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Quantitative Determination of Hemoglobin in Urine

1. The inhibitory effect of urine on the peroxidase-like activity of hemoglobin and on horseradish peroxidase[1])

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A method was developed to determine in urine microgram quantities of hemoglobin, based on its ability to catalyze peroxide oxidation of o-tolidine. It was recognized that urine contains potent inhibitors to the reaction. These inhibitors are of two classes: 1. high molecular weight (non-dialyzable) and 2. low molecular weight. The latter group contains substances such as uric acid and ascorbic acid. In order to measure micro amounts of hemoglobin in urine, it is necessary to separate it from some of the inhibitors by gel filtration through Sephadex G. 50-Some of the peroxidase-like activity is lost during the gel filtration unless haptoglobin is added to the urine before filtration. This is a loss in activity but not in protein. In the o-tolidine system as used in this report, haptoglobin apparently depresses the peroxidase-like activity of hemoglobin; while in a guaiacol system, haptoglobin enhances the activity. When sodium alginate is added to the o-tolidine system, haptoglobin potentiates the hemoglobin catalyzed reaction. A method based on these observations is suggested.

Es wurde ein Verfahren entwickelt, Hämoglobin im Harn in Mikrogramm zu bestimmen, das auf der Fähigkeit des Hämoglobins beruht, die Peroxyd-Oxydation von o-Tolidin zu katalysieren. Hierbei wurde festgestellt, daß Harn starke Inhibitoren gegen diese Reaktion enthält. Diese Inhibitoren unterteilen sich in zwei Klassen, nämlich in 1. hochmolekulare (nicht dialysierbare) und 2. solche von niedrigem Molekulargewicht. Die letztere Gruppe enthält Substanzen wie Harnsäure und Ascorbinsäure. Um kleinste Mengen von Hämoglobin im Harn zu bestimmen, ist es notwendig, es von einigen Inhibitoren durch Gelfiltration mit Sephadex G-50 abzutrennen. Etwas von der Peroxydase-ähnlichen Aktivität geht durch die Gel-Filtration verloren, es sei denn, daß Haptoglobin vor der Filtration dem Harn zugegeben wird. Dies ist ein Verlust an Aktivität, aber nicht an Eiweiß. Im o-Tolidin-System, wie es in dieser Arbeit verwendet wird, vermindert Haptoglobin offenbar die Peroxydase-ähnliche Aktivität des Hämoglobins, während Haptoglobin in einem Guajakol-System die Aktivität vermehrt. Wenn Natriumalginat zum o-Tolidin-System hinzugefügt wird, verstärkt Haptoglobin die von Hämoglobin katalysierte Reaktion. Ein Verfahren, das auf diesen Beobachtungen beruht, wird vorgeschlagen.

It was the aim of this investigation to develop a method for the quantitative determination of small amounts of hemoglobin[3]) (microgram-quantities) in urine. No completely satisfactory method for this purpose has yet been published. Chemical methods such as measurement of hemoglobin as cyanmethemoglobin (1) or pyridine-hemochromogen (2) are too insensitive detecting only miligram quantities, and there are certain restrictions in their application to urine. More sensitive methods are based on the so-called peroxidase-like activity of hemoglobin — its ability to catalyze the hydrogen peroxide oxidation of certain substance. The most widely used chromogen is benzidine (3) which will detect μg-quantities, but the reagent is unstable and the reproducibility of the determination is poor. o-Tolidine as the chromogen proved to be very satisfactory and was used for the development of the presented method. Just prior to our first publication (4) BEAU (5) described a method with o-tolidine, and recently LEWIS (6) reported on the successful use of this chromogen.

Methods

Standard method for the determination of „Hgb"

Solutions:

o-Tolidine-buffer-reagent: 0.05% o-tolidine-2HCl in 0·1M tartrate buffer pH 4,2
Hydrogen peroxide 15%

Test procedure

2 m*l* o-tolidine reagent and 1 m*l* Hgb-solution (1—10 μg Hgb) are pipetted into a 10 mm cuvette. After mixing with a polyethylene spatula the max. absorbance is set to 0.000 (635 mμ); 500 μl H_2O_2 are added quickly with a constriction pipette with simultaneuos mixing with a polyethylene spatula. The maximum absorbance which is reached after 2—3 minutes, serves as a parameter for the peroxidase-like activity of hemoglobin. The same procedure is applicable for myoglobin and horseradish peroxidase, although for the latter a different parameter for the calculation of the activity has to be used (see below).

Gel filtration with Sephadex

Sephadex G-50 columns (0.8×20 cm) were prepared with 0.9% saline. 1—5 m*l* urine or Hgb-solution were added to the columns and followed by saline. Two m*l* fractions were collected.

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[1]) Presented in part at the 143rd National Meeting of the *American Chemical Society*, Cincinnati, 1963.
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[2]) Career development awardee U. S. Public Health Service 5-K3-GM-15,1524.

[3]) *Abbrevations used in this work:* Hgb: hemoglobin; Hp: haptoglobin; HRP: horseradish peroxidase; Mgb: myoglobin.

Haptoglobin ("Hp")

Human Hp is prepared for us by Dr. Hs. NITSCHMANN and Mr. H. P. STAUFFER, Bern, Switzerland, under a research contract with Ames Company. The preparations tested so far contain 50—70% Hp and are essentially free of Hgb.

Results

Reaction curve

A typical reaction curve with Hgb, o-tolidine and H_2O_2 is given in figure 1. The color develops fast, reaches a maximum and is stable for a few minutes. Thereafter (not shown in the figure), the color begins to fade.

Relationship between max. absorbance and Hgb concentration

The relationship between the Hgb concentration and the max. absorbance is proportional (fig. 6). Although it would seem possible to use the initial velocity of the reaction as a parameter it is our experience that the slope of the tangents to the initial part of the curves is not in a straight relationship to the concentration of Hgb.

Reproducibility of the Hgb determination

The reproducibility of the determination depends largely on the mixing during the addition of H_2O_2. With proper mixing the coefficient of variation was $\pm$ 1% (n = 11) with 5 µg Hgb per test. When the mixing was done immediately *after* the addition of H_2O_2 the coefficient of variation was between $\pm$ 4 and $\pm$ 10%.

Determination of HRP activity

In contrast to Hgb (and myoglobin) the color development continues over a longer period of time and does not reach a maximum within 5—10 minutes (fig. 2). Under the conditions of the test the curves have a straight initial part. Readings are taken every 30—60 seconds or the curve is recorded, and Δ absorbance/min. is calculated from a tangent to the initial part of the curve. Δ absorbance/min. plotted against the concentration of HRP gives a straight line.

Application of the Hgb-test to urine

When the test was applied to urine, it became obvious that urine contains potent inhibitors for the peroxidase activity of Hgb, Mgb and HRP. When known amounts of Hgb (between 1 and 10 µg/ml) were added to urines and 1 ml was used per test, the inhibition ranged from 50 to 100%. Dialysis removed only some of the inhibitors, indicating that two kinds of inhibitors are present in urine, some that can be removed by dialysis and others that have probably a higher molecular weight and do not pass through the dialysis membrane (Visking tubing). To test this hypothesis, urines were fractionated on Sephadex-G-50 columns. After 5 ml of urine had entered the column, elution was carried out with 1% saline, 2 ml fractions being collected. Known amounts of Hgb or HRP were added to each fraction. The activity of both peroxidases was measured and expressed as per cent of the activity measured in saline. The results of such an experiment are shown in figure 3. Fractions 3—8 inhibit between 10 and 25% and fractions 12—16 almost 100%. The degree of inhibition with fractions 3—8

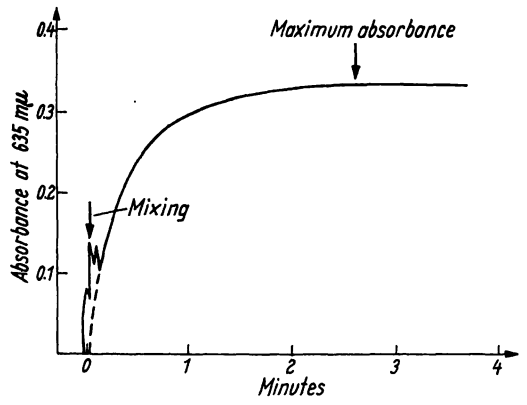

Fig. 1
Recorded reaction curve of Hgb-peroxidase activity. 4 µg Hgb

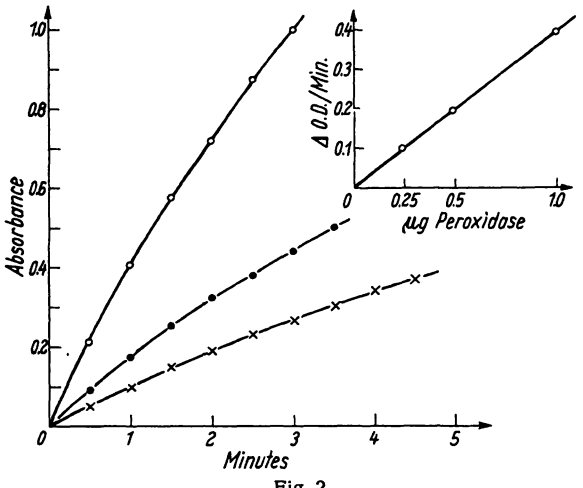

Fig. 2
Reaction curves of horseradish peroxidase. 1.0 (O), 0.5 (•) and 0.25 µg (×) peroxidase. Insert: activity expressed in Δ absorbance/min. vers. amount of peroxidase

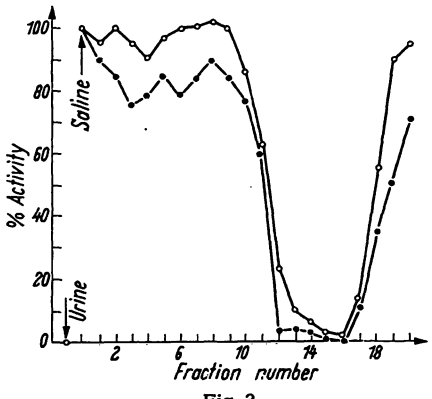

Fig. 3
Activity of Hgb-peroxidase (•) and horseradish peroxidase (O) in urine, saline and urinary fractions (2 ml) collected during gel filtration with a Sephadex 50 column. Activity in saline = 100%

varies somewhat from urine to urine and was found to be as high as 40% in some urines for Hgb. Although the activity of HRP is only slightly effected by these fractions; in other experiments the inhibition was of the same degree as with Hgb. Since we do not yet have knowledge of the chemical nature of the inhibitors, we can not decide whether or not Hgb and HRP are inhibited by the same substances. The experiment was repeated with a urine after dialysis against water. The inhibition in fractions 12—16 was no longer present, while the inhibition in the earlier fractions was unchanged. By dialysis the inhibitors in fractions 12—16

were removed, while the inhibitors in the earlier fractions were non-dialysable. This proves that two kinds of inhibitors for peroxidase are present in urine. Since Sephadex 50 was used, which excludes substances of mol. weight greater than 8—10000 from adsorption, the inhibitors in the earlier fraction must have a mol. weight of at least this order of magnitude.

The separation of Hgb from the inhibitors by gel filtration with Sephadex

According to its molecular weight Hgb passes rapidly through Sephadex 50 columns. The pattern of elution of the inhibitors suggested that a separation of Hgb from the inhibitors should be possible. In preliminary experiments with Hgb-solutions we found that the recovery of small amounts of Hgb from Sephadex columns is only 20—30%. The recovery was greatly improved when haptoglobin was added to the Hgb solution. The Hgb-Hp complex does not pass faster through the column than Hgb alone, but Hp seems to protect the peroxidase activity. This will be discussed in the next paragraph. To check if the separation of Hgb from urine is possible by Sephadex filtration the following experiment was done, as depicted in the flow diagram:

250 μg Hgb added to 5 m*l* urine → recovery of Hgb peroxidase 10%

↓	↓
put on Sephadex 50 column	500 μg Hp added (50% pure) and mixture put on column
↓	↓
eluted with 1% saline	eluted with 1% saline
↓	↓
2 m*l* fractions collected	2 m*l* fractions collected
peroxidase-like activity in fractions 5—8 with max. in 6. Recovery 38%, (in contrast, protein recovery was almost 100%).	Peroxidase-like activity in fractions 5—8 with max. in 6. Recovery 99%.

To fractions 9—13 20 μg of Hgb was added and the following inhibition was found: fractions 9 = 10%; 10 = 10%; 11 = 30%; 12 = 50%; 13 = 100%.

This experiment clearly demonstrates, that Hgb in urine can be separated from the inhibitors by gel filtration and the recovery can almost reach 100%. But it has to be pointed out that the recovery was not always that good and semed to vary from column to column for reasons unknown. Almost constant recoveries were obtained with one and the same column and the columns have to be standardized in regard to the recovery by using a standard Hgb solution. The recovery depends further on the concentration of Hgb. With 10 μg/m*l*, or 50 μg added to the column in 5 m*l*, the recovery was with most columns between 60 and 70% in the presence of Hp. The columns can be used repeatedly. Although Hgb comes through the column in the fractions which contain the non-dialysable inhibitors, these have no significant effect on the activity of the Hgb-Hp-complex. When Hgb-Hp is added to these fractions no inhibition is observed in contrast to the inhibition of Hgb alone.

The peroxidase-like activity of the Hgb-Hp-complex

It was known that Hp activates the peroxidase-like activity of Hgb, when guaiacol is used as a substrate (7). In contrast, we found an apparent inhibition in the test with o-tolidine. The max. absorbance was considerable lower in the presence of Hp (fig. 4). The initial velocity seems to be increased, but the curve bends rather sharply and the absorbance is stable for a few minutes before the blue color fades. The peroxidase-like activity of Hgb decreases with increasing amounts of Hp, until saturation of Hgb with Hp is reached (fig. 5). This is a reversed relationship as compared with the guaiacol test, where the activity increases with increasing amounts of Hp, until saturation is reached.

The max. absorbance in the presence of Hp is directly proportional to the amount of Hgb (as Hgb-Hp-complex) present and serves as a parameter of the Hgb-concentration (fig. 6). Lupovitch and Zack (8) also found inhibition of the Hgb-peroxidase in the presence of Hp with o-dianisidine as substrate. We will show in a paper in preparation that the inhibitory effect of Hp depends on the concentration of H_2O_2. With lower concentrations of H_2O_2 Hp activates the peroxidase activity of Hgb.

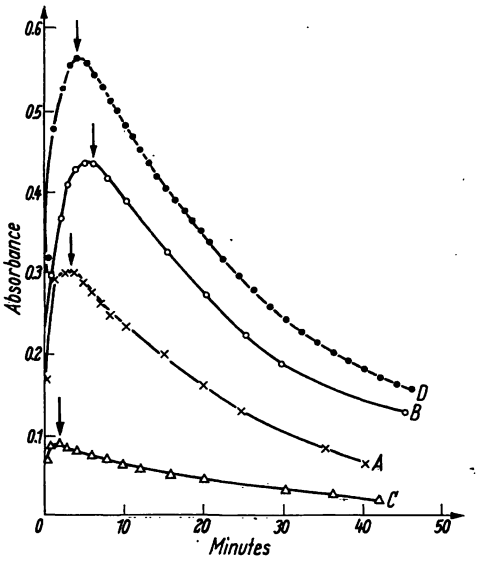

Fig. 4
Reaction curves of Hgb-peroxidase (3.3 μg) in the presence of saline (A), 0.025% Na-alginate (B), 6,6 μg haptoglobin, approx. 50% pure and free of Hgb (C) and haptoglobin Na-alginate (D)

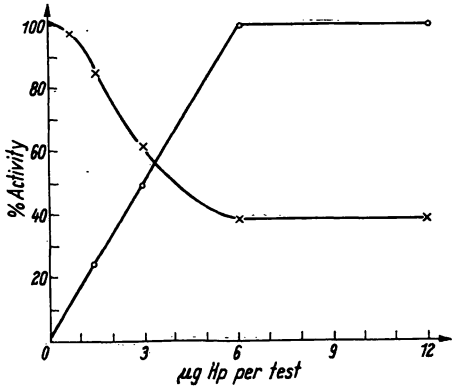

Fig. 5
Effect of haptoglobin (approx. 50% pure and free of Hgb) on the peroxidase activity of Hgb (3 μg), with o-tolidine (×) and guaiacol (○) as substrate

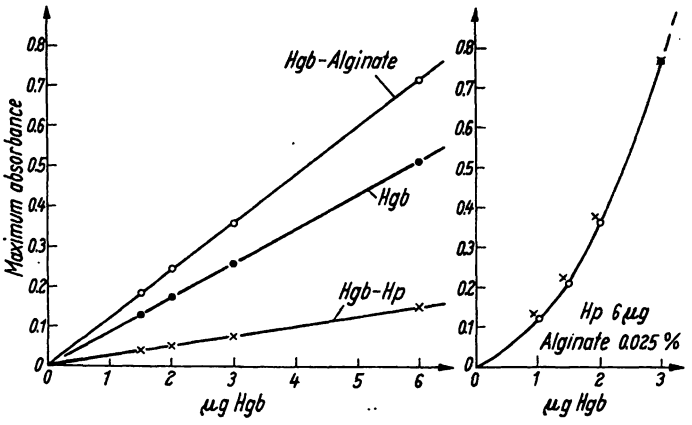

Fig. 6
a) Activity of Hgb-peroxidase (max. absorbance) with and without Hp (12 µg, approx. 50% pure, free of Hgb) or Na-alginate (0.025)%
b) Same as a) but with Hp and Na-alginate (2 experiments)

The effect of sodium-alginate on the peroxidase-like activity of Hgb and the Hgb-Hp-complex

From previous experience in our laboratories Na-alginate was known to stabilize the blue color developed from o-tolidine in the presence of H_2O_2 and a peroxidase. When added to our assay-system. Na-alginate delayed the color development in the presence of Hgb somewhat, led to a slightly higher max. absorbance and delayed the fading of the blue color (fig. 4 and 6). Na-alginate, when added to the assay system containing the Hgb-Hp-complex, counteracted not only the inhibition caused by Hp, but increased the initial velocity and led to a max. absorbance which was higher than with Hgb and Na-alginate alone (fig. 4). While the max. absorbance is directly proportional to the amount present of Hgb, Hgb-alginate and Hgb-Hp (fig. 6a), a non linear relationship was found with Hgb-Hp-alginate (fig. 6b). This curve is reproducible and serves as a standard curve for the calculation of Hgb concentrations.

About the nature of the inhibition

With horseradish peroxidase the reaction curves with fractions 13—17 show some peculiarities (fig. 7). No increase in absorbance occurs for the first few minutes, followed by a gradual increase in color development. Curves 13, 14 and 15 are S-shaped, which means that after the delay period some o-tolidine is oxidized, but the reaction then slows down again. Curves 16 and 17 reach a slope after the delay period, which is almost identical to the control curve without inhibitors. The delay period speaks for the presence of one or more substances that are oxidized preferably to o-tolidine. The delay period was found in all our experiments in the strongly inhibitory fractions. The length of the period varied from urine to urine from 1 to 5 minutes and we found that it depends on two factors, the amount of inhibitor present and the activity of the peroxidase. With less peroxidase added to the test the delay period increases, showing that the inhibitor(s) in question are enzymatically oxidized. The estimation of the activity in figure 3 was done by calculating the slope of the initial part of the curve, which in these fractions is slow because of the delay.

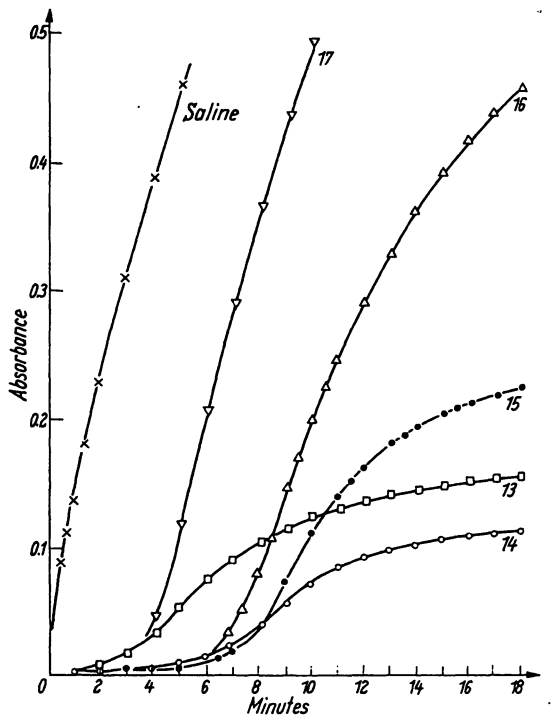

Fig. 7
Reaction curves of horseradish peroxidase, added to urinary fractions collected during gel filtration with a Sephadex 50 column. The fraction numbers correspond to the numbers in figure 3

With Hgb no "abnormalities" of the reaction curves were observed. The inhibition effect was just a lowering of the max. absorbance. Two known inhibitors of peroxidases occur in urine, uric acid and ascorbic acid. The effect of these two substances was therfore investigated.

Uric acid: The reaction curves with horseradish peroxidase have no delay period; they run straight for about one minute, after which time the speed of the reaction slows down. The inhibition was measured as the slope of the initial straight part of the curve (tab. 1). 1 m*l* of the uric acid solution of the highest concentration used contained 500 µg which is 50 mg/100 m*l* and is regarded as the mean concentration in a 24 hour urine specimen. Two effects of uric acid on the Hgb peroxidase were observed: 1. The blue color of the oxidized o-tolidine is not as stable as in the absence of uric acid and fades right after the max. absorbance has been reached; 2. The max. absorbance is lower, but is reached after a shorter period of time (table 2). Hp has no effect on the inhibition by uric acid.

Tab. 1
Inhibition of horseradish peroxidase by uric acid
Standard test conditions

µg peroxidase	µg uric acid	initial velocity absorbance/min.	% activity
.48	0	.195	100
	125	.110	56
	250	.080	41
	500	.060	31
.98	0	.390	100
	125	.220	56
	250	.160	41
	500	.105	27

Tab. 2
Inhibition of Hgb- and Hgb-Hp-peroxidase by uric acid
Standard test conditions. Hgb: 10 μg per test; Hp: 20 μg per test

μg uric acid	max. absorbance	min. to reach max. absorbance	% activity
Hgb only			
0	.870	3	100
125	.360	1.5	41
250	.290	1.5	34
500	.240	1.	28
Hgb-Hp			
0	.400	.5	100
62.5	.280	.5	70
125	.235	.5	59
250	.165	.5	41
500	.130	.5	32

Rapid fading of the color in the presence of uric acid after max. absorbance was reached.

Ascorbic acid: Both the reactions with horseradish peroxidase and with Hgb have a delay period. The curves for peroxidase (fig. 8) show a delay period which increases with the concentration of ascorbic acid. At the end of this period the slope of the curve is almost as steep as in the absence of ascorbic acid. With Hgb the max. absorbance after the delay period is depressed (tab. 3), higher concentrations of ascorbic acid inhibit the Hgb peroxidase completely. In the presence of Hp the Hgb peroxidase is more sensitive to the inhibition by ascorbic acid.

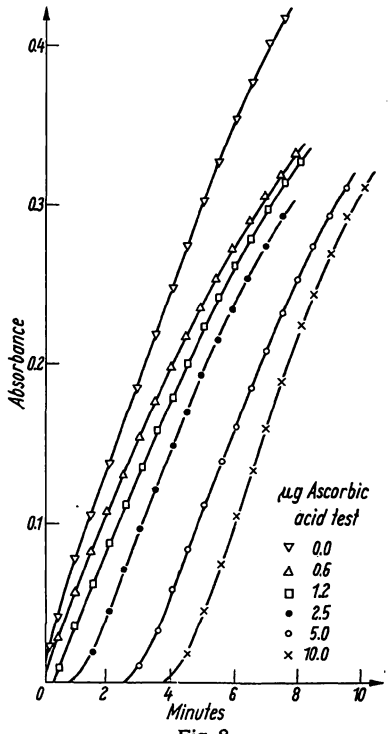

Fig. 8
Reaction curves of horseradish peroxidase in the presence of various amounts of ascorbic acid.

The curves found with HRP and Hgb added to the urinary fractions have some resemblance to the curves just described. With HRP there is a similar delay period as with ascorbic acid and with some of the fractions the curves show a slow down of the reaction as in the experiments with uric acid, but no ascorbic acid was detectable in any of the urinary fractions. Any ascorbic acid originally present was probably in the form of dehydroascorbic acid which does not effect the activity of horseradish peroxidase, but which has some effect

Tab. 3
Inhibition of Hgb- and Hgb-Hp-peroxidase by ascorbid acid and dehydroascorbic acid. Standard test conditions. Hgb: 10 μg per test; Hp: 20 μg per test

μg ascorbic acid	delay period (min.)	max. absorbance	% activity
Hgb only			
0	0	.870	100
6.25	0	.470	54
12.5	.5	.340	39
25	1	.200	23
50		0	0
100		0	0
Hgb-Hp			
0	0	.400	100
3.1	0	.250	62
6.25	0	.125	31
12.5		0	0
25		0	0
50		0	0
100		0	0
μg dehydro-ascorbic acid Hgb only			
0	0	.870	100
25	0	.635	73
50	0	.490	56
100	0	.370	42

on the Hgb peroxidase (tab. 3). The curves with Hgb and dehydroascorbic acid have no delay period, but the max. absorbance is depressed.

Fractions 13—16 (fig. 3) inhibit Hgb peroxidase and horseradish peroxidase almost completely, but uric acid alone cannot be responsible. The concentration of uric acid in these fractions, measured with the uricase method, was between 100 and 250 μg/ml (tab. 4). Since 1 ml of each fraction was added to the peroxidase test mixture, a direct comparison can be made with the results in tables 1 and 2. Even in the presence of 500 μg uric acid per test horseradish peroxidase and Hgb peroxidase still are about 30% active. Since no ascorbic acid was found in the fractions, other yet unidentified substances must add to the inhibition; dehydroascorbic acid might be one of them. Urea in concentrations present in urine has *no* effect on the peroxidase activities.

Tab. 4
Uric acid contents of the urinary fractions 11—18 (fig. 3)

Fraction Nr.	μg uric acid/ml
11	2
12	40
13	200
14	246
15	213
16	107
17	38
18	9
urine	429

From one experiment the urinary fractions 3—9 (non dialyzable inhibitors) and 12—16 (dialyzable inhibitors) were pooled, and lyophylized. The material was dissolved in a little water and spot tested. The non dialyzable group contained traces of protein, identified as albumin, which has no inhibitory effect. In addition other organic material was present but not yet identified. The second group contained various inorganic ions, including NH_4^+, none of which inhibited in concentrations present in urine. In addition reducing substances other than glucose were found, one of which was uric acid. As mentioned before, no ascorbic acid was found, neither in the dry material nor in the original fractions.

Discussion

The test is capable of detecting less than 1 μg Hgb/ml (0.1 mg/100 ml) solution, but inhibitors present in urine prevent the direct application to untreated urine. Dialysis removes only some of the inhibitors. Only if the amount of Hgb in urine is much higher (not less than 50 mg/100 ml) permitting a dilution of the urine of about 1:500 can the determination be done directly, because at such a dilution the inhibition is ineffective. The dilution of 1:500 is a safeguard. It is our experience that the degree of dilution necessary to eliminate inhibition varies from urine to urine and ranged from 1:100 to 1:500. Dialysis of solutions with low concentration of Hgb leads mostly to a considerable loss of Hgb. When urines containing 10 μg Hgb/ml were dialyzed overnight against saline or distilled water, up to 50% of the peroxidase-like activity was lost. This can be due either to inactivation of the peroxidase and/or adherence of the protein to the dialysis membrane (Visking tubing). The impermeability of this tubing for Hgb was tested by pressure filtration. For two reasons then, incomplete removal of the inhibitors and loss of activity, dialysis was ruled out for preparing urine for the determination of Hgb. The use of gel filtration with Sephadex is a time consuming procedure, but no better way to separate Hgb from the inhibitors has yet been found.

Crosby and Furth (3) used benzidine as substrate (chromogen). They use 20 μl of urine in a total of 2.02 ml, which is already a 1:100 dilution; in addition the urine was dialysed to remove urates, sulfates and other substances that form insoluble complexes and not only interfere with the color development but also cause turbidity. The authors do not mention inhibition of the peroxidase activity specifically, but it is our experience that the reaction with benzidine as chromogen is inhibited in the very same manner as with o-tolidine. We did not observe any turbidity with benzidine, but our test conditions were somewhat different. The recovery of Hgb added to saline or urine was 85% after dialysis and definitely better than in our experience. But the amounts of Hgb used in our experiments were 10 μg/ml versus 200 μg/ml in Crosby's work. A method very similar to Crosby and Furth (3) but with o-tolidine as substrate was published by Beau (5) shortly before our first report. Beau found dialysis unnecessary. The recovery of Hgb added to various urines ranged from 85 to 100%. Again the dilution in the test mixture was 1:100 and probably eliminated the inhibition in most of the urines. We added 100 μg Hgb/ml to 28 urines. After a dilution of 1:100 with saline the recovery was between 90 and 100% with only 10 urines. The recovery in the remaining urines was between 25 and 75%. Neither Crosby and Furth, nor Beau control the pH of the test mixture, which was measured to be around 2.5. They always include a Hgb standard for the calculation, thus eliminating changes due to variation in test conditions. We repeated Beau's method and found, not only a deviation from linearity of the standard curve (10, 20 and 40 mg Hgb/100 ml), but also a very poor reproducibility; duplicates varied up to 25%. It is our experience that the performance of the test at a controlled pH leads to reproducible results and permits calculation with a standard curve, obtained with Hgb solution from lyzed erythrocytes standardized with the cyanide method. — Both authors dilute the test mixture after incubation prior to photometry, thus decreasing the sensitivity of the test. In our system the absorbance is followed directly until the max. absorbance is reached after 2—3 minutes.

Still another modification of the method of Crosby and Furth (3) substituting o-tolidine for benzidine was recently published by Lewis (6). The author also incubates in a non buffered medium, which has a pH of about 2. At the end of the incubation period the medium is diluted with either 10% acetic acid (final pH of the mixture given with 2.2) or, when a greater sensitivity is required, with citrate buffer pH 4.96 (final pH 3.75). The absorbance is measured at 630 mμ. We measured the absorbance of the color complex at various pH at 635 and 435 mμ, which are the absorption maxima for the blue color at pH 3.75 and the yellow color resulting from acidification (fig. 9). It is obvious, that a measurement at 635 mμ with pH 2.2 is much less sensitive than with pH 3.75. The measurement with pH 2.2 at 435 mμ would not only be much more favorable but also more sensitive than at 635 mμ with a pH of 3.75. The same applies to the method of Beau (5). A more detailed discussion on the Hgb peroxidase reaction at lower pH and the pH-dependent color change will be included in our second paper, which is in preparation.

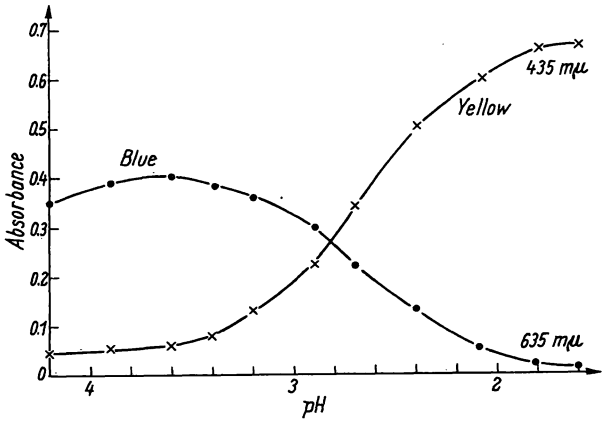

Fig. 9
Dependence of the absorbance of the primary reaction product of Hgb-peroxidase on pH at 635 (•) and 435 mμ (×)

The differences in the effect of haptoglobin on the hemoglobin catalyzed oxidation of guaiacol and that of o-tolidine by hydrogen peroxide has always been difficult for us to understand. Apparently the explanation lies in the instability of the blue oxidation complex of o-tolidine. Sodium alginate stabilizes the color so that the potentiating effect of haptoglobin as described for the guaiacol system can be observed in the o-tolidine system. This will be dealt with further in a later paper.

Recommended procedure for the determination of Hgb in urine

Solutions: as for the standard method, described under methods. In addition reagent-buffer with Na-alginate: To 100 ml 0.1M tartrate-

buffer pH 4.2 add 4.5 mg Na-alginate and heat until the alginate is dissolved. After cooling to room temperature add 50 mg o-tolidine 2 HCl.

Standard curve for Hgb: lyze freshly obtained erythrocytes with destilled water, centrifuge and determine Hgb-concentration in the supernatant with the cyanide method (1). Prepare dilutions from 1—10 µg Hgb/m*l* with saline and measure the peroxidase activity with the standard method. A straight line should be obtained by plotting max. absorbance over the amount of Hgb (compare fig. 6a).

Standard curve for Hgb-Hp in the presence of Na-alginate: Prepare Hp-solution with 8 µg pure Hp/1 m*l* dest. water (the actual amount of the Hp-preparation depends on its purity). Mix equal volumes of the Hp-solution and a standardized Hgb-solution, containing 8 µg Hgb/m*l*. 1 m*l* of the mixture contains 4 µg Hgb as Hgb-Hp-complex. Prepare dilutions 0.5—4 µg Hgb/m*l* and measure the peroxidase activity as described, but substitute the plain o-tolidine-tartrate-buffer with the alginate containing solution. In plotting max. absorbance over the amount of Hgb a curve as shown in figure 6b will be obtained.

Unknown urine: Dilute urine 1:500 with saline and use 1 m*l* in the standard method. If the max. absorbance is at least .1, read Hgb-concentration from standard curve and multiply by 500. If the Hgb-concentration in the urine is too low to permit a 1:500 solution (no measurable reaction), the urine has to be treated with sephadex.

Prepare a Sephadex-G-50 column (0.8×20 cm) with 1% saline. To 0.5 m*l* urine add 2.5 mg Hp (calculated as pure Hp) and mix well. Put the 5.0 m*l* on the Sephadex column, elute with 1% saline and collect 2.0 m*l* fractions (approx. 10). Take an appropriate aliquot (up to 1.0 m*l*) of each fraction and measure the peroxidase activity using the reagent-buffer with Na-alginate. With aliquots less than 1.0 m*l* make up the volume difference with saline. For each fraction the amount of Hgb per 2.0 m*l* fraction is calculated (standard curve c). The sum is the amount of Hgb per 5.0 m*l* of urine.

For each Sephadex column the percentage of recovery of Hgb-peroxidase is determined. Prepare Hgb-solution with 4 µg Hgb/m*l* saline and add an equal volume of a Hp-solution with 4 µg pure Hp/m*l*. From this mixture put 5.0 m*l* (= 10 µg Hgb as Hp-complex) on column and procede as described for urine. The percent recovery obtained from this determination is used to correct the value obtained for urines with this particular column. The columns can be washed with saline and used repeatedly.

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Über die Ausscheidung der Koproporphyrin-Isomere I und III bei Erkrankungen der Leber und des Blutes

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Mit extraktiven, hochspannungselektrophoretischen und papierchromatographischen Methoden wurde die Koproporphyrinausscheidung bei Gesunden, Leberkranken verschiedener Genese und einer Reihe von Bluterkrankungen untersucht und die Bestimmung der Isomeren I und III durchgeführt. Bei den Normalpersonen fanden sich 42—181 (88,6 ± 30,2) µg/24 Stdn. (Männer) bzw. 43—135 (70,8 ± 28,1) µg/24 Stdn. Gesamtkoproporphyrin im Urin. Koproporphyrin III überwiegt bei gesunden Männern und Frauen (64,8 bzw. 62,2%). Bei Lebererkrankungen zeigen die nichtalkoholischen Cirrhosen ein Vorherrschen des Isomerentyps I, die Alkoholcirrhosen dagegen eine Koproporphyrin III Vermehrung. Bei den Hepatitiden konnte nur bei einem Teil der Fälle ein Überwiegen von Kopro I nachgewiesen werden, die Mehrzahl der untersuchten Fälle klinisch abklingender Hepatitis näherten sich hinsichtlich der Isomerenverteilung der Norm. Alle überprüften Anämieformen schieden zum Teil beträchtlich gegenüber der Norm vermehrte Koproporphyrinmengen aus. Die Isomerentrennung läßt bei den sideroachrestischen Anämien, Eisenmangelanämien und Panmyelopathien eine signifikante Vermehrung von Kopro III erkennen, wogegen die hämolytischen Anämien eine eindeutige Vermehrung des Isomerentyps I zeigen.

The excretion of coproporphyrin in normal health, in liver ailments of various origin, and in a series of blood ailments was studied by extraction, high voltage electrophoresis and paper chromatography, and isomers I and III were determined. In normal persons the urinary level was 42—181 (88.6 ± 30.2) µg/24 hr. (males) and 43—135 (70.8 ± 28.1) µg/24 hr. total coproporphyrin. Coproporphyrin III was predominant in healthy men and women (64.8 and 62.2% resp.). In ailments of the liver, non alcoholic cirrhosis was accompanied by a prevelance of isomer I; alcoholic cirrhosis, however, caused an increase of coproporphyrin III. Isomer I only predominated in a few cases of hepatitis; most of the cases of clinical hepatitis showed a distribution of isomers similar to the normal. All cases of anaemia excreted considerably increased amounts of coproporphyrin. In sideroachrestic anaemia, iron deficiency anaemia and panmyelopathies, there was a significant increase of isomer III, while haemolytic anaemias showed a marked increase of isomer I.

Die Bestimmung der mit dem Harn, der Galle oder dem Stuhl ausgeschiedenen Porphyrine ist für die Diagnose und Klassifizierung der verschiedenen Formen der idiopathischen oder erworbenen Porphyrien von entscheidender Bedeutung. Diese Nebenprodukte der Haemsynthese werden vor allem in der Leber und dem

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