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THE EFFECT OF ENDOGENOUS COMPOUNDS, ISOLATED FROM SERA OF UREMIC PATIENTS, ON CHLORAMPHENICOL BINDING TO PROTEINS

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Abstract—Charcoal treatment (NORIT A) at pH 3 isolated hitherto unidentified endogenous compounds from uremic serum. These compounds were responsible for reduced binding of chloramphenicol to proteins in uremic patients. After treatment of uremic sera with NORIT, differences in the binding of chloramphenicol to serum proteins in healthy individuals and in uremic patients disappeared. The endogenous compounds were isolated from NORIT by elution using a mixture of 0.1 N HCl and ethanol (1:1). Eluates were then incubated with control serum for 2 hr at 37°C. The binding of chloramphenicol to control serum proteins decreased when eluates from uremic sera were added ($P < 0.01$). However, the eluate obtained from NORIT after the treatment of sera of healthy individuals did not affect the binding of chloramphenicol to serum proteins. To identify these endogenous compounds, we assessed the concentrations of free amino acids and lipids in the serum before and after NORIT treatment in both groups. Of the amino acids, uremic patients displayed significantly higher concentrations only of cystine, citrulline and 3-methylhistidine. These amino acids were adsorbed to an increased extent by NORIT ($P < 0.01$). However, when added to control serum, their enhanced concentrations did not affect the binding of chloramphenicol to proteins. Determination of lipid levels showed a decreased concentration of cholesterol ($P < 0.05$) and an increased concentration of triglycerides in uremic patients ($P < 0.01$). These lipids were not adsorbed by NORIT. There was no difference between free fatty acids levels in both groups. This finding suggests that free fatty acids did not cause the reduced binding of chloramphenicol to proteins in uremic patients, though they were adsorbed by NORIT.

At present, great attention is given to studies of the binding of drugs to serum proteins with chronic renal insufficiency. It has been recognized that the binding of most drugs, in particular organic acids in their nature, is decreased in uremic patients [1–9].

Many investigators are now trying to identify the reasons for reduced binding of drugs to proteins in uremic patients. Some authors propose that decreased binding in uremia is partly due to hypoproteinaemia [1, 2, 4], while other authors attribute the decreased binding to structural changes in the protein molecule [10, 11], or to blockade of the binding sites of proteins in uremic patients by unidentified endogenous compounds [12, 13].

It is technically a difficult task to demonstrate a structural change of the albumin molecule in uremic patients. In an earlier study, we attempted to compare the conformation of albumins isolated from normal and uremic sera by means of circular dichroism spectra (C.D. spectra), where dichroic bands reflect the secondary structure of albumin [14]. But like Sjöholm, we found no differences [12].

A likelier explanation, which agrees also with that of Sjöholm [12] and Craig [13], is that the binding capacity for drugs may be decreased in uremic sera due to the presence of hitherto unidentified compounds that occupy the binding sites of the albumin molecule and thereby alter its binding capacity for drugs. Using the method of Chen [15], Sjöholm [12] and Craig [13] managed to repair the binding capacity for some drugs in uremic patients.

In our earlier studies [14, 16] we have demonstrated decreased binding of chloramphenicol both to serum proteins and isolated albumin in uremic patients. Therefore, we tried to help clarify the mechanism leading to lower binding capacities of uremic sera using as a model the above mentioned experiments [14, 16].

MATERIAL AND METHODS

For our studies of the binding of chloramphenicol to proteins we used *fresh* blood sera obtained from healthy volunteers and sera obtained from the blood of uremic patients. Blood of uremic patients was collected before the start of hemodialysis prior to the administration of heparin. Blood collected from subjects of both groups was processed immediately.

The sera were diluted for the experiments (1:1) by 0.1 M phosphate buffer, pH 7.4. Binding to proteins was determined using the method of equilibrium dialysis as described in detail in our earlier paper [16]. The CUPROPHAN dialysis membrane originating from the hemodialysis coil DC 32 (Bemberg, Wuppertal, F.R.G.) was used for equilibrium dialysis performed at 4° under mechanical shaking for 48 hr. Bacterial contamination of the solutions was prevented by performing the dialysis at 4° and by the bacteriostatic concentration (20 μg/ml) of the used antibiotic. Judging from the identity of C.D. spectra of proteins recorded before and after dialysis [14], we assume that there were no conformation changes in the protein molecule during dialysis.

The mean extent of binding ($\bar{r}$) was calculated from the equation

$$\bar{r} = \frac{C_A - 2\,|A|}{C_P},$$

where C_A and C_P = total initial concentrations of antibiotic and protein, and $|A|$ = concentration of unbound (free) antibiotic as determined after equilibration on the protein free dialyzate compartment. Value $\bar{r}$ states the amount of chloramphenicol in milligrams bound to 1 g of serum proteins.

The binding of chloramphenicol to proteins expressed in per cent was calculated from the following relation:

$$\bar{r} = \frac{C_A - 2\,|A|}{C_A - |A|}.$$

Normalization of binding of chloramphenicol in uremic sera was induced by treatment of the sera with charcoal (NORIT A, Serva, Heidelberg, F.R.G.) at pH 3.0 using the method of Chen [15] as modified for serum by Craig [13]. We used always 5 ml serum to which 250 mg of NORIT were added.

The following procedure was developed to isolate endogenous compounds adsorbed to NORIT from uremic sera: NORIT was isolated from uremic sera by centrifugation at 12,000 rev/min in a Janetzki K 24 centrifuge. After decanting the sera, 3 ml of a mixture of 0.1 N HCl and ethanol (1:1) were added to NORIT, which was suspended in the mixture in a Wortex mixer and left standing overnight at 4° for elution. The following day the suspension was shaken on a mechanical shaker in an ice bath for 30 min. The eluates were separated from NORIT by centrifugation at 12,000 rev/min. This was repeated with another 3 ml of the mixture. The combined eluates were evaporated to dryness under a stream of nitrogen. The dry residues were dissolved in 4.5 ml of 0.1 M phosphate buffer of pH 7.4. This eluate was termed uremic eluate UE. Control sera from healthy volunteers were processed in the same manner and the eluates termed CE. Uremic and control eluates were mixed with 4.5 ml of control serum and the mixtures were incubated for 2 hr at 37°. After cooling to room temperature, 4 ml were pipetted into the dialysis chambers [22] and dialyzed against 4 ml of chloramphenicol solution (20 μg/ml in 0.05 M phosphate buffer, pH 7.4). The binding of chloramphenicol to control serum alone (CS) and to control serum mixed with eluate from uremic sera (CS + UE) and from control sera (CS + CE) was determined in each dialysis series.

To determine the serum concentrations of total proteins and albumin before and after charcoal treatment, we used the biuret method and electrophoresis on cellulose acetate. Using total protein concentrations a correction factor (f) was calculated (f = protein concentration before treatment divided by protein concentration after treatment). The serum concentrations of amino acids and lipids after charcoal treatment were multiplied by this factor to eliminate the effect of dilution of serum caused by adjustment of pH. Serum free amino acids were determined by ion exchange chromatography [21] using the automatic amino acid analyzers Unichrom and Multichrom (Beckman Co.). Serum cholesterol [18], triglycerides [19] and free fatty acids [20] were also determined.

RESULTS AND DISCUSSION

Our earlier study [16] and those of other investigators [5, 13, 27] indicated that low albumin content seen in the blood of uremic patients is not likely to be the main factor altering the binding of chloramphenicol. This hypothesis is supported by the observation that in uremic patients hemodialysis increased the binding to proteins, without any significant changes in albumin concentration.

The study of chloramphenicol binding to pure albu-

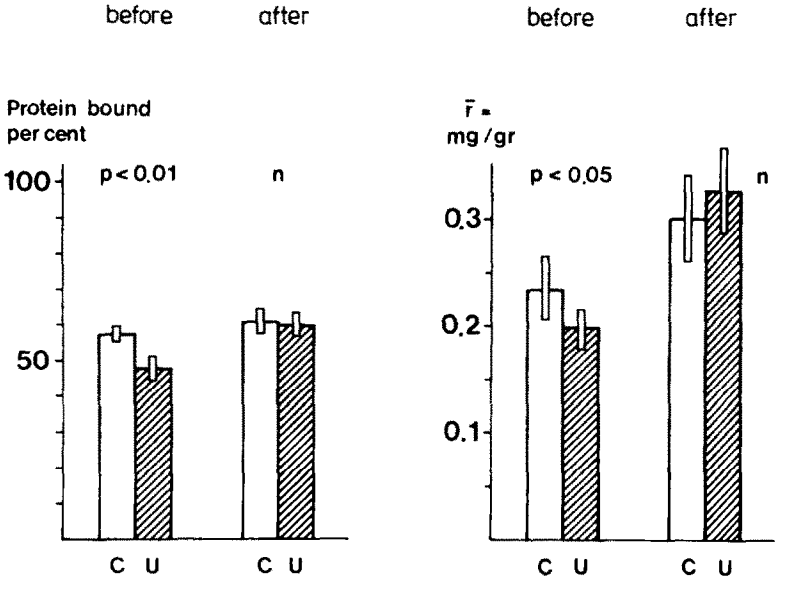

Fig. 1. The binding of chloramphenicol to serum proteins of healthy individuals (C) and uremic patients (U) before and after charcoal treatment at pH 3. left panel = binding in %; right panel = mg bound per g.

min isolated from the sera of both groups showed a similar decrease of chloramphenicol binding in uremic patients. However, binding of chloramphenicol to isolated pure albumins was higher than to serum proteins [14]. A difference between the binding to isolated serum albumin and to whole serum was observed also by other authors using different drugs [8, 9, 12].

We have therefore decided to use serum for investigation of the binding of chloramphenicol to proteins to approximate the conditions in the body. We attempted to normalize the binding of chloramphenicol in the sera of 10 uremic patients. The sera obtained from 8 volunteers (controls) were processed in the same way. The results are expressed for both groups in terms of means ± S.D.

Figure 1 shows that the lower binding of chloramphenicol in uremic patients ($P < 0.01$) was completely normalized after NORIT treatment, so that there was no longer a significant difference between the two groups. Studies of the binding of chloramphenicol to albumin isolated from the sera of uremic patients and healthy volunteers yielded analogous results [14].

These observations led us to infer that the decreased binding in uremic patients might be caused by the presence of unidentified compounds bound irreversibly to the albumin molecule at pH 7.4, thereby changing its binding capacities for chloramphenicol. The unknown compounds are removed from the molecule of albumin and evidently adsorbed to NORIT at pH 3.0.

We therefore tried to establish appropriate conditions for elution of these compounds from NORIT. As we used the method of Chen[15] to eliminate these substances from the uremic sera, we tried to discover whether the impaired chloramphenicol binding to proteins in uremic patients could be due to a competitive binding of some of the blood lipids [23, 24]. Therefore we determined the levels of cholesterol, triglycerides and free fatty acids in the sera of 9 controls and 15 uremic patients before and after treatment with NORIT at pH 3.0. These analyses showed (Fig. 2) a significantly decreased serum cholesterol level in uremic patients ($P < 0.05$). Cholesterol was not adsorbed to NORIT. Uremic patients displayed a significantly increased level of triglycerides ($P < 0.01$), which were also not adsorbed to charcoal. Like other authors [13, 25], we found no difference between the levels of free fatty acids in the two groups. This suggests that free acids did not cause the reduced binding of chloramphenicol to proteins in uremic patients although they were adsorbed by NORIT.

Then we determined the concentration of free amino acids in the sera of both groups before and after NORIT treatment (Table 1) to establish the potential presence of elevated concentration of one or more amino acids which would influence the binding of chloramphenicol. Uremic patients displayed lower levels of most amino acids than controls (Table 1). This was not true of cystine, citrulline and 3-methylhistidine whose concentrations in uremic sera were significantly higher (Fig. 3). Even though NORIT treatment effected a distinct decrease in their concentrations they remained significantly elevated ($P < 0.05$) in uremic patients versus controls. Our finding of higher concentrations of these amino acids in uremic patients agrees with the observations of Koppel [26]. Even though these amino acids were adsorbed to NORIT to a higher extent ($P < 0.01$), they did not affect the binding of chloramphenicol to proteins when added to control sera. Allosteric interaction is unlikely probably because of their small molecular size and low binding energy. Tryptophan was found to be the only amino acid having a significant affinity to proteins [34–36], but assessment of amino acid levels (Table 1) did not show elevated concentrations of tryptophan in uremic patients. It is therefore unlikely that tryptophan is capable of affecting the binding of chloramphenicol. On the contrary, according to reports in the literature [37, 38] the binding of tryptophan to proteins is also decreased in uremic patients, probably due to the presence of non-dialyzable compounds such as peptides or small solutes which are firmly bound to albumin. The presence of peptide-like compounds in uremic sera was demonstrated also by other authors [27–32]. They termed them 'middle molecules' and tried to isolate and characterize them.

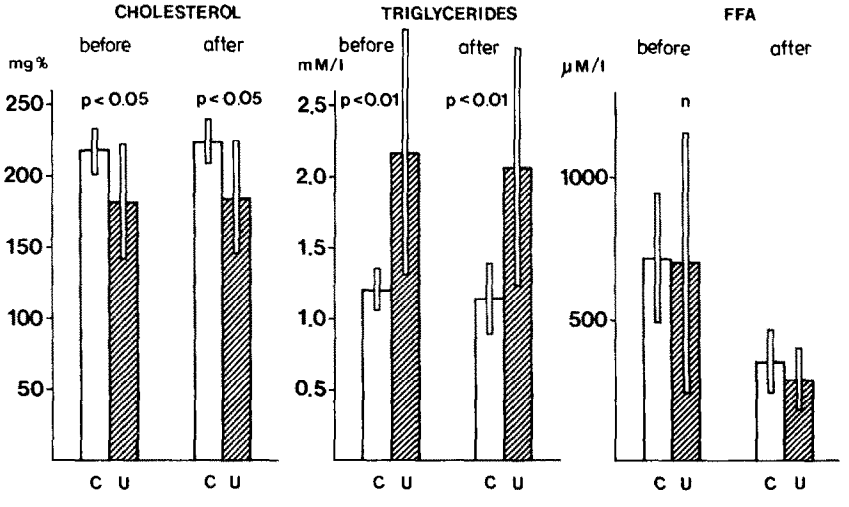

Fig. 2. Mean serum lipid levels in healthy individuals and uremic patients before and after charcoal treatment at pH 3.

Table 1. Mean serum amino acids concentrations (μmol/l ± S.D.) in uremic patients and healthy individuals before and after treatment with activated charcoal (NORIT A) at pH 3

Amino acids	Uremic patients (N = 7)		Healthy individuals (N = 5)	
	Before	After	Before	After
Aspartic acid	17.8 ± 6.2	14.7 ± 4.2	50.6 ± 16.3	42.1 ± 8.6
Serine	115.2 ± 51.4	100.3 ± 46.6	197.0 ± 35.5	194.6 ± 42.3
Proline	228.9 ± 80.5	177.0 ± 70.9	216.3 ± 16.6	159.2 ± 11.6
Glutamic acid	125.7 ± 48.5	56.7 ± 32.3	209.2 ± 61.2	97.6 ± 25.5
Glycine	247.0 ± 84.3	239.8 ± 82.4	321.5 ± 25.6	312.3 ± 46.6
Alanine	314.6 ± 172.9	297.2 ± 145.2	436.1 ± 43.9	428.3 ± 27.8
Cystine/2	171.1 ± 27.4†	45.7 ± 20.7*	52.6 ± 22.6	25.3 ± 11.7
Tyrosine	39.6 ± 12.7	1.6 ± 2.0	67.3 ± 9.8	1.6 ± 0.9
Histidine	71.5 ± 28.7	24.0 ± 6.0	122.9 ± 17.5	30.8 ± 20.6
Arginine	107.3 ± 14.0	20.6 ± 13.0	113.4 ± 19.5	17.8 ± 4.6
Threonine	89.7 ± 47.2	87.7 ± 44.5	157.0 ± 18.4	143.2 ± 15.0
Valine	147.4 ± 45.3	106.4 ± 52.8	239.5 ± 6.8	167.1 ± 7.6
Methionine	18.9 ± 7.7	4.0 ± 1.7	34.0 ± 6.0	5.4 ± 1.3
Isoleucine	50.6 ± 22.1	12.4 ± 8.4	84.3 ± 8.5	18.4 ± 4.1
Leucine	82.5 ± 28.1	22.7 ± 14.5	163.0 ± 22.3	40.3 ± 3.0
Phenylalanine	71.6 ± 25.1	2.0 ± 3.1	90.1 ± 13.5	2.0 ± 1.9
Lysine	134.4 ± 37.5	124.0 ± 52.2	211.4 ± 29.4	184.5 ± 21.1
Tryptophan	39.3 ± 10.9	2.4 ± 0.3	38.9 ± 7.0	4.7 ± 3.8
Taurine	66.8 ± 24.4	58.9 ± 21.1	154.7 ± 52.5	135.8 ± 44.3
Citrulline	107.7 ± 39.1*	31.1 ± 16.0*	46.8 ± 9.5	10.6 ± 6.5
α amino n-butyric acid	10.3 ± 4.9	8.4 ± 4.2	22.0 ± 6.7	23.2 ± 6.9
Ornithine	93.2 ± 22.1	94.5 ± 30.4	148.0 ± 34.2	140.0 ± 32.8
Asparagine + glutamine	667.0 ± 142.6	391.2 ± 72.5	650.1 ± 100.0	404.1 ± 44.5
3-Methylhistidine	35.8 ± 13.4*	8.8 ± 2.8†	12.1 ± 4.6	3.5 ± 0.9

* $P < 0.05$, † $P < 0.01$.

Judging by our results with lipids and amino acids, we speculated that the observed change in chloramphenicol binding in uremic sera may be due to the presence of pathological peptides [39]. We tried to eluate these hitherto unidentified endogenous compounds from NORIT with polar solvents at different pH. The best results were achieved with a mixture of ethanol with 0.1 N HCl (1:1) at pH 2.0. We did not use non-polar solvents because cholesterol and triglycerides were not adsorbed by NORIT, while free fatty acids were the same in both groups. Eluates obtained from NORIT after the treatment of control sera (CS + CE) did not affect the binding of chloramphenicol to proteins (Table 2), but addition of uremic eluates to control sera (CS + UE) significantly reduced the binding of chloramphenicol to serum proteins

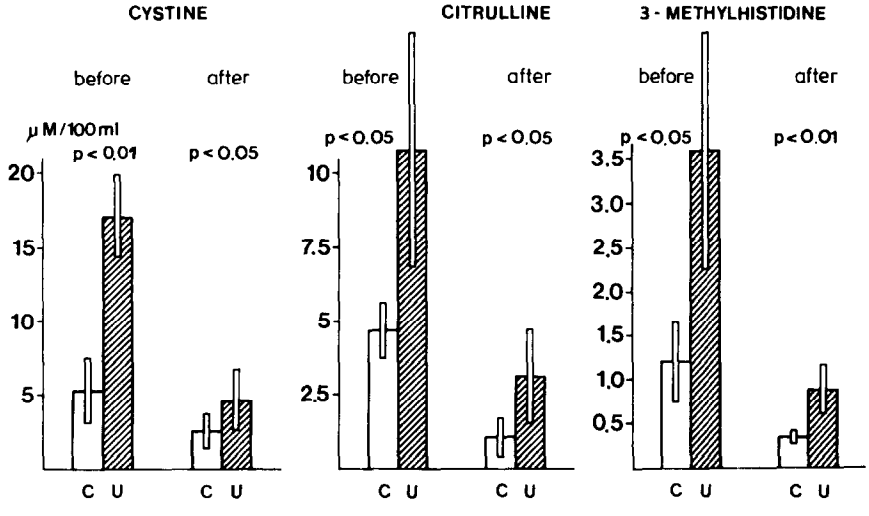

Fig. 3. Mean serum level of cystine, citrulline and 3 methyl-histidine in healthy individuals and uremic patients before and after charcoal treatment.

Table 2. Effect of endogenous substances eluted from NORIT A on *in vitro* protein binding of chloramphenicol

Binding of chloramphenicol							
Healthy individuals					Uremic patients		
Subject No.	Control serum (CS)		CS + CE		Uremic patients	CS + UE	
	$\bar{r}$ (mg/g)	%	$\bar{r}$ (mg/g)	%		$\bar{r}$ (mg/g)	5
1	0.201	52.3	0.203	52.7	J.P.	0.189	50.0
					Z.K.	0.179	47.9
2	0.212	55.8	0.213	56.0	M.K.	0.192	51.8
					J.P.	0.199	53.8
3	0.215	55.3	0.215	55.3	J.B.	0.196	53.2
					L.H.	0.184	52.2
4	0.222	58.1	0.226	58.8	E.M.	0.198	53.5
					J.S.	0.193	52.8
5	0.218	57.6	0.213	56.7	R.P.	0.205	55.0
					J.T.	0.193	52.8
Mean	0.214	55.8	0.214	55.9	Mean	0.193	52.3
± S.D.	0.01	2.04	0.01	2.01	± S.D.	0.01	2.0

CE = Compounds eluted from NORIT after treatment of control sera.
UE = Compounds eluted from NORIT after treatment of uremic sera.

($P < 0.01$). Uremic eluates were obtained from the sera of 10 uremic patients. Even though some patients received Furosemide, Prednisone or Digoxin, the binding of chloramphenicol decreased in all cases irrespective of the drug the patient used.

It can be inferred from the results of our experiments that uremic eluates contain hitherto unidentified endogenous compounds that either do not occur in the sera of healthy persons or do not occur there in a high enough concentration to alter the binding. These endogenous compounds are released from the binding to serum proteins at pH 3 and are adsorbed to NORIT. As soon as they were removed from uremic sera, the binding of chloramphenicol to proteins fully normalized and there was no difference in the binding of chloramphenicol between uremic patients and healthy persons. Incubation of uremic eluates with the control serum for 2 hr at 37° probably led to the binding of the endogenous compounds to proteins. This manifested itself by decreased binding of chloramphenicol to the control serum containing uremic eluate.

The results show that at pH 3 charcoal can remove from serum proteins not only free fatty acids [15] and bilirubin, as reported by Chignell [33], but also other hitherto unidentified endogenous compounds, which decrease the binding of drugs in uremic patients. These compounds are firmly bound to proteins since only a small part is removed from sera by charcoal treatment at pH 7.4 or by hemodialysis [8, 13, 16]. These endogenous compounds may interfere not only with the binding site for chloramphenicol on the albumin molecule [14] but also with the binding sites of other drugs [12, 13]. Once they are removed, the binding of drugs to proteins increases. The aim of our continued studies is to identify these compounds.

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