	22

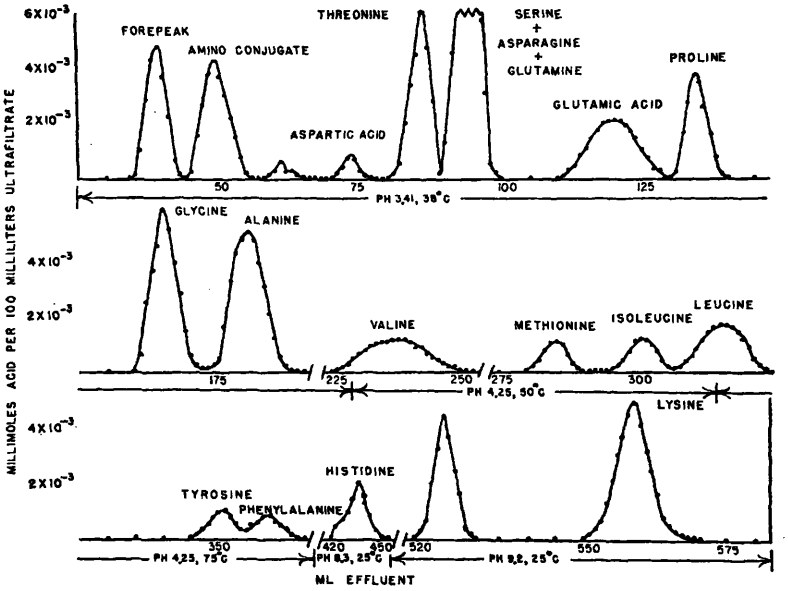

FIG. 4. Chromatographic pattern of normal rat plasma ultrafiltrate.

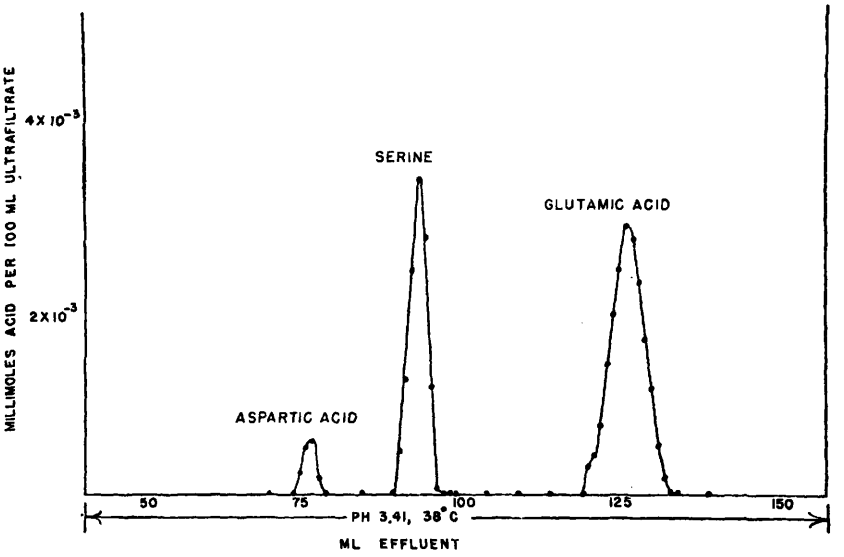

FIG. 5. Pattern of hydrolyzed "serine + glutamine + asparagine" peak isolated from normal rat plasma ultrafiltrate.

chromatographing plasma ultrafiltrates of injured rats are also listed in Table II. In general, the amino acid concentrations are elevated in the injured animals. The most striking effects are the increases of the forepeak and the amino acid conjugate. These two components account for 80% of the increase of amino nitrogen in rat plasma after burning.

About 20 ml of normal plasma were used for isolation of the amino conjugate. Hydrolysis of this fraction and rechromatographing yielded the pattern shown in Fig. 6. The predominant components are an acid stable fraction (as yet unidentified), glycine, and glutamic acid, and lesser amounts of alanine, proline, serine, threonine, valine, and leucine. The distribution of amino acids of the con-

TABLE II. Concentration of Amino Compounds in Normal and Thermally Injured Rat Plasma Ultrafiltrates. (mM amino acid/100 ml plasma ultrafiltrate.)

	× 10^{-3}			
	Normal			
	Chromatographic method	Microbiological method, Henderson *et al.*(14)*	Burned	% change
Forepeak (Taurine?)	39.0		149.8	+284
Amino conjugate	40.0†		127.6	+219
Aspartic acid	4.5		4.3	— 4
Threonine	37.7	37.6	31.7	— 16
Serine	21.8		25.9	+ 18
Glutamine	27.5		28.3	+ 4
Asparagine	3.7		5.2	+ 40
Glutamic acid	22.1		18.9	— 14
Proline	28.5	37.1	17.7	— 40
Glycine	52.7		45.4	— 15
Alanine	57.1		64.5	+ 12
Valine	24.9	22.8	20.1	— 20
Methionine	6.7	6.4	6.3	— 6
Isoleucine	10.3	10.0	15.1	+ 50
Leucine	17.3	20.3	25.6	+ 48
Tyrosine	8.7	12.3	17.6	+113
Phenylalanine	8.4	8.3	18.6	+125
Histidine	13.0‡	6.3	37.0	+185
Lysine	37.2	39.7	38.0	+ 2
Totals	461.1		697.6	

* These data appear as μg amino acid/ml plasma in Henderson *et al.*(14).
† Figures include *only* free amino nitrogen, calculated as leucine.
‡ Results probably high. See text.

jugate isolated from injured rats' plasma is similar to the normal. It is possible that the isolation and subsequent analysis of much larger amounts of the conjugate would reveal small amounts of other amino acids.

Discussion. The absorption spectrum of the ultrafiltrate of the sera from normal rats is typical of a mixture of amino acids, free or combined. The rise between 265-290 mμ is probably due in large part to amino acids containing the benzene nucleus(13). The absorption peaks of phenylalanine, tyrosine, and tryptophane are 260 mμ, 280 mμ, and 280 mμ, respectively. The ultrafiltrate of the sera from injured rats shows a markedly altered spectrum. There is an increase in absorption over the entire range of wavelength, but especially in the region of the original minimum at 255 mμ. This is consistent with a change, qualitative and/or quantitative in the sera amino acids free and combined, and with the appearance of purine containing compounds. At a pH of about 7 the absorption peaks of guanine, adenine, and xanthine are about 260 mμ while the main peak of uric acid is 293 mμ.

The changes observed in the spectral patterns after ultraviolet irradiations of the sera ultrafiltrates of the normal and injured rats are also compatible with the idea that there are changes in the purines and amino acids after injury. It has been shown that when proteins are irradiated with ultraviolet light the extinction coefficient increases over the whole range of wavelengths in the ultraviolet region, but especially in the region of the original minimum, around 250 mμ(15). McLean and Giese(15) have shown these effects can be attributed to modifications of the aromatic amino acids. When purines are irradiated with ultraviolet light, there is a progressive diminution of the absorptive patterns(16).

Proportionate concentration changes of the purines and aromatic amino acids cannot be inferred from the absorption curves (Fig. 1) since the extinction coefficients of these compounds are different, the purines, in general, having the higher absorption. Green and his associates(17) have found that various forms of trauma, including thermal injury, are accompanied by an increase of nucleotide metabolites in the plasma. This rise is quantita-

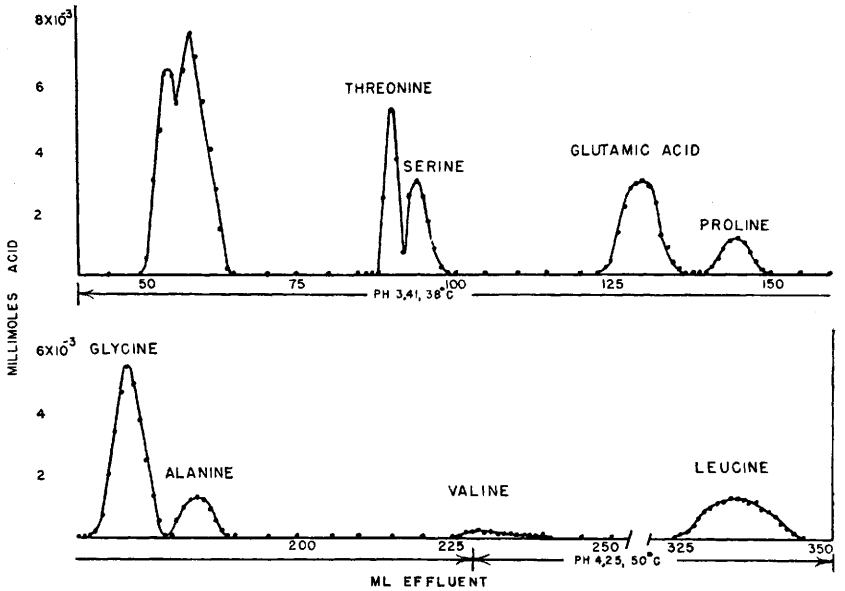

FIG. 6. Pattern of hydrolyzed amino conjugate isolated from normal rat plasma ultrafiltrate.

tively small in relation to that of amino nitrogen compounds. We have observed that almost the entire increase of filterable nitrogen in the sera of rats after burning consists of urea and free and combined amino nitrogen (Table I) in their normal relationships. As pointed out by Christensen(7) part of the increase in combined amino nitrogen might be due to heightened proteolytic activity and subsequent formation of peptides from the serum clot. An attempt was made to minimize this action by keeping the blood cold at all times, and centrifuging in the cold immediately after the blood was collected. Further, the value for hydrolyzable nitrogen (28.4 mg %) in the ultrafiltrate of serum from injured rats is almost identical with the "residual" nitrogen (30 mg %) found in plasma of rats given an identical thermal injury (Rosenthal and McCarthy(4)). This correspondence between plasma and serum values indicates that enzymatic formation of peptides from fibrin *in vitro* is not a significant factor in explaining the findings.

There is a close correspondence between our results for the amino acids of plasma ultrafiltrates of normal rats and those obtained by Henderson *et al.*(14) for rat plasma amino acids using microbiologic technics (Table II). Identical results are not to be expected, since a number of conditions were different in the two studies: 1) The microbiological measurements can be expected to include some peptide nitrogen. This would tend to raise the results for some acids, as compared with the chromatographic method, which, in this respect, is more specific. 2) The method by which protein is separated from the plasma is of fundamental importance in these determinations. Henderson *et al.*, who used the tungstic acid precipitation method, found that for given samples of plasma, the amino acid content increased, on the average, about 200% after acid hydrolysis. Our plasma ultrafiltrates yielded only an additional 50% of amino nitrogen after hydrolysis. It seems clear that our filtrates contained less combined amino nitrogen. 3) Our figures are reported as concentration of amino acids per unit of ultrafiltrate, while the above workers reported their results as concentration per unit of plasma. Our figures in this respect, then, will be higher than theirs.

Asparagine, isoleucine, leucine, histidine, tyrosine, and phenylalanine are increased in the sera ultrafiltrate of injured rats. The increases in the two aromatic amino acids are consistent with the spectrophotometric ob-

servations. Quantitatively, the largest part of the increase in amino nitrogen is made up of the forepeak (taurine?) and the amino acid conjugate. Some of the amino acids, for example proline and valine, are decreased in the injured animals.

The composition of the amino acid conjugate is striking, since it includes only a few of the common acids. Also, there is a large residual fraction unaffected by the hydrolysis procedure. The distribution of amino acids of the conjugate is similar in normal and injured rats. A peptide was isolated from a number of tissues of various animals, including the rat, by Borsook *et al.*(18). This peptide contains most of the common amino acids. It seems likely that the peptide fraction is a heterogeneous one, including large and small molecules. There is probably no sharp dividing line between "protein" and "non-protein" nitrogen in rat plasma. The efficiency with which the plasma is "deproteinized" is of fundamental importance in the interpretation of the results of "peptide" analyses. The heat coagulation procedure used by Borsook and his associates might account for the relatively large "peptide" residuum which they report.

Summary. 1. Ultrafiltrates of plasma and serum from normal and thermally injured rats were utilized in a study of non-protein nitrogen changes following thermal injury. The rats were burned deeply over about 30% of the body surface and the bloods were collected 11-12 hours after injury. 2. Absorption spectra were obtained before and after *in vitro* ultraviolet irradiation of the sera ultrafiltrates. The changes observed in the spectrum of the sera ultrafiltrate of the injured rats is consistent with a modification qualitative and/or quantitative in the sera amino acids, free and combined, and with the appearance of purine containing compounds. 3. Almost the entire increase in filterable nitrogen in the sera of rats after thermal injury consists of urea and free and combined amino nitrogen in their normal relationships. 4. Measurements of specific amino compounds were carried out by ion exchange chromatographic and colorimetric ninhydrin technics. The results obtained by these methods for the amino acids of plasma ultrafiltrates of normal rats are similar to values obtained by others for rat plasma amino acids using microbiologic technics. 5. In the plasma ultrafiltrate of injured rats, there are increases of some amino acids, particularly tyrosine, phenylalanine, and histidine, while other amino acids are decreased. Quantitatively, the largest part of the increase in amino nitrogen is made up of a forepeak (taurine?) and an amino acid conjugate. 6. The amino acid conjugate includes a large fraction unaffected by hydrolysis and a few of the common amino acids. The composition of the conjugate is similar in normal and injured rats.

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