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Production of uremic toxin methylguanidine from creatinine via creatol on activated carbon

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ABSTRACT

The mixture of creatinine, activated charcoal and water was stirred. As a result the conversion of creatinine into two products was observed. ^{1}H, ^{13}C NMR and HMBC spectra were recorded and the chemical shifts assigned. Two uremic toxins: creatol and N-methylguanidine were identified. To interpret the NMR data obtained, the optimum structure of creatol, which can exist in the forms of seven tautomers, has been calculated using the DFT B3LYP/6-311G(2d,p) method. The influence of the solvent was described by the polarizable continuum model (PCM). The calculated energy of the most energetically stable tautomeric form **A** is lower by 12.2, 16.9, 33.8, 81.5, 106.3, 130.4 kJ/mol in water than that of the tautomers **B**–**G**, respectively, which suggests that the **A** form of creatol should prevail in solution. In DMSO, the calculated energy of the most energetically stable tautomeric form **A** is lower than that of both **D** and **B** and the remaining tautomeric forms (**C**, **E**–**G**) are less energetically stable. Subsequently, we sought the correlations between the experimental and the calculated chemical shifts of protons and carbons-13 for the forms – **A**, **B** (in water) and **A**, **B**, **D** (in DMSO) – of creatol. The population of the **A** tautomer is predominant in both H_2O and DMSO. We have also recorded the spectra of creatol and N-methylguanidine at different pH. Our data are complete enough to be used in the analysis of body fluids.

1. Introduction

Creatol (5-hydroxycreatinine, **1**), first identified at the beginning of 1990 s [1], is the key precursor in the synthesis of N-methylguanidine uremic toxin (**2**) [2,3]. Diabetic patients with chronic renal failure accumulate the creatinine oxidative metabolites such as creatol and methylguanidine in their sera. Many toxic guanidino compounds are produced in the final stage of renal disease (ESRD). For example, guanidinosuccinic acid (GSA) has been identified as the major cause of bleeding tendency, a Na–K ATPase inhibitor and a reason of convulsions in ESRD. GSA is formed from argininosuccinic acid and the hydroxyl radical [4,5]. The significance of creatol lies in the fact that in vivo it is a direct product of the reaction of creatinine (**3**) with the hydroxyl radical [2,3]. The hydroxyl radical has the highest reactivity among various species of free radicals, although it is known to attack nucleic acids proteins, nucleotides, amino acids, saccharides and organic acids [6]. It is known that in position 5 of creatinine under oxidative conditions, a group containing an oxygen atom [7,8] or an amine group [8] substitutes one of the protons at C-5 of the imidazoline ring. On the other hand, it was reported previously that activated carbon has oxidative properties and can oxidise organic acids [9]. Furthermore, Tijsen [10] and Smith [11] have shown that creatinine, adsorbed on activated carbon, undergoes catalytic oxidation, but they have not identified the product of this reaction. This observation is important, as activated carbon, coated with different types of polymers, is being widely used as a sorbent in haemoperfusion techniques for the removal of various toxic products and metabolites from blood [12]. Taking this into account, we investigated the product of the reaction of creatinine with water in the presence of activated carbon. In this paper we report the results of this study and the NMR analysis of the creatinine derivatives.

2. Materials and methods

Commercial creatinine (Sigma, C-4255) and activated charcoal (Sigma, C-4386) were used in the syntheses without purification.

To a 50 ml round-bottom flask equipped with a thermometer, a reflux condenser and a magnetic stir bar, 0.5 g (4.4 mmol) of creatinine, 2 g of activated charcoal and 25 ml of water were added. The mixture was stirred at 40 °C for 25 h. The reaction progress was monitored by TLC (i-PrOH:H_2O:NH_4OH = 7:2:1) and NMR. After completion, the reaction mixture was filtrated, thoroughly washed with water and concentrated in an evaporator. The residue was separated via the preparative thin layer chromatography on 20 × 20 cm

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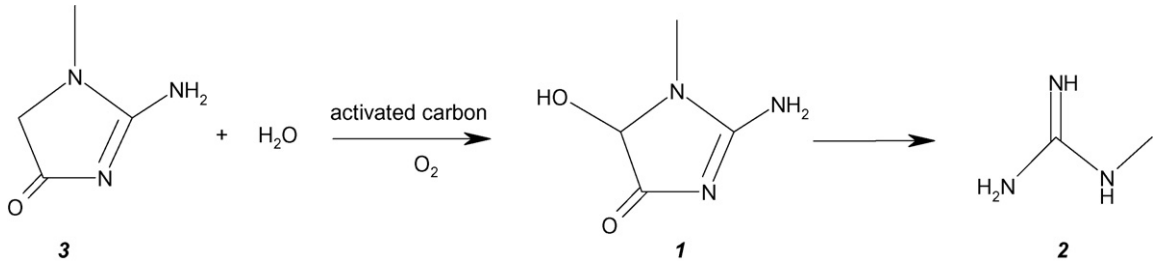
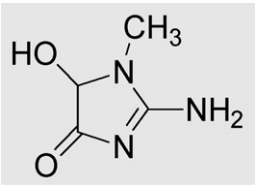


Fig. 1. Conversion of creatinine (**3**) into methylguanidine(**2**) via creatol(**1**).

Table 1
Experimental and calculated, using GIAO–DFT [B3LYP/6-311G(2d,p)] (PCM)method and the Gaussian 03 W program, ^{1}H and ^{13}C chemical shifts, δ [ppm], of 5-hydroxycreatinine.



Position	^{1}H chemical shifts δ [ppm] of proton number						^{13}C chemical shifts δ [ppm] of carbon number			
	3	**4**	**5**	**6**	**7**	**8**	**2**	**4**	**5**	**6**
EXP.*			5.14	3.08			171.32	190.50	85.98	30.63
CALC.** in H_2O for form A			5.52	2.98	4.93	5.66	175.63	195.51	90.32	28.37
CALC.** in H_2O for form B	8.00		5.7	3.00	4.68	5.94	160.19	180.33	88.87	27.87
CALC. in H_2O for form A from Eq (1).							170.81	189.95	88.66	29.00
CALC. in H_2O for form B from Eq (1)							168.68	190.23	92.39	27.13
Theoretical absolute values of carbon atom shielding constans in H_2O for form A			26.43	28.97	27.02	26.29	8.56	−11.32	93.87	155.82
Theoretical absolute values of carbon atom shielding constans in H_2O for form B	23.95		26.25	28.95	27.27	26.01	24.00	3.86	95.32	156.32
EXP***			4.95	2.98			171.22	188.41	84.87	29.87
CALC.** in DMSO for form A			5.35	2.92	4.13	5.29	175.06	194.55	89.09	27.61
CALC.** in DMSO for form B	7.93		5.53	2.94	4.38	5.98	159.76	181.05	87.68	28.03
CALC.** in DMSO for form D	5.82	5.63		2.97	4.17	6.43	164.33	84.01	182.26	25.52
CALC. in DMSO for form A from Eq. (1)							169.91	188.63	87.42	28.41
CALC. in DMSO for form B from Eq. (1)							167.12	189.76	90.46	27.03
Theoretical absolute values of carbon atom shielding constans in DMSO for A			26.56	28.99	27.78	26.62	8.50	−10.99	94.47	155.95
Theoretical absolute values of carbon atom shielding constans in DMSO for B	23.98		26.38	28.97	27.53	25.93	23.80	2.51	95.88	155.53
Theoretical absolute values of carbon atom shielding constans in DMSO for D	26.09	26.28		28.94	27.74	25.48	19.23	99.55	1.30	158.04

* Data (in H_2O with TSP-d4 sodium salt in pH 7.0).
** Data calculated from: $\delta_X^{calc}[ppm] = \sigma_X^{ref} - \sigma_X^{calc}$, for the ^{1}H and ^{13}C spectra, σ_X^{ref} is equal to 31.95 and 31.91 ppm and 184.19 and 183.56 ppm for H_2O and DMSO, respectively.
*** Data in DMSO-d_6.

glass plates coated with the 2 mm thick layer of silica gel 60 F_{254} (Merck 1.05717.0001) with i-PrOH:H_2O:NH_4OH = 7:2:1 as mobile phase. The purity was confirmed by NMR.

The conversion degree for creatinine was 76% and the yield for creatol was 9% (MS(EI, 70 eV): 129(M^+), 127, 101, 73, 56, 42), and for methylguanidine 67% (HRMS (EI): 73.06400 calcd for $C_2H_7ON_3$; found 73.06366).

NMR measurements were performed on 0.55 ml samples, each one consisting of 0.15 M solution of the compound **1, 2** or **3** in water, to which 50 μl of D_2O containing 3-trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP-d_4 sodium salt; Dr. Glaser AG Basel), was added as the spectrometer field lock and the internal chemical shift reference. The pH was controlled directly in an NMR tube using a pH electrode and a pH-meter (Aldrich). This measuring

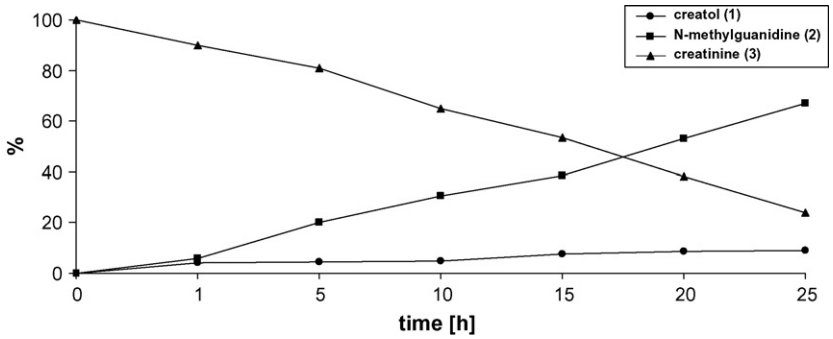
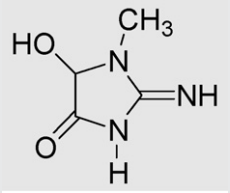
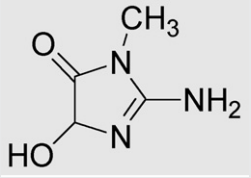
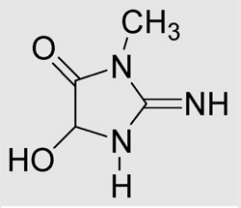
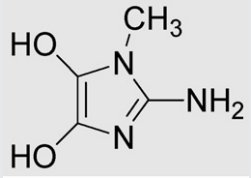
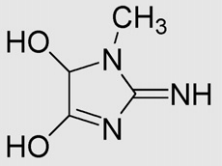
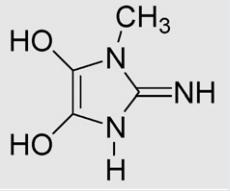
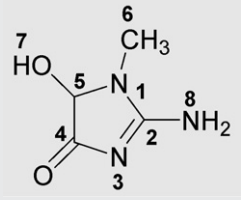


Fig. 2. Time course of creatinine (**1**) degradation and formation N-methylguanidine (**2**)(product) and creatol (**1**)) (intermediate) in the reaction creatinine $\xrightarrow{k_1}$ creatol $\xrightarrow{k_2}$ N-methylguanidine where $k_2 > k_1$.

Table 2
Total (a.u) and relative (kJ/mol) theoretical energy, calculated using DFT [B3LYP/6-311G(2d,p)] calculatated using PCM method and the Gaussian 03W program for 5-hydroxycreatinine tautomers.

Tautomers	In H_2O		In DMSO	
	Energy (a.u.)	Energy [kJ/mol]	Energy (a.u.)	Energy [kJ/mol]
A 	−471.540474	0	−471.538699	0
B 	−471.536021	12.2	−471.535465	8.8
C 	−471.534278	16.9	−471.533222	15.0
D 	−471.528108	33.8	−471.537748	2.6
E 	−471.510604	81.5	−471.509632	79.3
F 	−471,501547	106.3	−471.513748	68.1
G 	−471.492707	130.4	−471.491230	129.6

system was standardised using pH 1.68, 4, 7, and 10 (Cole-Parmer Instrument Co.) buffers. The pH of the samples was adjusted by adding small amounts of 1 M HCl or 0.55 M NaOH solutions. The proton and proton-decoupled ^{13}C NMR spectra were recorded using a Varian UNITY*plus* spectrometer operating at 11.7 T magnetic field. The proton spectra were recorded using the following measurement parameter set: pulse angle 30°, acquisition time 5 s. The water signal was suppressed by selective irradiation during the relaxation delay (5 s). 1000 scans were accumulated. This set of parameters guarantees that saturation effects are avoided. The standard measurement parameter set for ^{13}C spectra was: pulse width 7 μs (the 90° pulse width was 12.5 μs), acquisition time 1 s, spectral width 200 ppm, WALTZ 16 ^{1}H decoupling. 4000–8000 scans were accumulated and after zero-filling to 64 K, the FID signals were subjected

to Fourier transformation after applying a 1 Hz line broadening. The ^{1}H–^{13}C gs-HMQC 2-D spectra were also recorded using the standard Varian software. The measurements were performed at room temperature. The optimum ground-state geometries for all compounds were calculated using the density functional theory (DFT) and the proton and carbon chemical shifts by the GIAO–DFT method. At both calculation stages the B3LYP functional and 6-311G(2d,p) basis set were employed and the continuum model (PCM; Gaussian 03 W) [13,14] was used in order to simulate the effects of the solvent. The relative chemical shift of a given nucleus X in the molecule was defined as $\delta_X^{calc}[ppm] = \sigma_X^{ref} - \sigma_X^{calc}$. For the ^{1}H and ^{13}C spectra, σ_X^{ref} is equal to 31.95 and 184.19 ppm for H_2O and σ_X^{ref} is equal to 31.91 and 183.56 ppm for DMSO, as found on the basis of DFT [B3LYP/6-311G(2d,p)] geometry of the dual-reference standard (TMS).

3. Results and discussion

The mixture of creatinine, activated charcoal and water was stirred at 37 °C for 25 h. As a result we observed conversion of creatinine into two products (Fig. 1). We could detect the presence of the new compounds just after an hour (TLC). Smith et al. [11] had observed that when a creatinine solution was placed in contact with activated carbon in air, the concentration of the solution continued to decrease considerably over a number of days. The authors demonstrated that at pH of dialysate and at 37 °C the creatinine (the equilibrium creatinine–creatine was established in one day [15]) was rapidly oxidised. However, no creatine was produced simultaneously. In other words, the rate of oxidised creatinine on activated carbon was higher than equilibration—creatinine–creatine. On the other hand, Tijssen et al. [10] reported that they had observed an unknown product in the course of the contact of creatinine with activated carbon. Chemical oxidative conversion (with various active oxygen species) of creatol into N-methylguanidine is known and described in the literature [16]. We have analysed all the published information and our investigations into creatinine and activated carbon. ^{1}H and ^{13}C NMR and HMBC spectra of the new products were recorded and the chemical shifts assigned (in water and DMSO-d_6, Tables 1–3). We have identified creatol (**1**) and N-methylguanidine (**2**). Graphs of the concentration dependences of (**1**–**3**) on time can be drawn (Fig. 2). Once the intermediate (**1**) is formed, it is rapidly converted to the product (**2**). The magnitude of the intermediate (**1**) concentration is almost always low but steady and remains almost constant at this value until the reaction is virtually complete. This phenomenon takes place when $k_2 > k_1$. This course of reaction shows that creatinine in the presence of activated carbon is converted to N-methylguanidine via creatol.

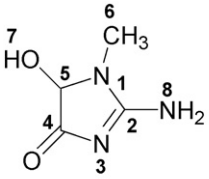
To interpret the NMR data obtained, the optimum structure of creatol, which can exist in the forms of seven tautomers, has been calculated using the DFT B3LYP/6-311G(2d,p) method (Table 2). The influence of the solvent was described by the polarizable continuum model (PCM) [13]. The calculated energy of the most energetically stable tautomeric form **A** is lower by 12.2, 16.9, 33.8, 81.5, 106.3, 130.4 kJ/mol in water than that of the tautomers **B**–**G**, respectively, which suggests that the **A** form of creatol should prevail in solution. This corresponds with the literature data concerning 5-substituted creatinines [8]. In DMSO, the calculated energy of the most energetically stable tautomeric form **A** is lower than that of both **D** and **B** (by 2.6 kJ/mol than that of **D** and 8.8 kJ/mol than that of **B**). The remaining tautomeric forms (**C**, **E**–**G**) are less energetically stable (Table 2). The calculations show that the difference between the forms **A** and **D** is very small. To get further evidence to verify this conclusion, the NMR parameters have been calculated for two (in water—**A** and **B**) and three (in DMSO—**A**, **B**, and **D**) tautomers of 5-hydroxycreatinine (Table 1). Subsequently, we sought the correlations between the experimental and the calculated chemical shifts of protons and carbons-13 simultaneously, for the forms – **A** and **B** (in water) and **A**, **B**, and **D** (in DMSO) – of creatol. The relative chemical shift of a given nucleus X in the molecule was defined as $\delta_X^{calc}[ppm] = \sigma_X^{ref} - \sigma_X^{calc}$. For the ^{1}H and ^{13}C spectra, σ_X^{ref} is equal to 31.95 and 184.19 ppm for H_2O and σ_X^{ref} is equal to 31.91 and 183.56 ppm for DMSO, as found on the basis of DFT [B3LYP/6-311G(2d,p)] geometry of the dual-reference standard (TMS). The correlations for the forms **A** and **B** in both solvents are satisfactory ($r > 0.99$). However, the best one is for the form **A** (compare $r = 0.9999$ for **A** to 0.9990 for **B** in water and $r = 0.9998$ for **A** to 0.9991 for **B** in DMSO). There is not any correlation for the form **D** ($r = 0.6160$). It has been found [17] that the experimental values of the carbon chemical shifts of creatol in water and DMSO can be reproduced, using the calculated absolute values of the shielding constants for both energetically stable tautomeric forms **A** and **B**, by the following equation:

$$\delta \exp = a[\sigma \mathrm{calc}_X - \sigma_0] \quad (1)$$

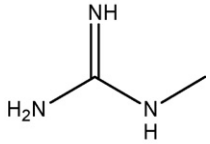
(in ideal situation $a = -1$, and $\sigma_0 = \sigma_{TMS}$)

The values of adjustable parameters for **A**, $a = -0.96297$ (± 0.01385), $\sigma_0 = 185.94$ (± 2.03) ppm, for H_2O (mean deviation = 1.61) and $a = -0.95966$ (± 0.01382), $\sigma_0 = 185.56$ (± 2.02) ppm, (for DMSO) (mean deviation = 1. 61102) were found using the least-squares method. For the tautomer **B** for both H_2O and DMSO the

Table 3
pH dependence of ^{1}H and ^{13}C chemical shifts δ [ppm] of 5-hydroxycreatinine (**1**) and N-methylguanidine (**2**) in water solution.



1



2

pH	^{1}H chemical shifts δ [ppm] of hydrogen number for compounds			^{13}C chemical shifts δ [ppm] of carbon number for compounds					
	1		**2**	**1**				**2**	
	5	6	CH_3	2	4	5	6	CH_3	CN
2.70	5.42	3.17	2.84	159.47	175.63	84.00	31.05	30.31	160.19
3.50	5.38	3.17	2.84	161.66	178.26	84.46	31.01	30.30	160.17
6.40	5.16	3.10	2.86	170.93	190.15	85.94	30.65	30.27	160.19
7.80	5.14	3.08	2.86	171.32	190.56	85.99	30.62	30.32	160.22
8.90	5.14	3.07	2.85	171.39	190.70	86.05	30.61	30.32	160.31
10.80	5.07	3.07	2.85	170.88	191.88	87.24	30.51	30.31	160.20

mean deviation is greater (3.88 and 3.81, respectively) and therefore it can be concluded that the population of the **A** tautomer predominates in H_2O and DMSO (Table 1). This information is important when using combined prolonged haemoperfusion (HP) and haemodialysis (HD) for severe dimethoate poisoning [18]. The combined HP–HD is performed within 24 h using the same charcoal column and dialyser. However, creatol is a product resulting from the reaction of creatinine with hydroxyl radical and is identified as a precursor of N-methylguanidine (MG). N-methylguanidine is a toxic compound and its amount increases in uremic patients.

As far as the diagnostic applications of ^{1}H and ^{13}C NMR [19–22] of 5-hydroxycreatinine and N-methylguanidine occuring in the urine of patients with chronic renal failure are concerned, we have also recorded the spectra of these compounds at different pH (Table 3). Our data are complete enough to be used in the analysis of body fluids.

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References

[1] K. Nakamura, K. Ienaga, Experientia 46 (1990) 470–472.
[2] S. Nagase, K. Aoyagi, M. Narita, S. Tojo, Nephron 44 (1986) 299–303.
[3] K. Nakamura, K.Ienaga,T. Yokozawa, N. Fujitsuka, Nephron, 68 (1994) 280–281; and Comment on: Nephron, 58 (1991) 42–46.
[4] K. Aoyagi, in: Yoshikawa, Toshikazu (Eds.), Free Radicals in Chemistry, Biology and Medicine, OICA International (UK) Ltd, London, 2000, pp. 396–402.
[5] K. Aoyagi, J. Artif. Organs 4 (2001) 3–7.
[6] G. Erenel, D. Erbas, A. Aricioglu, Mater. Med. Pol. 25 (1993) 37–43.
[7] K. Nakamura; K. Kawano, (Nippon Zoki Pharmaceutical Co., Ltd., Japan). Eur. Pat. Appl. EP 1195373 A2 10 Apr 2002, 9 pp. DESIGNATED STATES: R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO. (English). (European Patent Organization). CODEN: EPXXDW. CLASS: ICM: C07D233-88. ICS: G01N030-02. APPLICATION: EP 2001-123251 2 Oct 2001. PRIORITY: JP 2000-303172 3 Oct 2000. DOCUMENT TYPE: Patent CA Section: 9 (Biochemical Methods) Section cross-reference(s): 14.
[8] H. Krawczyk, A. Pietras, A. Kraska, Spectrochim. Acta A 66 (2007) 9–16.
[9] A. Kuhn, H. Gerhard, Kolloid Z. 103 (1943) 130–135.
[10] J. Tijssen, M.J.F.M. Kaptein, J. Feijen, A. Bantjes, A.W.J. VanDoorn, in: R.M. Kenedi, al. et (Eds.), Artificial Organs, Macmillan Press, London, 1977, pp. 158–163.
[11] M. Smith, S. Affrossman, J.M. Courtney, Carbon 17 (1979) 149–152.
[12] J.H. Henderson, C.A. McKenzie, P.J. Hilton, R.M. Leach, Thorax 56 (2001) 242–243.
[13] J. Tomasi, B. Mennucci, R. Cammi, Chem. Rev. 105 (2005) 2999–3093.
[14] J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, et al., GAUSSIAN 03, Revision B. 05, Gaussian Inc, Pittsburgh, PA, 2003.
[15] G. Edgar, H.E. Shiver, J. Am. Chem. Soc. 47 (1925) 1179–1188.
[16] K. Nakamura, K. Ienaga, T. Yokozawa, N. Fujitsuka, H. Oura, Nephron 58 (1991) 42–46.
[17] Program "a" written by professor Adam Gryff-Keller using the least-squares method.
[18] J. Nagler, R.A. Braeckman, J.L. Willems, G.A. Verpooten, M.E. de Broe, J. Appl. Toxicol. 1 (1981) 199–201.
[19] U.F.H. Engelke, S.H. Moolenaar, S.M.G.C. Hoenderop, E. Morava, Graaf van der, A. Heerschap, R.A. Weawers, Handbook of ^{1}H-NMR Spectroscopy in Inborn Errors of Metabolism: Body Fluid NMR Spectroscopy and in vivo MR sSpectroscopy, SPS Verlagsgesellschaft mbH, Heilbronn, 2007.
[20] H. Krawczyk, A. Gryff-Keller, W. Gradowska, M. Duran, E. Pronicka, J. Pharm. Biomed. Anal. 26 (2001) 401–408.
[21] H. Krawczyk, W. Gradowska, J. Pharm. Biomed. Anal. 31 (2003) 455–463.
[22] H. Krawczyk, W. Gradowska, J. Inherit. Metab. Dis. 30 (2007) 263.