PHARMACOLOGICALLY ACTIVE SUBSTANCES IN THE URINE OF BURNED PATIENTS

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KININ is found in normal human urine and the daily output is remarkably constant (Horton, 1959). Its presence in the urine may represent the renal clearance of kinin from the plasma or it may be formed in the kidney as a mediator of local vasodilatation. Evidence has accumulated which suggests that in disease considerable quantities of kinin are released from the tissues and plasma and that the amount excreted in the urine is increased. Goodwin and Richards (1960) showed that the urine of mice infected with protozoal and other infective organisms contains substances which have the pharmacological and biochemical properties of peptides of the kinin class. Tella and Maegraith (1962) found in monkey malaria that as the parasitaemia increased the urinary kinin increased and the amount of kinin-precursor in the α-globulin fraction of the serum decreased. Brocklehurst and Lahiri (1963) have found kinin in the serum of animals in anaphylactic shock.

When body protein is destroyed, short-chain peptides may be formed; if they escape degradation by the plasma and tissue enzymes they may pass through the kidney and appear in the urine.

In thermal injury tissue also undergoes rapid destruction and the onset is clear-cut; we have therefore studied the urine of patients with severe burns. The "toxaemia" of burns was recently reviewed (*Lancet*, 1960). The term toxaemia in this connexion is a convenient cloak for ignorance; we still do not know the causes of "toxaemia" and often do not know the exact cause of death of burned patients. However, there is evidence that pharmacologically active substances are liberated from burned tissues and that these substances may influence the clinical picture.

Cullumbine and Rydon (1946) showed that peptides with leucotaxic properties are released from burned skin; Keyser (1952) showed that the serum glycoproteins were raised in burned patients, and Baar (1956) found a peptide fraction in the urine which contained lactose, galactose and fucose, and was probably derived from glycoprotein. Balikov, Castello and Lozano (1957) followed the excretion of peptides in the urine of burned patients and demonstrated an outpouring of ninhydrin-positive substances which decreased in amount when the burned areas healed. Godfraind (1958) has shown that toxic peptides are formed in experimentally burned animals and Simonart (1958) has reported a variety of toxic substances in burn oedema fluid.

Fox and Hilton (1958) found that heat-vasodilatation in human skin was accompanied by the release of kinins. Spector and Willoughby (1959, 1960)

concluded from studies on experimental burns in rats that increased capillary permeability was initiated by release of histamine and maintained by other endogenous mechanisms such as activation of permeability-increasing globulins or peptides. More recently, Rosenthal, Hunter, Finamore and Roman (1960) and Roche e Silva and Rosenthal (1961) demonstrated the presence of kinin, adenosine derivatives and histamine in perfusates of subcutaneous air-pockets in rats when the overlying skin was burned, and Edery and Lewis (1962) have shown that lymph collected from the scalded hind-limb of the dog contains histamine and plasma kinin-forming-enzyme.

In the present study we have endeavoured to separate and identify some of the pharmacologically active substances in the urine of patients with severe burns.

MATERIALS AND METHODS

Urine from 25 patients was collected and examined. The patients were admitted to the Burns Unit of Queen Mary's (Roehampton) Hospital between March 1959 and August 1961. Skin areas affected by burns ranged from 6–90 per cent of the body-surface. Urine was collected in siliconized glass or polythene vessels containing N HCl as a preservative. When serial 24 hr. specimens were collected from the same patient, they were adjusted to standard volume (1500 ml.) with water so that concentration differences between samples did not give rise to error. Many of the samples were assayed for potassium, sodium, calcium and urea. In some experiments, catheter specimens were collected in siliconized vessels containing soya-bean trypsin inhibitor or alcohol, and were frozen immediately to minimize the enzymic production of kinin. Separated urine fractions were frozen and stored at $-15°$.

Blood was collected from several patients and the serum separated in siliconized or plastic apparatus immediately after admission to hospital.

Pharmacological methods.—Urine and fractions prepared from it were tested for activity on guinea-pig ileum in Tyrode solution at 37°, rat duodenum and rat uterus in de Jalon at 32° and hen rectal caecum in Tyrode with low potassium at 37°. Atropine (10^{-6}) was used in most experiments to depress spontaneous movements. Perfusions of rat hind-limbs were made and the outflow was recorded by Stephenson's (1948) method. Some of the extracts were injected intravenously into cats, rats and rabbits anaesthetized with chloralose or pentobarbitone sodium, and chickens anaesthetized with phenobarbitone sodium; the blood pressure was recorded. Frog hearts were prepared by the Straub method.

Active fractions were tested in the presence of mepyramine, triprolidine or chlorcyclizine, atropine, eserine, iproniazid and lysergic acid diethylamide, usually in concentrations of 10^{-4} in the isolated organ bath.

Fractions were tested after incubation with trypsin, pepsin, papain, chymotrypsin, peptidase or histaminase. Appropriate controls were set up in every experiment, with normal urine fractions and with the enzymes and reagents alone.

Electrophoresis.—Cellulose acetate membrane filter strips and sheets (Kohn, 1958) were found to be the most satisfactory supporting medium. Electrophoresis was carried out at 200 V. 0·4 mA./cm. width for 30 min. on a 12 cm. long strip, using a veronal buffer (pH 8·6, 0·06 M). The sample was applied approximately in the centre of the strip.

Best results were obtained with alcoholic extracts of urine. Urine was evaporated to dryness, the solid residue extracted with alcohol and the alcoholic extract evaporated again to dryness and reconstituted with distilled water to 1/10 of the original volume; 10–20 μl. of this concentrate was applied. A parallel section of the strip was cut off and stained with ninhydrin, the unstained segments of the strip corresponding to the stained bands were cut out and eluted with de Jalon solution. The eluate was then tested for biological activity. The unstained strips were also viewed under U.V. light as it was found that the highest activity was close to one of the fluorescent bands on its cathode side.

Preparation of urine fractions.—The urine was adjusted to pH 6·0 and passed through a column of Amberlite CG50, type I ion-exchange resin (10 ml. wet resin per 100 ml. urine). The resin had been prepared by the method of Hirs, Moore and Stein (1953) and equilibrated at pH 6·0 with phosphate buffer.

The column was washed with a volume of water equivalent to the volume of the urine. The total effluent was evaporated to dryness *in vacuo* at 60–90°, the residue extracted with boiling ethanol and the extract filtered. The filtrate was evaporated to dryness. The ethanol-insoluble residue was designated Fraction A and the soluble residue Fraction B. The Amberlite column was then treated with a volume of N HCl equal to that of the urine, the effluent collected, evaporated and extracted with ethanol as before. The ethanol-soluble residue was Fraction C. For pharmacological tests, the dried extracts were dissolved in the appropriate Ringer solution.

In later experiments extraction with ethanol was carried out before column chromatography. The ethanol-soluble material (Fractions B and C) was dissolved in water and passed through a column of resin in the H^+ form. This made it possible to use more concentrated solutions and 25 ml. of wet resin was sufficient for the extract from 1500 ml. of urine. Fraction C was found by paper chromatography to contain several ninhydrin positive substances. This fraction was further purified by first eluting the column with N acetic acid to give Fraction C_1, followed by N HCl to give Fraction C_2.

Paper chromatography of Fractions C_1 and C_2.—All papers were washed overnight in running tap water to remove pharmacologically active contaminants. Fraction C_1 was chromatographed using *n*-butanol/pyridine/water (1 : 1 : 1). A corresponding fraction from normal urine was used for comparison. Chromatograms were also made from Fraction C_1 prepared from urine to which histamine had been added. The papers were treated with ninhydrin.

Fraction C_2 was chromatographed in two directions using butanol/acetic acid/water (60 : 15 : 25) and butanol/pyridine/water (1 : 1 : 1) as solvents. It contained at least 2 substances which, like bradykinin, gave positive reactions when exposed to chlorine and treated with potassium iodide and *o*-tolidine (Reindel and Hoppe, 1954). One of these (C_3) had the same R_f values as synthetic bradykinin.

Fraction C_3 was hydrolysed by heating for periods from 14 hr. to 4 days in a sealed tube at 110° with 8 N HCl. The hydrolysate was chromatographed with various solvents, standard amino-acids being used for comparison.

RESULTS

The urine of patients with severe burns contained substances which relaxed the isolated rat duodenum. Fig. 1 shows the effects of samples from five patients and from normal individuals of similar age and sex. Normal urine usually caused a small relaxation followed by a contraction of the gut ; burn urine caused a profound relaxation. Urine from severely burned patients was usually more active in this respect than that from patients with less extensive tissue destruction. As the lesions healed, the effect of 24-hr. specimens of urine became progressively less. The effect was independent of differences in urea or electrolyte concentration in the samples of urine.

The relaxing substances were present in specimens obtained by catheter and collected in siliconized tubes containing soya-bean trypsin-inhibitor, acid or alcohol and frozen without delay ; they were therefore unlikely all to have been artefacts produced by the action of enzymes on constituents of the urine after it had left the bladder. When 2·5 per cent of normal serum was incubated with either normal or burn urine, an increase in the activity of the extract was observed, but the increase was very small compared with the original activity of urine collected from the burned patient.

Serum collected with precautions against the production of artefacts also caused relaxation of the rat duodenum. The serum from one patient who had a burn affecting 80 per cent of the body surface caused a profound relaxation at a concentration of 1 : 1000 in the bath. Normal serum collected and tested in the same manner had only a slight effect at a concentration of 1 : 100. Blister fluid

from this burned patient also contained a large amount of duodenum-relaxing activity. Blister fluid caused a profound fall in blood pressure when injected intravenously into anaesthetized cats.

Electrophoretic strips of normal and burn urine showed several bands which fluoresced in U.V. light ; these bands showed no obvious connexion with pharmacological activity. When stained with ninhydrin, electrophoretic strips of normal urine showed several positive bands. With burn urine the bands stained more deeply and were more numerous. The duodenum-relaxing material in burn-urine moved from the line of application towards the cathode. The activity was most

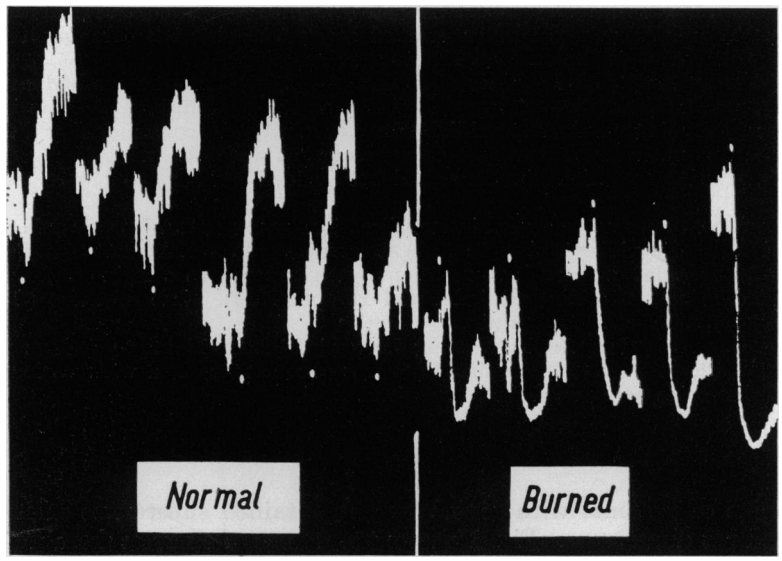


FIG. 1.—Effect upon the isolated rat duodenum of urine from normal and burned people. Normal urine causes a contraction ; burn urine a relaxation.

readily demonstrated when alcoholic extracts of the urine were used for electrophoresis. The main ninhydrin-positive band was inactive pharmacologically. The sample of synthetic bradykinin used in these experiments produced a ninhydrin positive spot on the electrophoretic strip. The area corresponding with this spot showed duodenum-relaxing activity. The active material in burn urine moved at the same rate as synthetic bradykinin (Fig. 2).

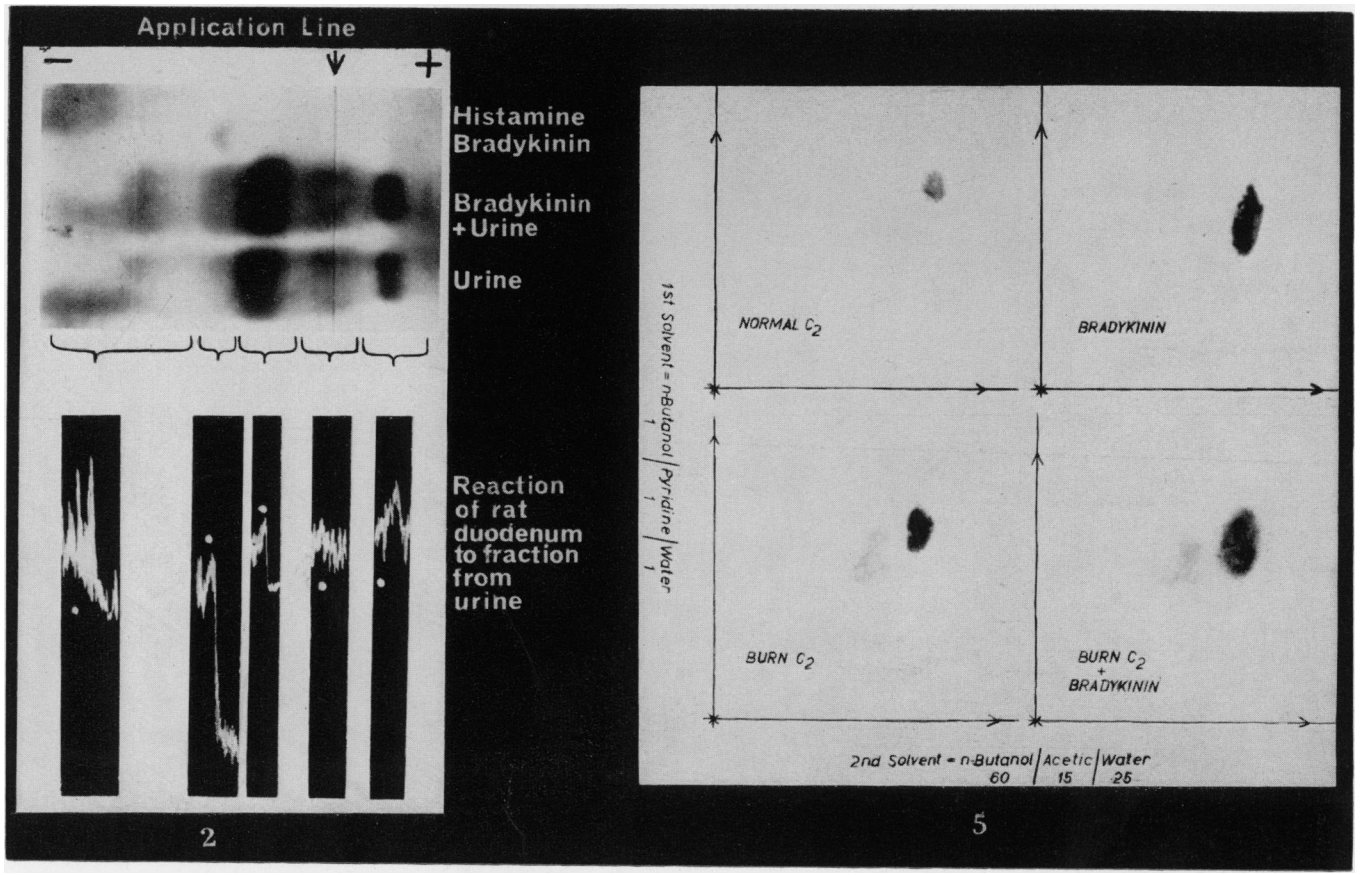
EXPLANATION OF PLATE

FIG. 2.—The effect on the rat duodenum of fractions separated from an alcoholic extract of burn urine by electrophoresis on cellulose acetate. The relaxing substance moves towards the cathode and appears in the same region as bradykinin.

FIG. 5.—Two-dimensional paper chromatograms showing that an extract of burn urine yields a spot with the same R_f values as synthetic bradykinin. Normal urine contains a small amount of material which stains with the Reindel and Hoppe reagent. Burn urine extract contains more than one substance.

R_f values for bradykinin by this method :

1st solvent	0·40
2nd solvent	0·48



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Tests made upon fractions separated by column chromatography showed that Fractions A and B (see Methods) from the urine of normal and burned individuals were similar in activity. Fraction A caused relaxation of the rat duodenum ; the response was modified but not abolished by the action of proteolytic enzymes. Fraction A was probably a mixture of substances, some of which may have been proteins or peptides. Fraction B (ethanol-soluble) caused a relaxation of the rat duodenum which was unaffected by treatment of the fraction with proteolytic enzymes. It has not yet been studied fully.

Fraction C_1, eluted from the column with acetic acid, caused contraction of the rat duodenum and guinea-pig ileum and had properties similar to those of histamine. The R_f value using butanol/pyridine/water was equal to that of histamine ; it appeared in the same fraction when histamine was added to normal urine before extraction ; it was destroyed by histaminase and antagonised by antihistamine drugs. Table I shows the results of parallel assays on a selection of pharmacological preparations ; agreement is sufficiently close to indicate that C_1 was probably histamine. The amount of histamine activity in extracts of the urine of burned patients (assayed on the guinea-pig ileum) was 0·5 to 25 μg./ml. Normal human urine contained 0·05 to 0·15 μg./ml. when extracted and assayed by the same methods. In the course of preparation of Fraction C_1 all conjugated histamine is hydrolysed to the free form. Further hydrolysis of the fraction by refluxing for 1½ hr. with hydrochloric acid did not increase histamine activity.

TABLE I.—*Histamine-activity of Fraction C_1 from Burn and Normal Urine*

	μg. Histamine equivalent to extract of 1 ml. of urine		
		Burn urine	
Test organ	Normal urine	(1)	(2)
Guinea-pig ileum.	0·05	0·4	25
Hen rectal caecum	0·08	0·4	20
Rat hind-legs	0·15	0·5	8
Blood pressure, Cat	<0·2	1·0	15
Blood pressure, Hen	—	1·0	20

The normal urine was a pooled 24-hr. specimen from 7 normal adults ; burn urine (1) was pooled from 7 patients with extensive burns ; burn urine (2) was a 24-hr. specimen from a patient with a 30 per cent burn, who died within a few days.

Fraction C_2, eluted from the column with hydrochloric acid, had the properties of a kinin. It caused relaxation of the rat duodenum and contraction of the rat uterus and the guinea-pig ileum. Its effects were unchanged by atropine, antihistamine drugs, eserine, iproniazid or lysergic acid diethylamide. C_2 extracted from burn urine had an activity 5 to 40 times as high as a comparable fraction from normal urine. When assayed against synthetic bradykinin on rat duodenum, rat uterus and guinea-pig ileum it was about 2·5 times as active on the rat uterus as upon the other two tissues (Table II). If the activity of Fraction C_2 is due to its bradykinin content, interfering substances must also be present. Fraction C_2 arrested the isolated frog heart, dilated the vessels of the rat hind-limbs and

TABLE II.—*Parallel Assays of Fraction C_2 from the Urine of Patients with Severe Burns*

Test organ	μg. of synthetic bradykinin equivalent to extract of 1 ml. original urine	
	Sample 1	Sample 2
Guinea-pig ileum . .	200	160
Rat duodenum . .	200	200
Rat uterus . . .	540	500

The samples were prepared from the pooled urine from two groups of 4 patients.

caused a fall of arterial pressure in the anaesthetized cat, rat and rabbit. Fig. 3 shows the hypotension which followed intravenous injection of C_2 from burn urine in a cat which had been treated with chlorcyclizine to abolish histamine responses. Fraction C_2 was unaffected by incubation with trypsin or pepsin but

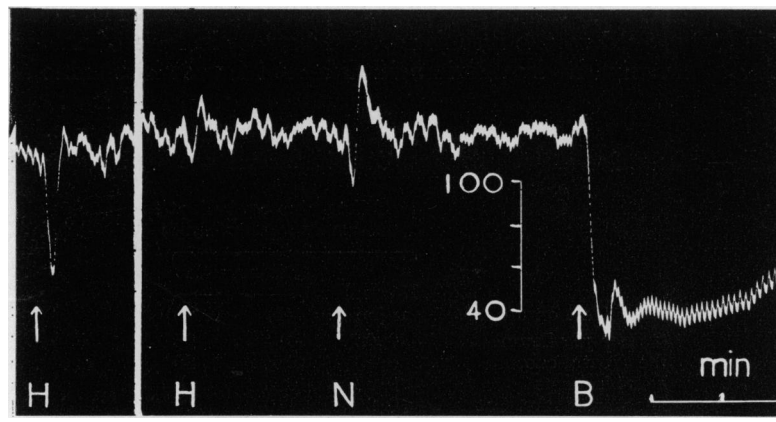

FIG. 3.—Effect of Fraction C_2 from normal and burn urine on the cat blood pressure. Chloralose.
At H ; 2 μg. histamine.
Between the 2 doses of histamine, 0·5 mg. chlorcyclizine hydrochloride.
At N ; Fraction C_2 from normal urine (equivalent to 2 ml. urine).
At B ; Fraction C_2 from burn-urine (equivalent to 2 ml. urine).

its action was destroyed by papain (Fig. 4), chymotrypsin and peptidase. It was not affected by boiling for 10 min. with N/10 HCl, but its activity was reduced to one-half by similar treatment with N/10 NaOH. The active material dialysed readily through cellophane. On several occasions, treatment of the guinea-pig ileum with C_2 potentiated the effect of histamine, sometimes more than fifty-fold. This effect did not always occur.

By paper chromatography C_2 separated into several fractions, all of which gave the colour-reactions of peptides or amino-acids. The pharmacological activity was found to reside in the most densely-staining spot (C_3) which had the same R_f values as synthetic bradykinin (Fig. 5). However, it was found that while the

intensity of the colour produced by the locating reagent indicated a value equivalent to 1 mg./ml. of bradykinin solution, the pharmacological activity was equivalent to about 1 μg./ml. of bradykinin. An inactive peptide must have been present ; this was confirmed by electrophoresis, gel filtration and hydrolysis.

When C_3 was hydrolysed, arginine, phenylalanine, glycine, proline, lysine, alanine, glutamic acid, valine, leucine and/or isoleucine were identified. Apart

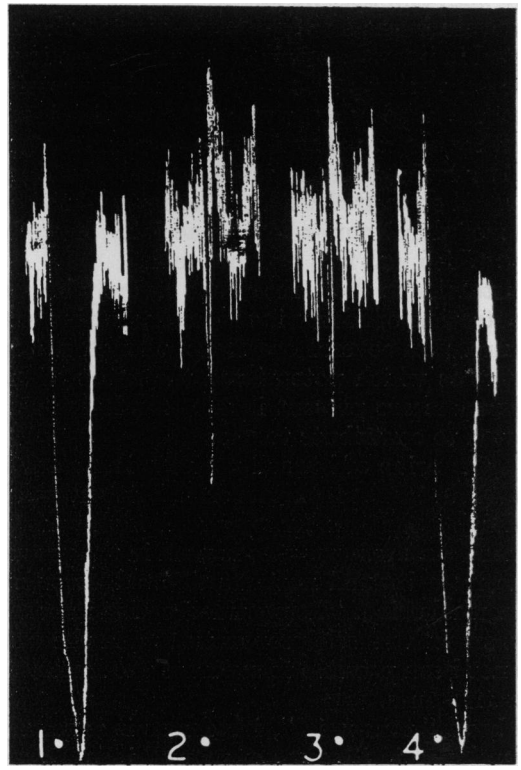

FIG. 4.—Effect of papain on Fraction C_2. Rat duodenum.

1—Fraction C_2.
2—Fraction C_2 after incubation for 24 hr. with papain and cysteine.
3—Fraction C_2 after incubation for 1 hr. with papain and cysteine.
4—Fraction C_2 after incubation for 1 hr. without papain.

The relaxing property of the extract is destroyed by papain.

from the absence of serine and aspartic acid, which have so far not been found, this corresponds to the aminoacids of bradykinin together with the inactive peptide described by Hamberg (1962). If threonine should also prove to be present, the amino-acids would correspond with those of Substance P (Zuber and Jaques, 1962).

The other ninhydrin-positive constituents of Fraction C_2 have not been studied in detail.

DISCUSSION

A review of burn toxaemia (*Lancet*, 1960) expresses the view that " whether the toxicity of oedema fluid found experimentally is clinically important depends on whether the toxic products enter the circulation (this seems likely), how quickly they enter, how and how quickly the body deals with them, and their rate of destruction and elimination."

The present work suggests that histamine and active peptides released during the early stages of severe burns survive destruction in the tissues and the circulation and are excreted in pharmacologically active forms in the urine.

The urine of burned patients frequently contains protein and this could act as a source of kinins. We have shown that active peptide is present in urine which has been obtained with precautions against the formation of artefacts and that the amount of additional active material which is formed when burn urine is incubated with serum is very small compared with the activity of the original urine. Moreover, large amounts of duodenum-relaxing activity have been found in urine from burned patients which was free from protein.

The amount of histamine in the urine of one severely burned patient was about 500 times the concentration of histamine in normal urine extracted and assayed by the same procedure. On several occasions, though not invariably, the urine also contained substances which potentiated the effect of histamine on the test organs ; if such substances are present in the circulation as a result of burning, they would be expected to contribute to shock.

Peptides occur in the urine of burned patients in quantities which far exceed the peptide content of normal urine. Ansorge, Fittkau and Hanson (1961) have identified 20 peptides by chromatography and high-voltage electrophoresis in normal urine. By the less sensitive methods used in the present study the peptide constituents of urine from burned patients differed quantitatively and qualitatively from those of normal urine. The peptide fraction in burn urine which showed high activity against smooth muscle preparations had the characteristic effects of kinin. So far, it has not been possible to separate the fraction into its constituents and to identify the active ones.

The situation in burn " toxaemia ", insofar as it is related to peptide formation, may well be paralleled in illnesses of many kinds. Drugs which antagonise pharmacologically active peptides or prevent their liberation, would be of value as supportive measures to more specific treatment. Spector and Willoughby (1959, 1960) have shown that increase of capillary permeability in acute inflammation is suppressed by anti-esterases such as salicylate, DFP, chloroquine and the cinchona alkaloids. A preparation of pancreas is reported by Koslowski and Waschkeit (1960) to be of value in the treatment of the early stages of burn toxaemia, and an enzyme-antagonist from potatoes has been used in experimental burns in animals (Hladovec, Horáková and Mansfeld, 1961). These results require confirmation.

It is too early to speculate upon the clinical significance of the results obtained in the present study and it may be that peptides play a relatively minor rôle in burn " toxaemia ". Nevertheless, the pharmacological potentialities of peptides released from body proteins are now becoming recognised ; the stage may be set for developments in medical treatment which will reduce the injury that a patient can inflict upon himself with the breakdown products of his own tissues.

SUMMARY

Urine, serum and blister-fluid were collected from patients with severe burns with precautions to minimize the production of artefacts. They contained substances which relaxed the isolated rat duodenum.

Electrophoresis of alcoholic extracts of burn urine on cellulose acetate showed the presence of ninhydrin-positive bands which differed qualitatively and quantitatively from those given by normal urine. The duodenum-relaxing activity was present in a band which moved at the same rate as synthetic bradykinin.

Fractionation of alcoholic extracts of burn urine by column chromatography showed that it contained up to 500 times the histamine activity of normal urine. A peptide fraction from burn urine had from 5 to 40 times the activity of a corresponding fraction from normal urine.

By paper chromatography, the R_f values of the peptide fraction were similar to those of bradykinin, but quantitative pharmacological assays showed that other substances were present. The aminoacids liberated from the peptide fraction by hydrolysis corresponded approximately with those of bradykinin plus the inactive peptide described by Hamberg.

It is suggested that in burned patients, histamine and pharmacologically active peptides released into the circulation as a result of the injury may possibly contribute to " toxaemia " and shock.

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