A color-producing reagent for determining blood sugar comprising o-tobutidene, thiourea, an ethylene glycol mono-lower alkyl ether, phthalic acid, and dimethyl formamide as a solubilizing agent, is outstandingly sensitive even to very low blood sugar contents, as in hypoglycaemia, and exhibits prolonged color stability.

7 Claims, 3 Drawing Figures
Fig. 1

\[ \Delta E_{578\text{nm}} \]

\[ \text{(1mm = 0.04 OD)} \]

STANDARD CURVE FOR GLUCOSE DETERMINATION WITH PHTHALIC ACID / TOLUIDINE

Fig. 2

\[ \% \Delta E_{578\text{nm}} \]

COLOR STABILITY (578nm) AT ROOM TEMPERATURE (200 mg % GLUCOSE)

O-TOLUIDINE/PHTHALIC ACID

O-TOLUIDINE/GLYCOLIC ACID
Fig. 3

CORRELATION BETWEEN O-TOLUIDINE/PHTHALIC ACID METHOD AND HEXOKINASE METHOD

STATISTICAL EVALUATION:

\[ y = 2.76 + 0.9804x \]

\[ s_yx = 2.875 \]

\[ r = 0.981 \]
REAGENT FOR DETERMINING BLOOD SUGAR

The present invention is concerned with a reagent for the determination of blood sugar by the o-toluidine method, which reagent does not contain glacial acetic acid.

The determination of blood sugar with o-toluidine in glacial acetic acid is standard method in clinical laboratories. According to this method, the blood sample, after deproteinization, is mixed with about the tenfold amount of a reagent solution which contains o-toluidine and thiourea dissolved in glacial acetic acid. The mixture is heated, a green colored material being formed which is measured in a photometer at 630 nm.

This known method suffers from two important disadvantages which present considerable problems, especially in large laboratories in which routine analyses are carried out. On the one hand, the sensitivity is comparatively low, i.e., the possibility of error in the lower region of sensitivity is rather high. On the other hand, the use of glacial acetic acid as the reaction medium gives rise to a strong and very unpleasant smell, not only when pipetting the reagents but also when heating the samples in a boiling waterbath, as well as when cleaning the glass reaction vessels used. Furthermore, the corrosion of the photometer by the glacial acetic acid vapors is increased considerably.

Attempts have, therefore, already been made to overcome these disadvantages by the use of another solvent and reaction medium. Thus, it has already been proposed to use semi-concentrated acetic acid. However, this does not result in the disadvantages being overcome.

It has been proposed by A. Hartel et al (Z. Klin. Chem. u. klin. Biochem., 7, 14/1969) to replace the glacial acetic acid with a mixture of glycolic acid and malic acid, water, methanol and ethylene glycol monomethyl ether. A reagent of this type is also commercially available.

In the case of another commercially available reagent, the glacial acetic acid is replaced with organic esters and salicylic acid.

The two above-mentioned glacial acetic acid-free reagents overcome the problem of malodor and also somewhat improve the sensitivity of the test. This sensitivity is, in the case of the usual normal values for the blood sugar content in whole blood of 60 - 100 mg. percent, below the value of E ≤ 0.100 at the measurement wavelength of 578 nm usually employed.

However, in the case of very low blood sugar values, such as occur in the cases of hypoglycaemia, the extinction differences are of the order of the blank. Therefore, there is a need for the provision of a reagent which still further increases the sensitivity of the method.

A further disadvantage of the two above-mentioned known reagents is the instability of the solvent mixture which is due to the content of readily volatile components. The increased evaporation rate is of significance because the reagent must be heated for 8 minutes in a boiling waterbath for the development of the coloration. In the case of the known salicylic acid-containing reagent, even after standing for about 1 hour in the air, the dissolved substances start to crystallize out.

Finally, another disadvantage is that the substances used instead of glacial acetic acid are considerably more expensive and thus the economic competitiveness of such reagents is impaired.

It is, therefore, an object of the present invention to provide a new reagent for the determination of blood sugar which avoids the above-mentioned disadvantages of the known reagents and is dependable, very sensitive and economical.

The reagent according to the present invention for the determination of blood sugar contains o-toluidine, thiourea, an ethylene glycol mono-lower alkyl ether, and an acid and is characterized in that it contains phthalic acid as the acid and dimethyl formamide as a solubilizing agent.

From A. Hartel and H. Lang (Arztl. Lab., 15, 60/1966), it was known that non-hydroxylated dicarboxylic acids could not replace the glacial acetic acid because they prevented the formation of the desired colored material. It is, therefore, most surprising that phthalic acid is an outstanding catalyst for the aldose-specific color reaction of o-toluidine, whereas, for example, isophthalic acid and terephthalic acid are useless for this purpose.

The reagent according to the present invention preferably comprises 0.05 - 2.5 percent by weight thiourea, 5 - 33 percent by weight phthalic acid, 25 - 65 percent by weight dimethyl formamide, 10 - 50 percent by weight of an ethylene glycol mono-lower alkyl ether and 2.5 - 15 percent by weight o-toluidine, based on total weight of composition. The sum total of dimethyl formamide and ethylene glycol mono-lower alkyl ether advantageously does not exceed 75 percent by weight, based on total weight of composition.

An especially preferred composition consists of 0.075 - 0.15 percent by weight thiourea, 20 - 25 percent by weight phthalic acid, 35 - 55 percent by weight dimethyl formamide, 20 - 40 percent by weight ethylene glycol mono-lower alkyl ether and 7.5 - 10 percent by weight o-toluidine, based on total weight of composition.

Within the scope of the present invention, the term "lower alkyl" is to be understood to mean an alkyl radical containing up to six carbon atoms.

The reagent according to the present invention can contain up to about 5 percent water, without its usefulness being thereby impaired.

The reagent according to the present invention does not result in any unpleasant smell nor is there any danger of a loss of solvent when heating in order to bring about formation of the colored material. The reagent is colorless to pale yellow in color and the viscosity is low so that there is no impairment of the pipetting due to air bubbles or after-runnings. The after-runnings in the case of a normal pipetting amount to ≤ 1 percent, i.e., in the case of 2.0 ml. of reagent, are ≤ 0.02 ml. The reagent is also storage stable although, in the case of comparatively long periods of storage at an elevated temperature (33° C.), an intensification of the yellow color can occur. However, this influence neither the measurement sensitivity to any considerable extent nor the stability of the color formed.

An especial advantage of the reagent according to the present invention is its outstanding sensitivity, even in the case of low glucose contents. Thus, even in the case of the range of 20 - 400 mg. percent (and even up to 1 g. percent) glucose content, there is obtained a very good linear proportionality between the extinction values at 578 nm and the glucose concentration. In this regard, reference is made to the accompanying drawings in which:

FIG. 1 is a plot extinction values against glucose concentration;
FIG. 2 is a plot of color intensity of an incubated test sample against time; and
FIG. 3 presents a correlation between two glucose determination methods.

With reference to FIG. 1, when the glucose content is over 400 mg. percent, then the sample is expeditiously diluted, for example, in the ratio of 1:10, since even in the case of this content, an extinction of 0.65 is measured.

The sensitivity of the reagent according to the present invention is considerably greater than the sensitivity of the known reagents, as can be seen from the following Table 1:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>E0 blank</th>
<th>E0 sample</th>
<th>E0% referred to (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-toluidine</td>
<td>0.084</td>
<td>0.201</td>
<td>100%</td>
</tr>
</tbody>
</table>

TABLE 1

Comparison of the Sensitivity of Various Blood Sugar Determination Methods

Sensitivity=200 mg% glucose
The superior sensitivity of the reagent according to the present invention, which can be seen from the last column of the above Table 1, is of great practical importance since it is hereby possible, even in the cases of hypoglycaemia, as well as in the cases in which only very little blood is available, i.e., in the case of blood sugar measurement values of 30 - 50 mg. percent, to obtain realizations of the order of 0.050 to 0.082 at 578 nm, measured against a blank, so that errors of measurement in the reading off of the photometer are very considerably reduced.

A further advantage of the reagent according to the present invention lies in the fact that in the region of the especially preferred composition, it represents a so-called "optimum composition". This means that all individual components of the mixture are present in the concentration in which the sensitivity, the period of incubation of the test and the stability of the resultant color have their maxima and a slight alteration of the conditions does not cause any significant differences in the course of the test or in the measurement operation. The working errors are, therefore, in the case of this composition, extremely low.

The color which arises in the case of the o-toluidine reaction is, in principle, unstable. It is assumed that an unknown secondary reaction follows by means of which the extinction at 578 nm, the maximum, and at the measurement wavelength of 578 nm, decreases in the course of time. This instability of the color is reduced in the case of the reagent according to the present invention. FIG. 2 of the accompanying drawings shows the remaining color intensity as a percentage of the original value, in dependence upon time, which the sample had at ambient temperature at the end of the incubation. The glycollic acid reagent was used as comparison reagent. There can clearly be appreciated the superior stability of the reagent according to the present invention. This improvement represents an important advantage when a large series of tests are to be measured one after the other.

A further advantage of the reagent according to the present invention is its cheapness. Thus, the cost of phthalic acid is only about one-ninth of the cost of glycollic acid.

The following Examples are given for the purpose of illustrating the present invention:

**Example 1**
0.2 grams thiourea and 45.0 grams phthalic acid were dissolved in 100 ml. dimethyl formamide (b.p. 152 - 155° C.). 5 ml. ethylene glycol monoethyl ether were then added thereto and finally 18.0 ml. o-toluamide was stirred in.

For the purpose of deproteinization, 0.1 ml. of blood was pipetted into 1.0 ml. 3 percent trichloroacetic acid, mixed and, after standing for a few minutes, centrifuged.

From the supernatant, there was taken 0.2 ml. and this was mixed with 2.0 ml. of the above-described reagent according to the present invention. The mixture of supernatant and reagent was heated for 8 minutes on a boiling waterbath. After cooling in cold water, the resultant green colored material was measured in a photometer at 578 nm against a blank (trichloroacetic acid and reagent). A glucose standard (100 mg. glucose/100 ml. 0.2 percent benzoic acid solution) was treated in the same manner. The blood sugar content was calculated from the following equation:

\[
glucose\ content\ of\ the\ sample\ (mg.\ %) = \frac{E_{sample}}{E_{standard}} \times 100
\]

The determination was repeated with a series of blood samples of differing glucose content. The results obtained are set out in FIG. 1 of the accompanying drawings. They show the outstanding linear proportionality of the results which are obtained with the reagent according to the present invention.

In the above-described manner, the standard deviation of the determined blood sugar values from one another was ascertained by means of 10 determinations on the same serum. The standard deviation, which serves for the assessment of the distribution of the individual values around the arithmetic mean, was calculated from the following equation:

\[
s = \sqrt{\frac{(X - \bar{X})^2}{N-1}}
\]

In the present case, \( s = 1.25 \) mg. percent. As arithmetic mean value (\( \bar{X} \)) in the case of the 10 determinations, there was found 163.3 mg. percent glucose. The exactitude which can be achieved with the reagent according to the present invention can also be expressed by the following equation:

\[
\bar{X} \pm 1 s = 163.3 \pm 1.25 \text{ mg. percent}
\]

The variation coefficient (\( VC \)), which is an expression of the standard deviation in percent of the mean value, amounted to ±0.76 percent, calculated from the following equation:

\[
VC = \frac{s \times 100}{\bar{X}}
\]

**Example 2**

The o-toluidine method, with the use of the reagent according to the present invention, was compared with the known enzymatic, highly specific blood sugar determination method, using the system hexokinase/intermediate enzyme. In this case, 25 different whole blood samples were tested in the manner described in Example 1. The same number of blood samples were determined enzymatically with hexokinase/intermediate enzyme. The blood sugar values obtained by both methods were compared. The results obtained are shown in FIG. 3 of the accompanying drawings. The good agreement of the values which were obtained by the two methods was obvious. The distribution of the comparable individual values was, on the average, ± 2.0 percent.

What is claimed is:
1. A reagent composition for determination of blood sugar which composition comprises o-toluidine, thiourea, an ethylene glycol mono-lower alkyl ether, phthalic acid, and dimethyl formamide as a solubilizing agent.
2. A reagent composition as claimed in claim 1 in which water is contained in an amount of up to 5 percent by volume.
3. Method for the determination of blood sugar which comprises mixing a blood sample with a reagent composition as claimed in claim 1 and measuring the resulting mixture photometrically as an indication of the blood sugar content.
4. A reagent composition as claimed in claim 1 which comprises 0.05 - 2.5 percent by weight thiourea, 5 - 33 percent by weight phthalic acid, 25 - 65 percent by weight dimethyl formamide, 10 - 50 percent by weight ethylene glycol mono-lower alkyl ether and 2.5 - 15 percent by weight o-toluidine, based on total weight of composition.

5. A reagent composition as claimed in claim 4 which comprises 0.075 - 0.15 percent by weight thiourea, 20 - 25 percent by weight phthalic acid, 35 - 55 percent by weight dimethyl-formamide, 20 - 40 percent by weight ethylene glycol mono-lower alkyl ether and 7.5 - 10 percent by weight o-toluidine, based on total weight of composition.

6. A reagent composition as claimed in claim 4 wherein the total volume of dimethyl formamide and of alkylene glycol mono-lower alkyl ether does not exceed 150 parts by volume.

7. Method of the determination of blood sugar which comprises mixing a blood sample with a reagent composition as claimed in claim 4 and measuring the resulting mixture photometrically as an indication of the blood sugar content.