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Development and Improvement of a Commercial Uric Acid Enzymatic Determination Kit on a Centrifugal Fast Analyzer

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Summary: The performance of a uric acid determination kit has been evaluated for five months, under routine conditions, in a General Hospital Biochemical Laboratory.

An anomalous increment in fresh serum blanks was noted in the kit when first introduced.

This interference, probably due to alcohol dehydrogenase contamination, was corrected by addition of 50 mmol/l $(NH_4)_2SO_4$ to the reagent. Results obtained with this modified reagent correlate perfectly with those obtained with modified kits by Smith Kline Instruments (SKI), and with many other determination methods.

Correlations are discussed and explanations for differences in statistical data are offered.

Within run and between run precision data are presented. The kit fits perfectly with the needs of centrifugal fast analyzers and discrete micro analyzers, on account of its speed, reliability and precision.

Entwicklung und Verbesserung eines kommerziellen Testbestecks zur enzymatischen Harnsäurebestimmung an einem Zentrifugalanalysator

Zusammenfassung: Die Brauchbarkeit eines Testbestecks zur Harnsäurebestimmung wurde unter Routinebedingungen im biochemischen Laboratorium eines allgemeinen Krankenhauses über fünf Monate geprüft.

Als das Testbesteck neu eingeführt wurde, wurde ein regelwidriger Anstieg in frischen Serum-Leerwerten bemerkt.

Diese Störung, die wahrscheinlich auf einer Kontamination mit Alkoholdehydrogenase beruht, wurde durch Zugabe von Ammoniumsulfat (50 mmol/l) zum Reagenz korrigiert. Die mit diesem modifizierten Reagenz erzielten Ergebnisse korrelierten völlig mit den mit modifiziertem Testbesteck von Smith Kline Instruments und mehreren anderen Bestimmungsmethoden erhaltenen.

Die Korrelationen werden kommentiert und eine Erklärung von Unterschieden der statistischen Daten wird versucht.

Die Präzision in der Serie und von Tag zu Tag wird dargestellt. Das Testbesteck erfüllt die Erfordernisse von Zentrifugalanalysatoren und diskreten Mikroanalysatoren hinsichtlich Geschwindigkeit, Zuverlässigkeit und Genauigkeit vollkommen.

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Introduction

The rapid and reliable routine determination of uric acid in biological fluids seems to have been for many years an insurmountable problem for Clinical Chemists. Many papers have appeared and a large number of chemical principles have been exploited; some of these are non-specific: phosphotungstate reduction (1), iron (III) reduction (2, 3, 4), copper (II) reduction (5, 6), others are more specific, using uricase in conjunction with redox systems (7–12).

The *Kageyama* method (13) has proved very popular, but it is difficult to apply to fast analyzers, particularly the centrifugal ones; in addition, there are problems of recovery (14).

For the latter type of instrument the only method so far applicable is the determination of uric acid by measurement of the decrease in absorbance at 293 nm due to the destruction of uric acid by uricase (15, 16, 17); this method is troublesome and extremely imprecise under normal routine conditions.

Very recently, a six year old colorimetric method proposed by *Barham & Trinder* (18) has been revived by different workers (19, 20, 21, 22).

Problems of interference by bilirubin and hemoglobin (21–24) and of ascorbic acid (19) in the "*Trinder*'s reaction" have been solved in different ways.

In recent months different versions of a new kind of determination based on the *Haeckel* technique and data (25) have been reported (26, 27, 28). This technique does not suffer from bilirubin, ascorbate and hemoglobin interference. In this paper we discuss the problems encountered in setting up a commercial kit for the uric acid determination[1]) on a CentrifiChem System 300, and we present solutions for these problems.

Another very recently reported method (29), for the same kind of instrument, does not appear to exhibit the same high performance.

Material and Methods

Reagents

- SpinChem reagent test for uric acid, product No. 89517 Smith Kline Instruments (SKI), Inc. Sunnyvale, CA, USA distributed in Italy by Smith Kline & French S. p. A., Milan (SK & F): different lots of trial products, and kit lots No. 615911 and 633111.
- Urica-quant test-combination, enzymatic colorimetric test, product No. 124761 (Boehringer-Biochemia S. r. l. – Divisione Diagnostici – Milan).
- Sera-Pak uric acid, enzymatic colorimetric test, product No. 6379 (Ames-Miles Italiana S. p. A., Cavenago Brianza)[2])
- Uri-300-C, direct colorimetric method for uric acid determination, product No. 60551 (Elvi S. p. A., Division Euro-Chima, Milan).
- Uricase (EC 1.7.3.3) from hog liver 9 U/mg, solution in 500 ml/l glycerol, pH 10.2, 50 mmol/l glycine, 0.13 mol/l sodium carbonate; glutamate dehydrogenase (EC 1.4.1.3) from beef liver, 120 U/mg (= 1.2 MU/l) solution in 500 ml/l glycerol, pH ca. 7; alcohol dehydrogenase (EC 1.1.1.1) from yeast, ca. 400 U/mg enzyme protein; all the above mentioned enzymes were purchased from Boehringer-Biochemia S. r. l. – Divisione Diagnostici –Milan.
- Boric acid, sodium hydroxide, ammonium sulfate, glycerol, glycine, sodium carbonate, all from Carlo Erba – Milan.
- "Glutestere" (γ-ethylester of *L*-glutamic acid, 150 g/l solution) (Maggioni & Co. S. p. A. – Milan).
- Standards: uric acid (Merck) 100 mg dissolved in 50 ml of distilled water containing 60 mg of lithium carbonate, sonicated and made up to a volume of 100 ml (stock uric acid standard, 1 g/liter) stored in 2 ml aliquots at – 20 °C for not more than two months, then discarded; uric acid standard, 357 µmol/l (Ames-Miles Italiana S. p. A., Cavenago Brianza) containing a patented preservative interfering in none of the enzymatic methods tried.

Instrumentation

A CentrifiChem System 300 (Union Carbide, Tarrytown, USA, distributed in Europe by Roche, Basle, Switzerland) was used during the present evaluation.

The reference spectrophotometer was a Unicam SP 1700 provided with a Linear Recorder Unicam AR 25 and with a thermostated cuvette holder (by means of a Thecne C-100 circulating bath) (all provided by Philips S. p. A., Sez. PIT, Monza).

In some instances a Vickers D-300 (Vickers Medical, Basingstoke, England, distributed in Italy by Logos S. p. A., – Milan) was used.

A P6060 Personal Minicomputer (Olivetti, Ivrea, Italy) was used to process the statistical data.

Test procedures

The operation protocol for the CentrifiChem S-300 with the SKI reagents (tab. 1) used during this research was the one suggested by *Alliguie* et al. (31) and independently set up by us, as reported previously (22). The same protocol was also used for on the spectrophotometer, using proportional volume modifications to allow for the characteristics of the instrument.

The characteristics of the uricase from *Aspergillus flavus* (used in the SKI kit) have been outlined by *Laboureur* et al. (30) and by *Tiffany* et al. (16). The same enzyme is used in the Ames Sera-pak uric acid kit.

The protocol for the 293 nm method, as performed on the CentrifiChem, was according to Roche Diagnostica (32) with home-made reagents and hog liver uricase, while the manual 293 nm method was performed according to *Scheibe* et al. (33).

The colorimetric enzymatic Ames test was performed manually according to the procedure suggested by the producer.

The colorimetric (Fe^{++}/*o*-phenanthroline) test was performed on a Vickers D-300, according to the procedure suggested by the manufacturer (2).

The Urica-quant manual test was performed according to the producer (13).

Results

In February 1978, when we started to evaluate the SKI kit on the CentrifiChem, we discovered that data obtained with this procedure were on average 120 µmol/l higher than the values we had previously obtained with

[1]) SpinChem reagent for uric acid – Smith Kline Instruments, Inc.

[2]) *Fossati, P., Prencipe, L. & Berti, G.*, Use of 3,4-dichloro-2-hydroxibenzesulfonic acid/4-aminophenazone chromogenic system in the enzymatic direct determination of serum and urine uric acid: Clin. Chem., in press.

Tab. 1. Procedure for the uric acid determination with SKI reagent on the CentrifiChem S. 300.

Reagent		
Contents of reagent vial are dissolved in 16.0 ml of diluent; about 15 µl uricase solution is added per ml of reagent.		
Pipettor	*Sample ring*	
	Samples and control sera	positions 1–27
	Standard	positions 28–29
	Last-sample	position 0
	Volume (µl)	
	Sample	25
	Sample + diluent	75
	Reagent	250
Analyzer	*Settings*	
	All memory control switches in up-position	
	Temperature:	30 °C
	Filter	340 nm
	Programming controls:	auto blank (write) terminal oper. absorb.
	Absorbance: according to calibration or 500, resp.	
	No. of prints:	5/last
	Concentration:	calculation
	ΔT time interval	1 min
	T_0 time delay	3 s
Calculation	Concentration factor = $\frac{c(S)}{A(S)}$	
	c(S) = Uric Acid concentration of the standard in mg/dl or µmol/l	
	A(S) = mean of absorbances of cuvette 28 and cuvette 29	
	Set concentration of the factor obtained, switch absorb. to conc. and press PRINT.	

the 293 nm method on the CentrifiChem. By performing a serum blank with distilled water in place of the reagent, in the same proportions used in the proper test, and holding this blank in the instrument memory before we performed the test, the difference was lowered, giving a correlation y = 49 + 0.93 x (x = 293 nm method, y = SKI method), r = 0.93 (22).

This approach was, however, completely arbitrary, and the situation therefore remained unsatisfactory. As the blank increment with sera was also observed in properly cleaned glass cuvettes in the spectrophotometer, the hypothesis was rejected of a carry over between different reactants on the teflon lining of the CentrifiChem rotor (this does occur for some inorganic substances e. g. NH_4^+ and phosphate).

We noticed that sera from patients with liver disease, with glutamate dehydrogenase particularly elevated (from 50 to 80 U/l at 25 °C) and infused with "Glutestere", showed an abnormal increase in the reaction blank in comparison to sera containing lower glutamate dehydrogenase concentrations (fig. 1). When we allowed the reaction (without uricase) to continue for a long time (12–20 hours), the spectra shown in figure 2 were obtained by scanning the reaction product against a blank of the same serum. Analysis of these spectra suggests that they are due to NAD(P)H.

The absorbance ratio A 334 nm/A 340 nm agrees with the ϵ ratio obtained from recent data: A 334 nm/A 340 nm = 0.97 and ϵ 334 nm/ϵ 340 nm = 0.98 (34). Presumably the reaction was:

$$\text{Glutamate} + \text{NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{glutamate}} \text{2-oxoglutarate} + \text{NADPH} + \text{NH}_4^+$$

which otherwise is not very favoured (35). We therefore added, to the diluent, $(NH_4)_2SO_4$ at concentrations up to 50 mmol/l.

With this solution we prepared both the blank solution and the reaction mixture (uricase added).

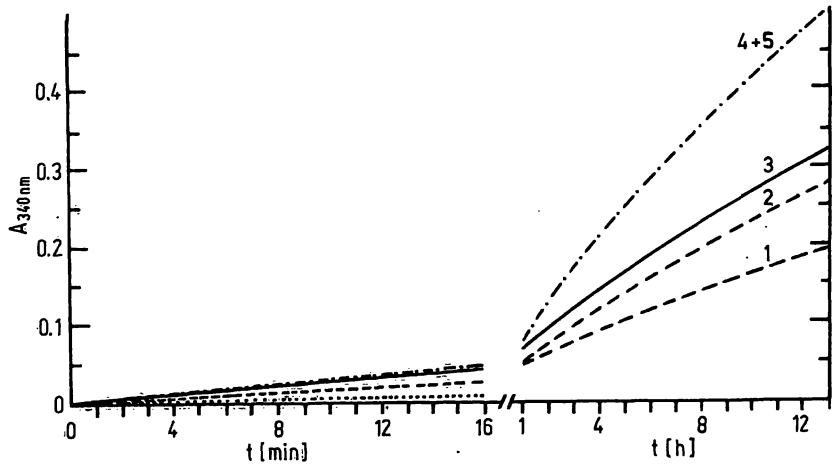

Fig. 1. Reaction rates of sera from normal persons and from patients with liver disease, determined spectrophotometrically with the unmodified SKI reagent without uricase. Glutamate dehydrogenase activity (U/l at 25 °C):
——— serum No. 1 (10 U/l); – – – serum No. 2 (6 U/l); —— serum No. 3 (14 U/l); —·—·— serum No. 4 (50 U/l); —·—·— serum No. 5 (80 U/l); · · · · · Reagent blank.

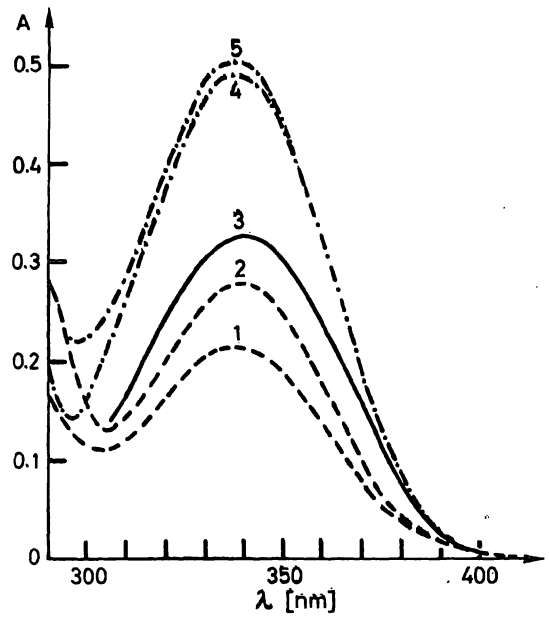

Fig. 2. Spectra of the reaction product of the sera in fig. 1 scanned against their own serum blank, prepared at the moment of scanning, ca. 12 hours after reaction start.

As shown in figure 3 the average blank on 35 sera obtained with reagents as provided by SKI and with reagents modified according to us, have completely different reaction rates on the CentrifiChem. Otherwise the blank reaction rate was independent of the uric acid concentration in the sample.

Moreover, with the unmodified SKI reagent, the blank reaction rates of fresh sera were different from the blank reaction rate of aqueous standards, dialyzed sera with added uric acid and some control sera (fig. 4). Hence the reaction rate with the two reagents, with uricase added, seemed to behave differently (fig. 3 and 4).

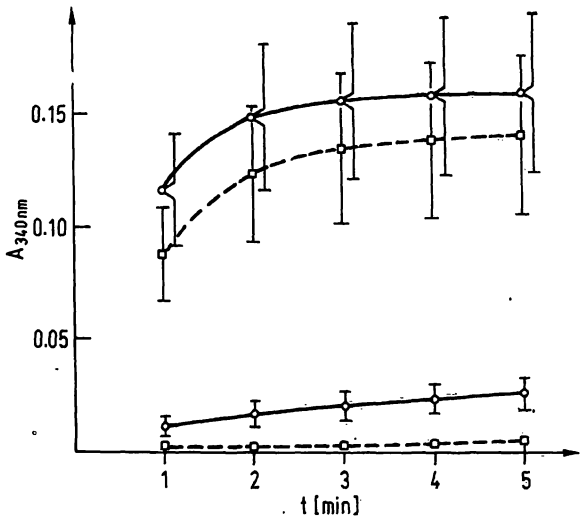

Fig. 3. Average reaction rates for 35 sera:
o—o—o unmodified SKI reagent lot. No. 615911
□—□—□ modified SKI reagent (50 mmol/l $(NH_4)_2SO_4$ added) lot No. 615911;
bottom: sera in reagents without uricase, top: sera in reagents with uricase. 1 SD (±) is shown.

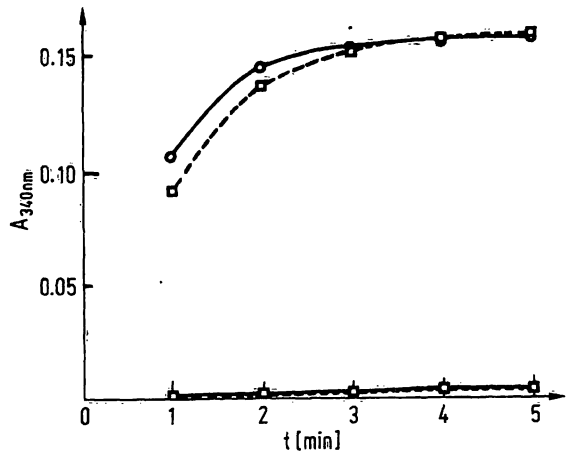

Fig. 4. Reaction rates for 357 µmol/l standards; same symbols as in fig. 3; bottom: standards in reagents without uricase top: standards in reagents with uricase.

Using our modification, excellent correlations were obtained for the SKI kit, when the test was performed (with the same kit) with serum blank subtraction ($A^{5\,th\,min}_{serum\,+\,reagent\,+\,uricase} - A^{5\,th\,min}_{serum\,+\,reagent}$), and when the new kit lot (lot No. 63311) as modified by SKI (table 2) was used.

Tab. 2. Correlations between values obtained with the modified SKI reagent (50 mmol/l $(NH_4)_2SO_4$), lot No. 615911 (y) and values obtained from the same kit (with serum blank subtraction) (procedure A) and values obtained with the new kit, as modified by SKI, lot No. 633111 (procedure B).

	Procedure A (x)	Procedure B (x)
No. of data pairs	35	57
Concentration range (µmol/l)	149 ÷ 476	131 ÷ 786
Regression equation	y = 0.8 + 1.00 x	y = 4.9 + 0.99 x
Correlation coefficient, r	0.985	0.998
SD_d (µmol/l)	11.9	7.7

Comparative studies: precision and accuracy

We compared the data obtained with the modified reagent (diluent plus $(NH_4)_2SO_4$ 50 mmol/l) with different uric acid determination procedures at present available, according to the statistical procedures suggested by *Westgard & Hunt* (36).

None of the compared procedures seems to behave in exactly the same way as the SKI kit. With respect to the official reference method (293 nm method), comparison shows an overestimation by the SKI method in the low concentration range (fig. 5).

This could be due, perhaps, to an equilibrium arising in the reference method procedure, particularly sensitive in the lower concentrations of uric acid, apart from the many other possible pitfalls in the 293 nm test (37).

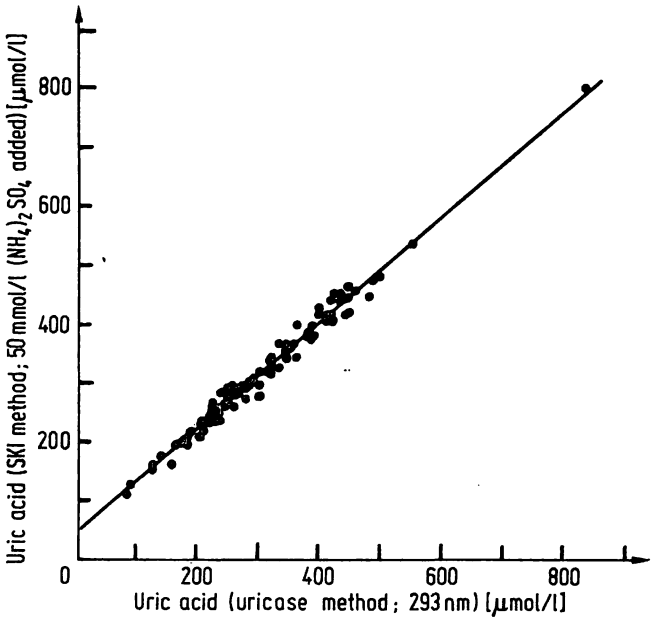

Fig. 5. Correlation between the modified SKI method and the 293 nm manual method.
y = 42 + 0.90 x, r = 0.991, n = 105, SD_d = 17

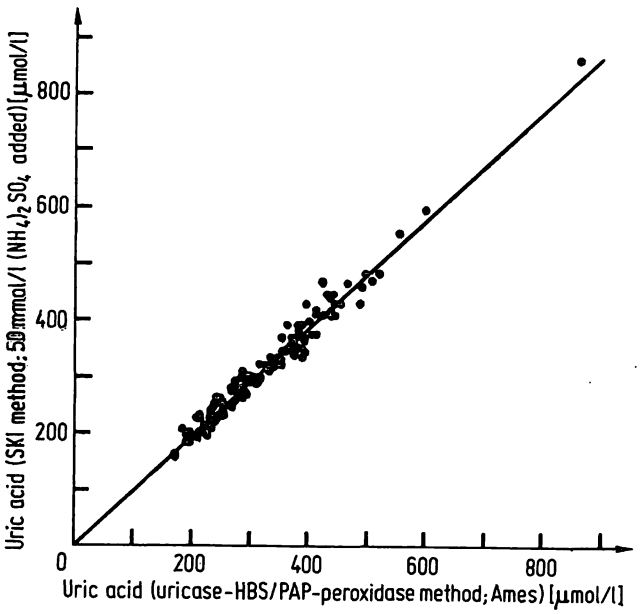

Fig. 7. Correlation between the modified SKI method and the manual Ames Sera-pak colorimetric enzymatic method.
y = 2 + 0.97 x, r = 0.985, n = 125, SD_d = 17.
HBS = 3, 4-dichloro-2-hydroxybenzene sulfonic acid
PAP = 4-aminophenazone

Urica-quant and Sera-pak uric acid seem to give systematic slightly overestimated values throughout the concentration range examined (fig. 6 and 7) in respect to the SKI kit.

There may be several reasons for this: some concern the indicator reactions (e. g. non specificity of catalase and of peroxidase) and others might be ascribed to the SKI test: e. g. incomplete recovery of uric acid in sera (denied by *Haeckel*'s data (25)), or reaction not completed in five minutes. This last possibility was not confirmed by our experimental results.

The comparison with a non specific test, the one using Fe^{++}-*o*-phenanthroline as indicator, is still good (fig. 8). As far as accuracy is concerned, very few control sera are so far available for estimating the test accuracy.

Precilip (Boehringer-Biochemia), Seronorm (Nyegaard & Co. A/S, Oslo) and Target normal and abnormal (SKI) control sera were evaluated on the CentrifiChem. The values were extremely dispersed. Better results were obtained on pooled sera over a period of 80 days (tab. 3). The "in the run" precision is shown in the same table.

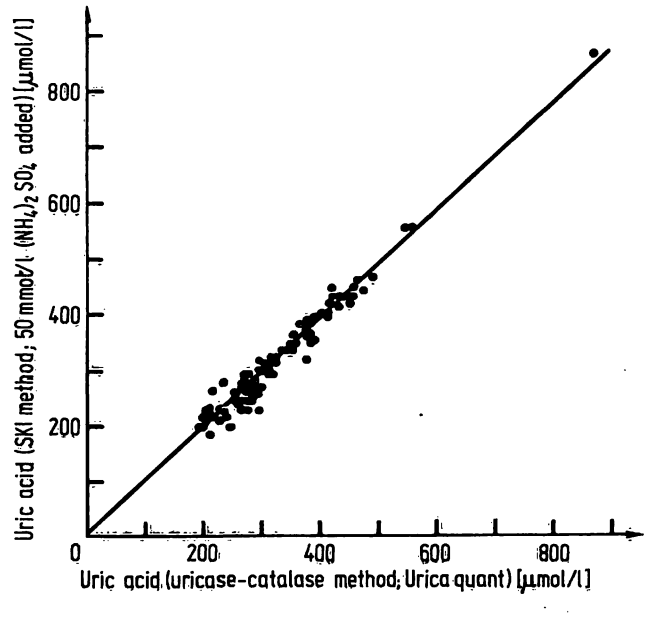

Fig. 6. Correlation between the modified SKI method and the manual Urica-quant method.
y = 3 + 0.97 x, r = 0.979, n = 107, SD_d = 20

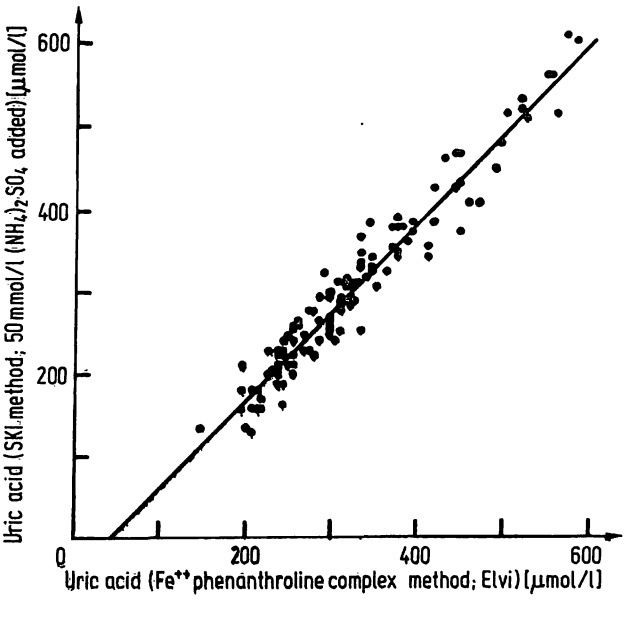

Fig. 8. Correlation between the modified SKI method and a non-enzymatic redox determination method.
y = 43 + 1.08 x, r = 0.970, n = 125, SD_d = 26

Tab. 3. Precision studies for the uric acid determination on CentrifiChem with the modified SKI reagent.

	n	27	27	27
Within-run precision	$\bar{x}$ (μmol/l)	163	346	619
	SD (μmol/l)	8.3	4.8	5.4
	CV (%)	5.1	1.4	0.9
	n	80	12	
Day to day precision	$\bar{x}$ (μmol/l)	273	671	
	SD (μmol/l)	6.5	11.9	
	CV (%)	2.4	1.8	

Discussion

Haeckel pointed out (25) that alcohol dehydrogenase could interfere in the test, when present in sera of hepatic patients; but the same interference would be observed if alcohol dehydrogenase were present as a contaminant in the reagent.

With the aim of demonstrating the validity of this last hypothesis, we added in high serial concentrations alcohol dehydrogenase (from yeast) to pooled normal sera and we obtained the reaction rates which are shown in figure 9. Nevertheless the reaction trend is different for the two reagents, i. e. the unmodified (fig. 9) and

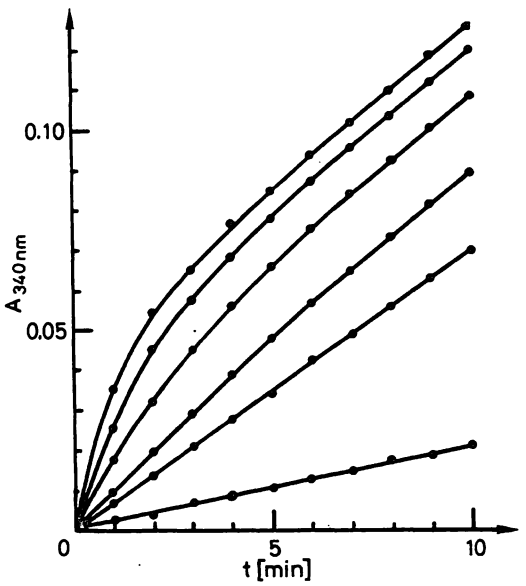

Fig. 9. Reaction rates on CentrifiChem for pooled fresh sera with added alcohol dehydrogenase (from yeast) in unmodified SKI reagent; alcohol dehydrogenase added (U/l): 0 (bottom curve); 250; 500; 1000; 2000; 4000 (top curve).

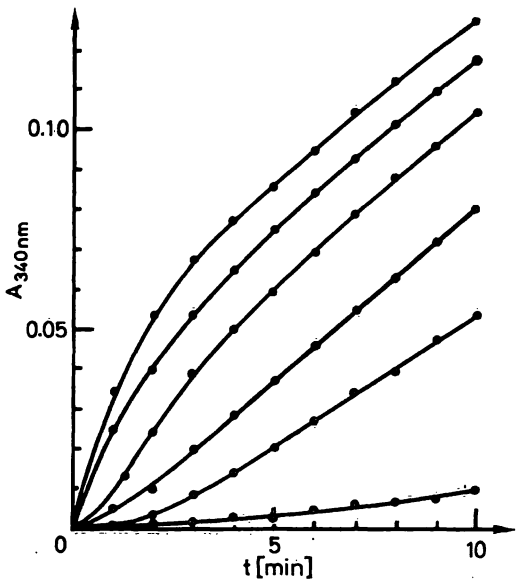

Fig. 10. Reaction rates on CentrifiChem for pooled fresh sera with added alcohol dehydrogenase (from yeast) in modified SKI reagent (50 mmol/l $(NH_4)_2SO_4$ added). Same alcohol dehydrogenase additions as in fig. 9.

the modified (fig. 10), particularly at lower alcohol dehydrogenase concentrations. One possibility is that the reaction lag-phase is longer in the presence of $(NH_4)_2SO_4$ 50 mmol/l at moderate alcohol dehydrogenase concentrations.

Alcohol dehydrogenase uses as its proper coenzyme NAD^+, but can also utilize $NADP^+$, particularly when the enzyme is present in large excess (38).

We presume, however, that when alcohol dehydrogenase is present in short supply, its elective coenzyme should be NAD^+.

This nucleotide, present in low concentrations in sera, should be necessary, in this case, to carry on the reaction sequence shown in figure 11.

Glutamate dehydrogenase provides the reaction catalyzed by alcohol dehydrogenase with reformed NAD^+.

When NH_4^+ is present in excess, a larger quantity of oxidized nucleotide is formed in the reaction catalyzed by glutamate dehydrogenase, delaying for some time the appearance of the reduced nucleotide. 2-oxoglutarate has a similar effect to NH_4^+, with respect to the blank increase, but the addition of this substrate gives extremely high absorbances at 340 nm, when used at the same concentrations as $(NH_4)_2SO_4$.

This fact, apart from the lower stability of such a substrate, makes the use of NH_4^+ preferable.

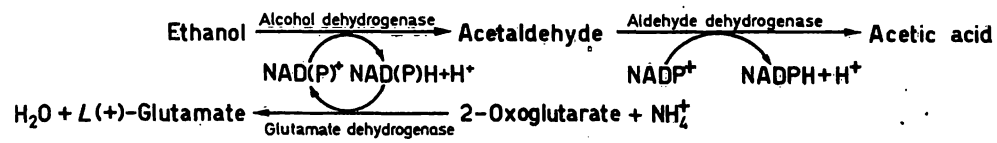

Fig. 11. Proposed mechanism of alcohol dehydrogenase/glutamate dehydrogenase interference and interplay of NH_4^+ ions.

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