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From the Medizinisch-Chemisches Institut and Pharmazeutisches Institut, University of Berne, Berne (Switzerland)

Determination of Disulfiram and its Metabolites in Human Blood

By A. M. Sauter, W. Wiegrebe, and J. P. von Wartburg

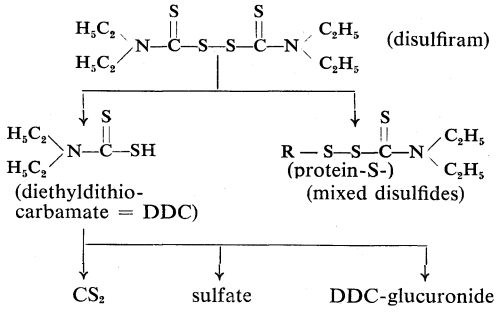
Summary: *This work was initiated by the lack of a sensitive method for the determination of disulfiram and its metabolites in blood of patients treated with this drug. A method is described which allows the separate determination of carbon disulfide, free diethyldithiocarbamate and disulfides derived from disulfiram with adequate precision in 10 ml patient blood. It is based on a spectrophotometric determination of a yellow compound formed by trapping carbon disulfide produced from diethyldithiocarbamate and disulfiram in an ethanolic solution of diethylamine and copper(II)-acetate. Good quantitation of disulfiram and diethyldithiocarbamate in blood was achieved by trapping carbon disulfide produced when formic acid and cystein were added to the samples. During daily administration of 200 mg disulfiram to humans, concentrations of zero to 0.6 µg carbon disulfide and 0.2 to 1.0 µg diethyldithiocarbamate per ml blood were found using this method.*

Zusammenfassung: *Nachweis von Disulfiram und seinen Metaboliten im menschlichen Blut*

Nach unserer Erfahrung gibt es keine Methode mit ausreichender Empfindlichkeit, um Disulfiram und seine Metaboliten im Patientenblut zu bestimmen. Wir beschreiben ein Verfahren, mit dem CS_2, Diäthyldithiocarbamat und vom Disulfiram stammende Disulfide getrennt mit hinreichender Genauigkeit aus 10 ml Blut bestimmt werden können. Grundlage ist die spektrophotometrische Bestimmung einer gelben Verbindung, die aus CS_2 in einer äthanolischen Lösung von Diäthylamin und Cu(II)-acetat entsteht. CS_2 wird aus Diäthyldithiocarbamat und Disulfiram durch Ameisensäure bzw. Ameisensäure/Cystein freigesetzt. Bei Tagesdosen von 200 mg Disulfiram wurden 0 bis 0,6 µg CS_2 und 0,2 bis 1,0 µg Diäthyldithiocarbamat pro ml Patientenblut gefunden.

1. Introduction

Disulfiram (tetraethylthiuramdisulfide, TETD, Antabus®*)) has become a popular drug for treatment of alcoholism since its discovery in 1948 [1]. It is usually administered p.o. in daily doses of 100—400 mg or implanted as tablets containing 1000—1600 mg. The absorption from the gastrointestinal tract seems to be incomplete, 5—20% appearing in the faeces [1—4]; nothing is known on the rate of absorption of implanted disulfiram. Scheme 1 represents the known metabolic pathways of disulfiram or its reduced form diethyldithiocarbamate (DDC). Using ^{35}S-labelled TETD Strömme demonstrated the in vitro and in vivo formation of mixed disulfides with serum or liver proteins



Scheme 1: Main pathways of disulfiram metabolism.

*) Manufacturer: E. Tosse & Co. mbH, Hamburg.

[5—9]. No free, unmetabolised TETD could be detected by various authors in serum, liver or urine of experimental animals and man [1, 3, 7, 8]. After i.p. application to rats free DDC could be detected in serum only for a short time and mixed disulfides were formed with protein, indicating rapid interconversions between DDC, TETD and mixed disulfides in vivo. TETD and DDC are further metabolized to CS_2 [10, 11], sulfate [7, 10] and DDC-S-glucuronide [7, 12]. A significant part of administered TETD is eliminated in expired air as CS_2 [10, 13].

In view of a wide-spread therapeutic use of disulfiram in general and an increased application of the implantation-technique it was of interest to determine TETD and its metabolites in patients. Several methods for the determination of disulfiram have been proposed [3, 14—16]. These methods rely on the formation of a yellow copper — TETD — compound which is subsequently extracted with an organic solvent and determined photometrically. When these methods were applied to samples of human blood in our laboratory they proved to lack the necessary sensitivity. High blank values restrict their application to concentrations of TETD or DDC far above those expected in patients. Furthermore they do not account for the formation of mixed disulfides and CS_2. Similar problems were encountered when we utilized the recently proposed colour-reaction of TETD with ethanol and cyanide [17]. Hence a method was developed which allows to determine CS_2, free DDC and DDC liberated from disulfides (mixed disulfides and/or TETD).

2. Materials and methods

All reagents were of the highest commercial grade from Fluka, Buchs, Switzerland, or Merck, Darmstadt, Germany, and used without further purification. The Viles-reagent was modified as follows: Cu(II)-acetate 0.1 g, diethylamine 35 g, triethanolamine 10 g, demineralised water 75 ml, ethanol abs. ad 1000 ml. Excess solvent was removed by the use of a rotary evaporator Rotavapor Büchi, Flawil, Switzerland. Solutions were filtered with a syringe-filter apparatus from Sartorius (Göttingen, Germany) with solvent resistant and inert cellulose membrane filters SM 11 604. The spectra were recorded on a Zeiss PMQ II and absorbances determined on a Unicam Sp 1800.

3. Results and discussion

3.1. Evaluation of published methods

Previous methods [3, 14—16] to determine TETD and DDC were based on the fact that these compounds form yellow complexes with cupric ions. Possible structures of these have been reported by Geldmacher et al. [18]. Although the precise nature of these copper-addition compounds is not yet known, they will be referred to as copper-complexes. From their study these authors concluded that the complexes formed from DDC and TETD cannot be distinguished on the bases of several physico-chemical parameters. As shown in Fig. 1, identical spectra were obtained

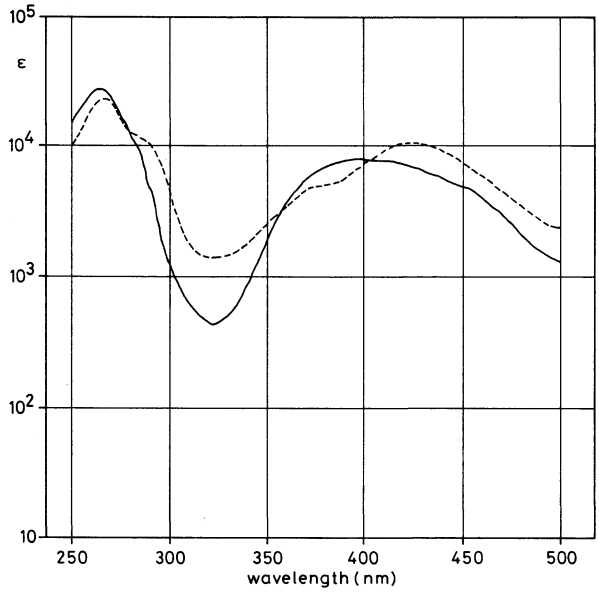


Fig. 1: Spectra of disulfiram-, DDC- and CS_2-copper complexes ——— solvent: 85% ethanol containing 5×10^{-4} M Cu-II-acetate. Identical spectra were obtained with 4×10^{-5} M disulfiram or 8×10^{-5} M DDC-Na. — — — solvent; modified Viles reagent (cf. 2). Identical spectra were obtained with 4×10^{-5} M disulfiram, or 8×10^{-5} M DDC-Na, or 8×10^{-5} M CS_2. The molar extinction coefficients ε were calculated assuming that 1 mole disulfiram forms 1 mole Cu-II-complex, while 2 moles DDC-Na or CS_2 form 1 mole Cu-II-complex.

by the addition of copper(II)-acetate to DDC and TETD in two solvent-systems. For the formation of the TETD-complex the samples were heated to 50° C for 15 min to enhance the rate of complex formation. As reported previously [14, 18], two absorption maxima are observed at 266 nm and 400 nm in ethanol with molar extinction coefficients of 25 000 and 7200, respectively. The addition of diethylamine and triethanolamine leads to a shift of the second absorption maximum to 425 nm and increases the molar extinction coefficient to 10 300. Our results show that with the previous methods TETD and DDC cannot be detected separately. Divatia et al. [14] observed that the extraction of the TETD-Cu-complex into dichlorethane is independent of the pH, while the extractability of the DDC-Cu-complex decreases with increasing pH. Since this finding could not be reproduced attempts were made to separate the free compounds before formation of the copper complex. As suggested by Domar et al. [3], DDC is ionized at pH 9 and should not be extractable with a lipophilic solvent in contrast to TETD. Subsequent adjustment to an acid pH should allow extraction of DDC. Attempts to separate DDC and TETD in this way failed because DDC was found to contaminate TETD by approximately 30% at pH 9. Furthermore DDC decomposes to CS_2 and diethylamine both in alkaline and acidic solution. Identification of TETD by means of thin-layer chromatography has been proposed by Farago [16]. However, a separation and quantitation of TETD and DDC could not be achieved by this technique. Although the two compounds were separated with $CHCl_3$ 55, benzene 18, methanol 15, butanol 10, NH_3 25% 2 (vol. parts) as solvent system, such a procedure could not be used for subsequent quantitation due to losses of DDC by decomposition.

3.2. Determination of CS_2 and DDC

Since CS_2 has been identified as a major metabolite of TETD [10, 11, 13, 19] it seemed appropriate to establish a method for its determination in human blood. DDC decomposes rapidly and quantitatively to CS_2 and diethylamine in acidic solution and hence can be determined by measuring the CS_2 evolved [20—22]. A special apparatus was employed (Fig. 2) for this purpose.

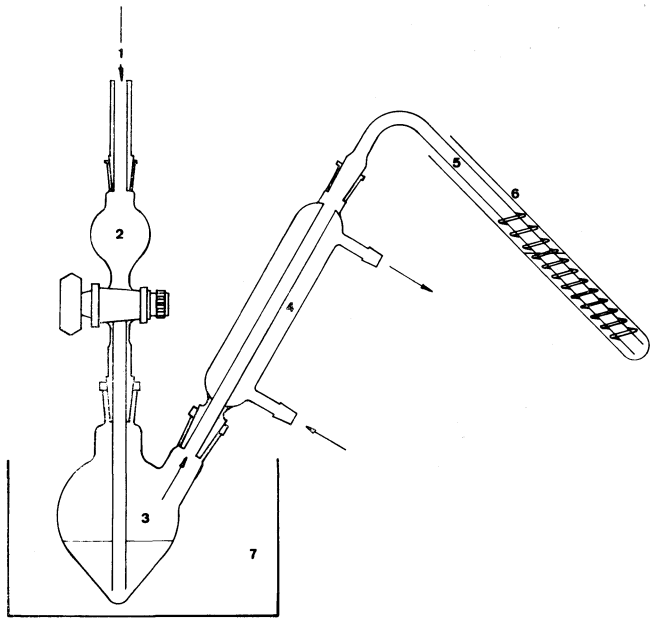


Fig. 2: Apparatus employed for the determination of CS_2, DDC and disulfiram. **1**: inlet for carrier-gas (nitrogen); **2**: side flask containing formic acid; **3**: reaction flask containing sample; **4**: water cooling; **5**: glass tube with external spiral glassrod; **6**: trap (test tube) containing reagent solution; **7**: waterbath (50° C).

The sample of citrated blood, serum or urine was diluted and placed in the reaction flask (for details cf. 3.6.1.). Free CS_2 was flushed with a stream of nitrogen from the reaction flask and trapped with a reagent containing Cu(II)-acetate, diethylamine, and triethanolamine [19]. The yellow-coloured compound formed has a spectrum identical with those of the DDC-copper and TETD-copper complex (Fig. 1). Furthermore, the same molar extinction coefficients were found on both absorption maxima. An increase in concentration of diethylamine was necessary because the original composition of the reagent [23, 24] yielded a maximal colour intensity only after 20 min and the recovery of CS_2 was incomplete. In order to increase the sensitivity of the method, the content of the trap was transferred into a flask with ethanol and benzene and evaporated to dryness. The residue was taken up in methylacetate-acetone, because this solvent proved to give the highest colour yield. The copper complex was stable within the condition of

this procedure and for at least 24 h at room temperature in solution. Absorbance was determined with 4 cm pathlength at 426 nm; measurements at 270 nm were less reliable because of high reagent blanks. A molar extinction coefficient of 13 120 was obtained by adding CS_2 directly to the reagent in the trap and using the procedure described (see below). Hence, a concentration of 1 μg CS_2/ml sample should yield an extinction corrected for reaction blank of 0.862.

3.3. The calibration curve for CS_2

A calibration curve for CS_2 was obtained by adding various amounts of CS_2 to 10 ml samples of urine, serum and blood (Fig. 3). The absorbance of 0.866 for a sample concentration of 1 μg CS_2/ml calculated from the slope of the calibration curve is in good agreement with the value obtained from the molar extinction coefficient; these results indicate a full recovery of CS_2 added to blood, serum and urine in a range of 0.05 to 1.5 μg/ml.

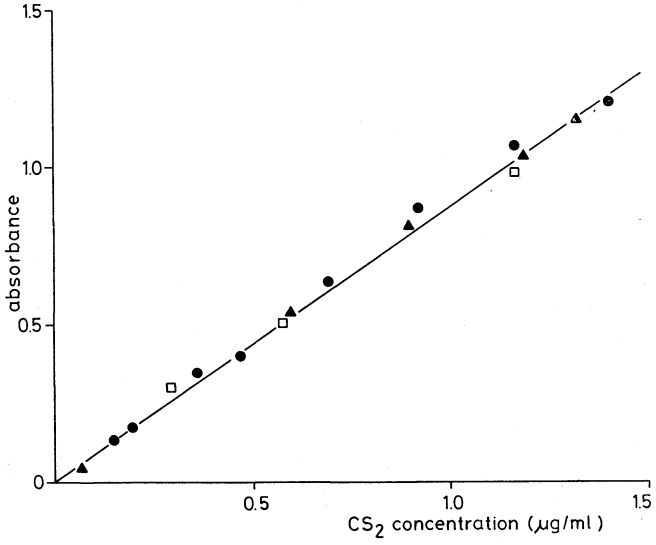


Fig. 3: Calibration curve for CS_2. Various amounts of CS_2 added to 10 ml blood (●), serum (▲) or urine (□) were analysed as mentioned in 3.6.1. The absorbance is corrected for the reagent blank. y = 0.866 x + 0.014; R = 0.998.

3.4. The calibration curve for DDC (Fig. 4)

Various amounts of DDC were added to blood, serum and urine and the CS_2 evolved from the addition of formic acid was determined as described. One mole of CS_2 could be recovered from one mole of DDC added to urine. However, recoveries varying between 30 to 80% were found with serum and virtually no CS_2 was liberated from DDC added to blood under these conditions. Since free DDC cannot form mixed disulfides it seems that it is oxydized to TETD. By addition of cystein to reduce disulfides full recovery was obtained. 1 μg CS_2/ml is equivalent to 1.96 μg DDC/ml. For this DDC concentration of 1.96 μg/ml an extinction of 0.875 could be calculated from the slope of the calibration curve. This is in good agreement with the value obtained for CS_2.

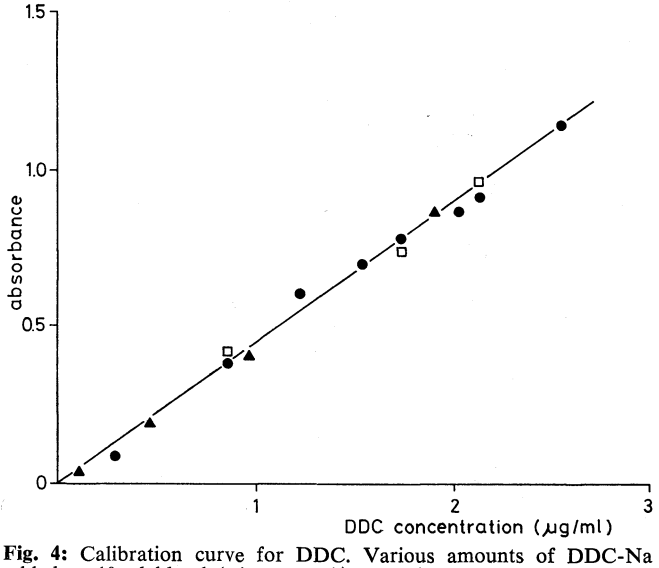


Fig. 4: Calibration curve for DDC. Various amounts of DDC-Na added to 10 ml blood (●), serum (▲) or urine (□) were analysed as mentioned in 3.6.1. The absorbance is corrected for the reagent blank. y = 0.447 x — 0.006; R = 0.997.

3.5. The calibration curve for TETD (Fig. 5)

Cystein and formic acid were added to the samples of blood, serum and urine containing TETD (Fig. 5). No CS_2-formation was detectable without cystein, confirming that no reduction of TETD took place. This method does not discriminate between TETD and possibly formed mixed disulfides. Again good agreement was found between the slopes of the calibration curve for TETD with that for CS_2. Since it has to be considered that 2 moles CS_2 are formed from one mole of TETD, 1 μg CS_2/ml corresponds to 1.95 μg TETD/ml and yields an extinction of 0.850.

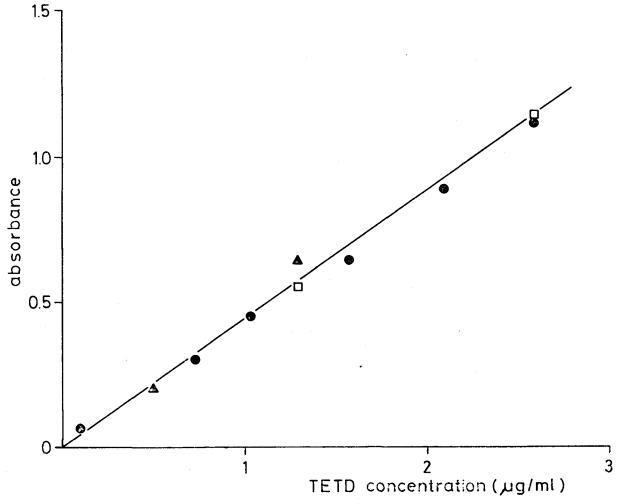


Fig. 5: Calibration curve for TETD. Various amounts of TETD added to 10 ml blood (●), serum (▲) or urine (□) were analysed as mentioned in 3.6.1. The absorbance is corrected for the reagent blank. y = 0.436 x — 0.002; R = 0.997.

3.6. Determination of CS_2, DDC and TETD in the same sample

Various amounts of all three compounds were added to water to yield mixtures within the range of concentrations used in the calibration curves. Free CS_2 was flushed and trapped in a first reagent solution. CS_2 subsequently liberated from DDC by addition of formic acid was captured in a new trap. Finally the remaining TETD was reduced with cystein and determined like DDC. All three compounds could be recovered separately within the accuracy obtained in the calibration curve.

3.6.1. Procedure

Mix 10 ml blood, serum or urine with 20 ml water and 0.1 ml n-octanol (antifoaming agent). Flush free CS_2 with N_2 at room temperature for 30 min with 100 ml N_2 per min. Trap the CS_2 in 6 ml of the modified Viles reagent (see 2.). Transfer the yellowish reagent solution into a flask and rinse the trap successively with 3 ml ethanol and 5 ml benzene. Evaporate the combined solution at 50° C and 15 Torr. Dissolve the residue in 2 ml of a mixture of methylacetate-acetone (7:3 vol.), filter and read the absorbance at 426 nm in 4 cm cuvettes using the solvent mixture as the reference.

To determine free DDC and disulfides use a new trap with fresh reagent and add 5 ml formic acid and 0.1 g cystein-HCl. Heat

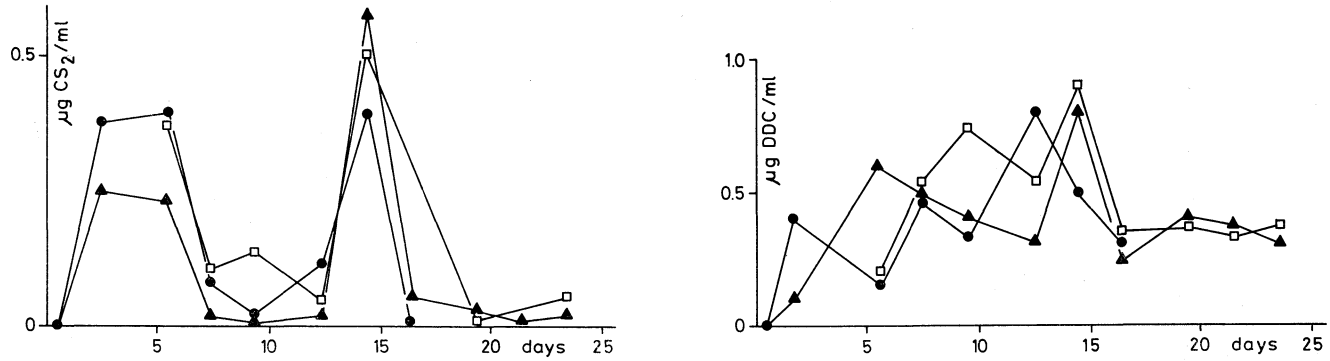


Fig. 6: Blood levels of TETD-metabolites. CS_2 and mixed-disulfide levels (expressed as DDC) in blood of 3 patients taking 200 mg of TETD p.o. per day between 8 and 10 p.m. Blood was taken for analysis at 8 a.m.

the sample to 50° C in a waterbath for 30 min. Determine the CS_2 evolved from DDC and disulfides as mentioned. A differentiation between free DDC and disulfides (TETD and/or mixed disulfides) is possible by adding only formic acid at first liberating CS_2 from free DDC only. Subsequent addition of cystein liberates DDC from its disulfides.

3.7. Within-day precision and recovery

Table 1 shows the results of within-day estimates of precision and recovery for 3 different concentrations of DDC-Na added to blood-samples and for reagent blanks obtained from different blood samples or water. The same values for the reagent blank were obtained when various blood samples were used instead of water. The reagent blanks showed a relatively large variation but remained constant for one batch of reagents and for solutions stored for 2 weeks. A CV of 21 was found for a DDC-Na concentration of 0.1 µg/ml blood. At these low blood levels the method is limited by the large contribution of the blank to the absorbance values of tests. Table 2 shows the good recovery of both CS_2 and DDC-Na added simultaneously to various blood samples.

Table 1: Within-day precision and recovery of DDC added to blood. All analyses were carried out as described under 3.6.1. The figures represent absorbance at 426 nm. [a]) RB water = Reagent blank with 10 ml of distilled water. [b]) RB blood = Reagent blank with 10 ml of blood. Samples 1,2 and 3,4 = 2 blood conserves; samples 5,6 and 7,8 = 2 fresh blood samples. [c]) DDC-Na was added to 10 ml of blood. A value of A = 0.123 was subtracted to correct for reagent blank.

Sample no.	RB[a]) water	RB[b]) blood	DDC-Na concentration µg/ml[c])		
			0.3	1.0	2.0
1	0.118	0.126	0.087	0.267	0.567
2	0.128	0.126	0.091	0.307	0.557
3	0.120	0.122	0.091	0.307	0.577
4	0.130	0.128	0.091	0.307	0.587
5	0.124	0.110	0.096	0.297	0.567
6	0.128	0.128	0.086	0.287	0.567
7	0.126	0.120	0.096	0.287	0.557
8	0.110	0.126	0.096	0.267	0.587
Mean	0.123	0.123	0.092	0.291	0.571
SD	0.007	0.006	0.004	0.017	0.012
CV	5.7	4.9	4.3	5.8	2.1
Recovery %	—	—	105.3	100.1	97.2

Table 2: Recoveries of CS_2 and DDC added to various blood samples. 4.2 µg of CS_2 and 25 µg of DDC-Na were added to 10 ml of fresh blood. Both compounds were determined from the same sample.

Blood sample		CS_2		DDC	
No.	Sex	found (µg/ml)	recovery (%)	found (µg/ml)	recovery (%)
1	M	0.44	105	2.6	104
2	M	0.44	105	2.5	100
3	F	0.42	100	2.5	100
4	F	0.44	105	2.4	96

3.8. Analyses of patient blood

In order to test the practicability of the method within the range of concentration of CS_2 and total DDC in the blood of patients, daily doses of 200 mg TETD were administered between 8 and 10 p.m. to three individuals and blood was taken at 8 a.m. for analysis. The results are shown in Fig. 6. CS_2-concentrations ranged from zero to 0.6 µg/ml and DDC-concentrations from 0.2 to 1.0 µg/ml. It is of interest to note that the CS_2-concentrations varied over a broad range simultaneously in all these subjects. Although the reason for this phenomenon is not known, it seems to be of physiological origin, since experimental errors were excluded by analyses of standard solutions.

4. References

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For the authors: Prof. Dr. J. P. von Wartburg, Medizinisch-Chemisches Institut, Universität Bern, Bühlstr. 28, CH-3000 Bern (Schweiz)