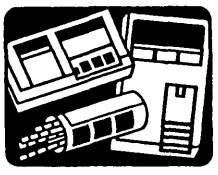
Technical Section



Development of Paper-strip Test for 3-Hydroxybutyrate and its Clinical Application

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A rapid paper-strip test for the semiquantitating 3-hydroxybutyrate (3-OHBA) has been developed. The color develops within 5 min after applying the serum to the paper strip, and the purple color was read either visually or by reflectance meter. This can detect 3-OHBA levels as low as 0.1 mmol/L up to 2.0 mmol/L. The more concentrated sample can be measured on serial dilution. Clinical usefulness has been tested in a summer camp for insulin-dependent diabetic children as well as in a routine diabetes clinic. Serum 3-OHBA levels ranged from > 100 μmol/L to 4 mmol/L in all the subjects before breakfast in a summer camp. In four subjects, 3-OHBA was elevated to the level of 2–4 mmol/L, and only one of these four subjects exhibited ketonuria by nitroprusside test. In a diabetes clinic, a new paper-strip test for 3-OHBA has revealed ketonemia in 34 (74%) of 46 diabetic subjects, while nitroprusside test revealed ketonemia in only 4 (13%). The present paper-strip test for 3-OHBA is sensitive enough to detect levels as low as 0.1 mmol/L and is clinically useful for rapid detection of ketosis proneness as well as for monitoring of diabetes control. DIABETES CARE 1984; 7:481–85.

Since acetone was first shown to be elevated in the blood of uncontrolled diabetic subjects in 1857 by Petters,[1] acetoacetate (AcAc) and 3-hydroxybutyrate (3-OHBA) have been noted to increase in both decompensated and controlled diabetic patients.[2,3] In diabetic ketoacidosis, 3-OHBA is a major ketone body that increases in blood.[4] MacGillivray et al. recently reported that 57% of the urine tests that were negative for ketone bodies by acetest were associated with elevated plasma 3-OHBA in insulin-dependent diabetes.[5]

Ketosis proneness is one of the most important features of type I diabetes.[6] Therefore, determination of serum levels of ketone bodies, especially 3-OHBA, would be valuable for the diagnosis of type I diabetes and as markers monitoring diabetes control.[7] However, at present, clinical evaluation of ketosis depends on nitroprusside tests in most diabetes clinics. The test detects AcAc and acetone, to some extent, but does not react with 3-OHBA.

Therefore, development of a rapid semiquantitative paper-strip test to estimate 3-OHBA is warranted for clinical use. We have attempted to develop a paper strip for the semiquantitation of 3-OHBA. The clinical usefulness of this paper strip has been evaluated in a summer camp for insulin-dependent diabetic children as well as in our diabetes clinic.

Note to Readers: Because the treatment of diabetes involves the use of materials and devices, there is an obligation to report their performance data. Often papers in this area do not elucidate any new physiologic principle nor substantially advance the means of treatment. However, the Editors believe that the readership should be informed regarding technologic advances relevant to the care of diabetes. This section will be devoted to such papers. Each will have undergone the usual peer review process. Only those that provide new information will be published.

PATIENTS AND METHODS

Subjects

Control subjects. Ninety-one normal subjects, aged 18–65 yr, were used as a control for adult diabetic subjects. The normal ranges for AcAc and 3-OHBA were 14–68 μmol/L and 0–74 μmol/L, respectively. Eight healthy children aged 5–11 yr were also studied. Serum levels of ketone bodies seemed to be slightly higher in the children, but 3-OHBA concentration fell below 100 μmol/L.

Diabetes camp study. Nineteen boys and 28 girls aged 6–12 yr were studied. Mean duration of diabetes was 3 yr. They were treated with 0.32–1.2 U/kg (mean, 0.63 U/kg) of in-

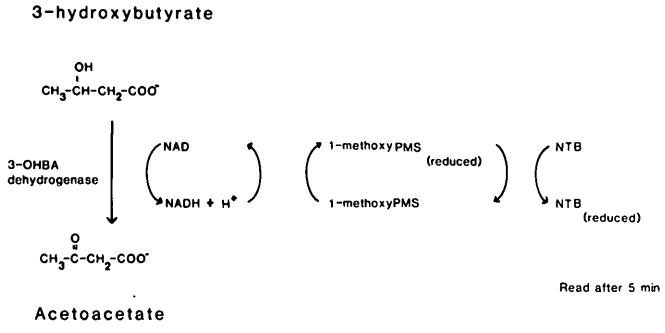

FIG. 1. Reaction sequence of the color development for 3-hydroxybutyrate. PMS: phenazine methosulfate; NTB: nitroblue tetrazolium.

termediate insulin before breakfast. In 23 subjects, additional intermediate insulin (0.06–0.55 U/kg; mean, 0.23 U/kg) was administered before dinner. Eleven subjects received short-acting insulin by either single or divided dose before breakfast and/or dinner. Mean (±SE) HbA_1 value was 10.8 ± 0.4%. Blood was drawn before each meal and before sleep (9 p.m.). Urine specimens and blood samples were obtained coincidentally.

Diabetes clinic. Both maturity-onset type I and non-insulin-dependent diabetic subjects were studied. Blood was obtained after overnight fasting unless specifically mentioned.

Methods

Glucose was determined in plasma by an immobilized enzyme method (Model GA-1000, Sankyo, Osaka, Japan); HbA_1 was determined by the method of Trivelli et al.[8]

Paper-strip test for 3-OHBA. The reaction sequence for the color development is depicted in Figure 1. 3-Hydroxybutyrate is enzymatically converted to AcAc, and the H^+ is conveyed to nitroblue tetrazolium (NTB, reduced form), which has a purple color with absorbance at 600 nm. In order to exclude the color contribution from lactate, an LDH inhibitor, oxamic acid, is added to the reaction mixture. Forty milligrams NAD, 40 mg NTB, 400 U 3-OHBA dehydrogenase, 0.5 g oxamic acid, and 0.5 mg 1-methoxy PMS were dissolved in this order in 20 ml 0.1 M sodium tetraborate:monopotassium phosphate buffer (pH 8.0). A 10 × 15-cm sheet of filter paper, Toyo No. 65 (Toyo-Roshi Co., Higashi-Ku, Osaka, Japan), was dipped in the above enzyme solution in a developing dish. The paper was then dried at 25°C for 2 h with a fan in a dark room. Four hundred sheets of test-paper tips (5 × 7 mm) were obtained. Each tip was attached to the plastic strip (5 × 80 mm). Twenty microliters of serum was applied to the paper strip. Alternatively, a paper tip was covered with a drop of serum, immediately wiped with tissue paper, and then allowed to stand for 5 min. The purple color developed was read either visually from the standard chart or by reflectance meter with a scale of 0–2 mmol/L.

The reflectance reached the plateau at 4–5 min; therefore, the color was read 5 min after the sample application. The color intensity increases up to 5 mmol/L, but the increase between 2 and 3 mmol/L was not so remarkable. Therefore, the samples that exceeded 2.0 mmol/L were judged after the dilution.

Photometric determination of ketone bodies. AcAc: One-tenth milliliter of serum was mixed with 0.5 ml water and 0.1 ml 15% PCA; then the mixture was centrifuged at 3000 rpm for 10 min. An aliquot of the supernatant (0.5 ml) was mixed with 0.5 ml citrate buffer (pH 3.9) and then with 0.5 ml diazonium solution. After the incubation at 37°C for 10 min, 0.5 ml of 1.5 mol/L NaOH was added and the absorbance at 645 nm was determined 5 min later.[9]

Total ketone bodies and 3-OHBA: One-tenth milliliter of serum was mixed with phosphate buffer (pH 8.0), NAD, pyruvate, and enzyme (3-OHBA dehydrogenase, LDH) solution. The mixture was incubated at 37°C for 10 min. After deproteinization with PCA by centrifugation, an aliquot of the supernatant was processed for AcAc determination. The difference between the above total and AcAc was calculated to be 3-OHBA.[9] The assay kit can be obtained from Sanwakagaku Kenkyusho (Higashi-Ku, Nagoya, Japan). For a conventional semiquantitation of ketone bodies, Ketostix (Miles, Sankyo Co., Tokyo, Japan) was used. In order to evaluate Ketostix and the present paper-test for 3-OHBA, serum levels of ketone bodies were determined by both photometric and paper strips in patients with type I or type II diabetes who visited our diabetes clinic. Since AcAc was stable at −70°C for 3 wk,[9] either fresh sera or sera frozen at −70°C within 1 wk was used.

Reagents: 3-OHBA dehydrogenase (S.A. 30), which was purified, is now available from Sanwakagaku Kenkyusho; and other chemicals were from Nakarai Chemicals (Kyoto, Japan).

RESULTS

Correlation between serum 3-OHBA levels determined manually and by paper-strip test. Serum levels of 3-OHBA were determined by both quantitative (photometric) and semiquantitative (paper-strip test) methods in sera obtained from 76 subjects with both types of diabetes. A good correlation ($r = 0.97$) was observed between the two methods from 0.1 to 5 mmol/L 3-OHBA (Figure 2A). The paper-strip test can detect levels as low as 0.1 mmol/L 3-OHBA. A good correlation was also observed ($r = 0.97$) between the results obtained by both visual and reflectance meter using paper-strip tests (Figure 2B).

Comparative sensitivity of Ketostix and paper-strip test for 3-OHBA in the same sera obtained from subjects with type I or type II diabetes. Six of 46 diabetic subjects were positive (13%) and 2 equivocal by Ketostix (Figure 3A). A simultaneous determination of AcAc by the diazonium method revealed that a "positive" reading with Ketostix was greater than 0.2 mmol/L. Thirty-four of 46 diabetic subjects were positive (74%) by the newly developed paper-strip test for 3-OHBA (Figure 3B).

Fasting serum 3-OHBA levels determined by paper-strip test in juvenile type I diabetic subjects. Serum levels of 3-OHBA

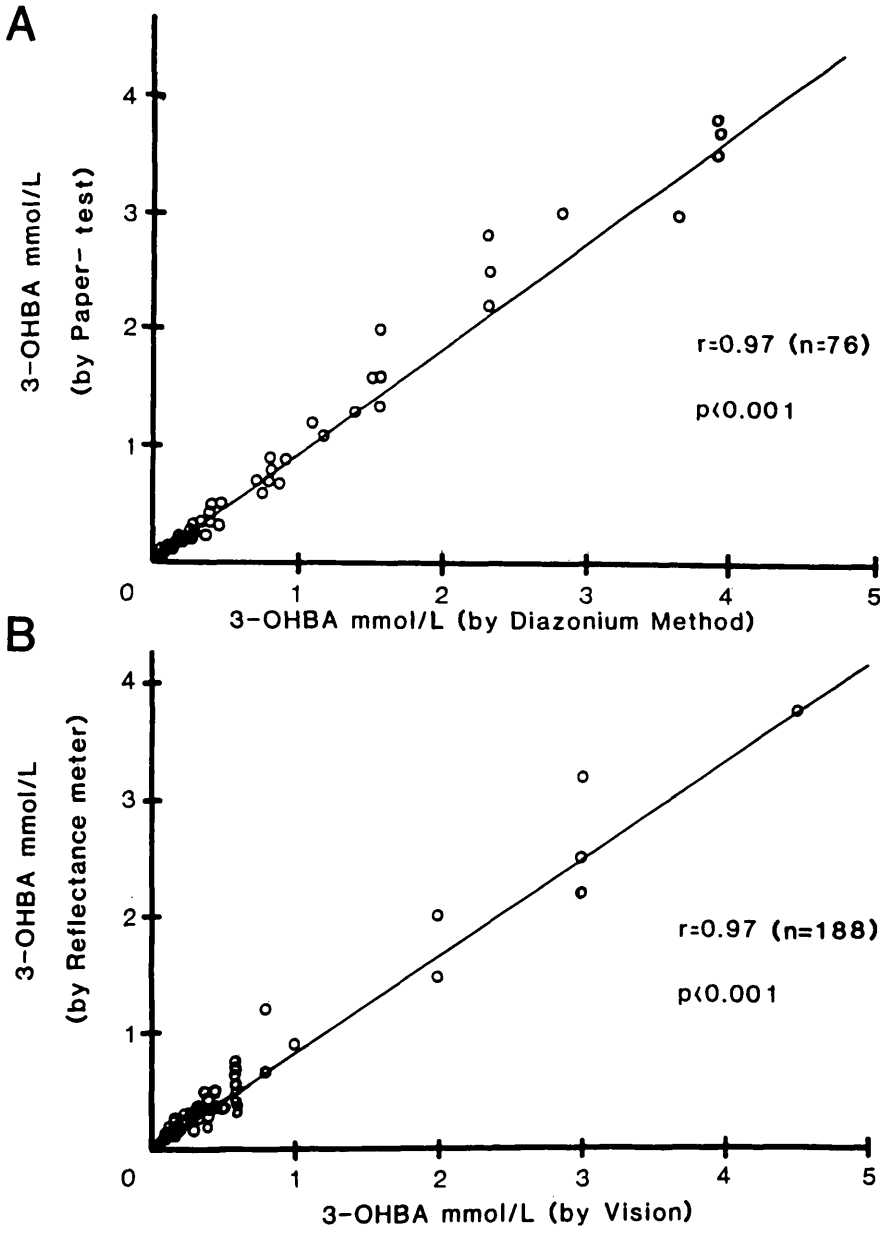

FIG. 2. Correlation between serum 3-OHBA levels determined by manual (diazonium method) and paper-strip tests in sera obtained from diabetic subjects. (A) Correlation of 3-OHBA levels determined by diazonium and paper-strip test. (B) Correlation of 3-OHBA levels determined visually and by reflectance meter using paper-strip test.

were all greater than 100 μmol/L (Figure 4). Significant, although not close, correlation was observed between 3-OHBA and FPG levels (r = 0.61). Fasting plasma glucose (FPG) ranged from 50 to 400 mg/dl. Four subjects showed severe ketosis exceeding 2.0 mmol/L. Those who received insulin injection twice a day seemed to have less ketosis before breakfast than those who received a single-dose insulin injection, although apparently not significant.

Relationship between degree of ketonuria detected by Ketostix and serum 3-OHBA levels by paper-strip test before breakfast in juvenile type I diabetic subjects. Ketonuria (+1 to +3) was revealed in only 8 of 47 subjects by nitroprusside tests. In these subjects, 3-OHBA ranged between 0.4 and 3.5 mmol/L. Elevation of serum 3-OHBA ranging from 0.1 to 4 mmol/L was observed even in those who were negative for ketonuria by nitroprusside test (Figure 5).

DISCUSSION

A paper-strip test for semiquantitation of 3-OHBA has been developed and its clinical significance has been tested in a summer camp and in a routine diabetes clinic. The strip could detect 3-OHBA exceeding 0.1 mmol/L up to 2.0 mmol/L without serum or plasma dilution and was very sensitive. The color was read 5 min after applying serum either visually or by reflectance

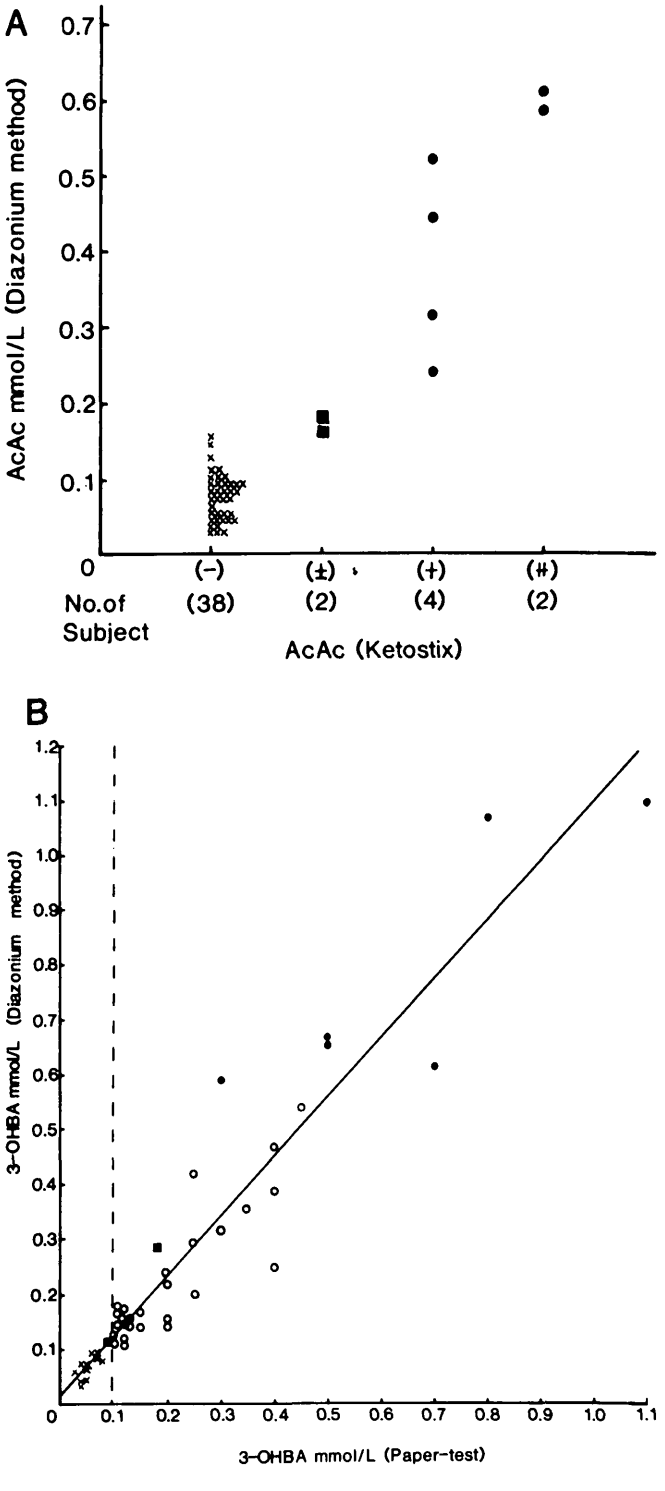

FIG. 3. Comparative sensitivity of Ketostix and paper-strip test for 3-OHBA in the same sera obtained from both type I and II diabetic subjects. (A) Relationship of serum AcAc levels determined by Ketostix and diazonium methods. Four subjects with AcAc greater than 0.4 mmol/L were type I diabetic subjects. (B) Relationship of serum 3-OHBA levels determined by paper-strip test and diazonium methods. ●, + to + + by Ketostix; ■, equivocal (+) by Ketostix; ○, positive by paper-strip test for 3-OHBA, but negative by Ketostix; x, negative by paper-strip test for 3-OHBA.

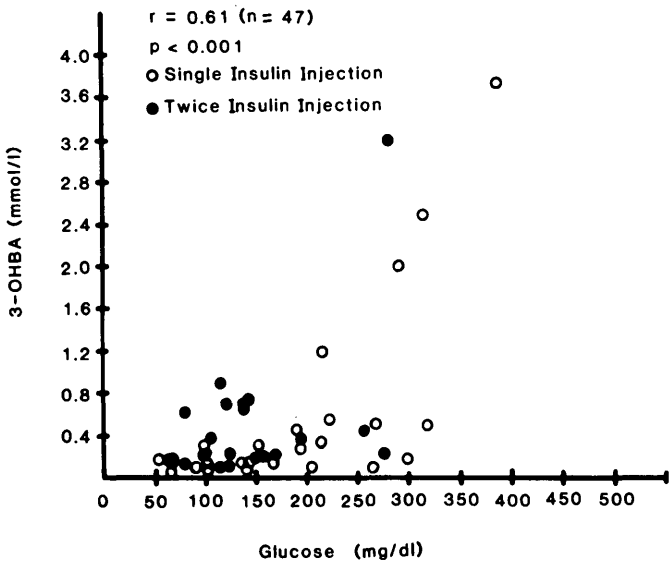

FIG. 4. Relationship between fasting plasma glucose and serum 3-OHBA levels determined by a paper-strip test in type I diabetic children.

meter. The method is accurate since a good correlation was observed between the values obtained by photometric determination and by paper-strip test.

In 46 arbitrarily selected diabetic subjects who visited our diabetes clinic, the newly developed paper-strip test for 3-OHBA was about fivefold more sensitive in detecting ketonemia. This was attributable to the greater elevation of 3-OHBA over AcAc or to the higher sensitivity of the present test paper compared with Ketostix (Figure 3, A and B).

Among 47 type I diabetic children, all showed higher levels of serum 3-OHBA exceeding 100 μmol/L in sera obtained before breakfast. Four subjects showed ketosis with 3-OHBA between 2 and 4 mmol/L. Among them, three subjects were not ketonuric by Ketostix. Serum levels of 3-OHBA were higher before breakfast compared with during the daytime or before sleep in these diabetic children, probably because of insufficient serum levels of insulin in the early morning hours. Ketonemia was often observed in normoglycemic subjects, and dissociation between serum levels of ketone bodies and glucose was observed. Therefore, normalization of both ketone bodies and glucose in serum may be a useful marker for the monitoring of diabetes control.

In type II diabetic subjects treated with diet or sulfonylureas, serum 3-OHBA was shown to fluctuate in a manner roughly inversely proportional to the serum levels of insulin, while AcAc did not show clear diurnal changes.[7] Since the 3-OHBA/AcAc ratio increases in diabetes according to the severity of diabetes in most of the diabetic subjects, semiquantitation of 3-OHBA reflects total ketone bodies in blood.[4,7] In urine, AcAc concentration is greater than in serum and tends to comprise major ketone bodies in most of the diabetic subjects.[7] Therefore, the nitroprusside test may be valuable for the detection of ketonuria, but not sensitive enough to detect mild ketonemia.

Use of LDH inhibitor (oxamic acid) and the stabilization of 3-OHBA dehydrogenase activity on the paper strip were

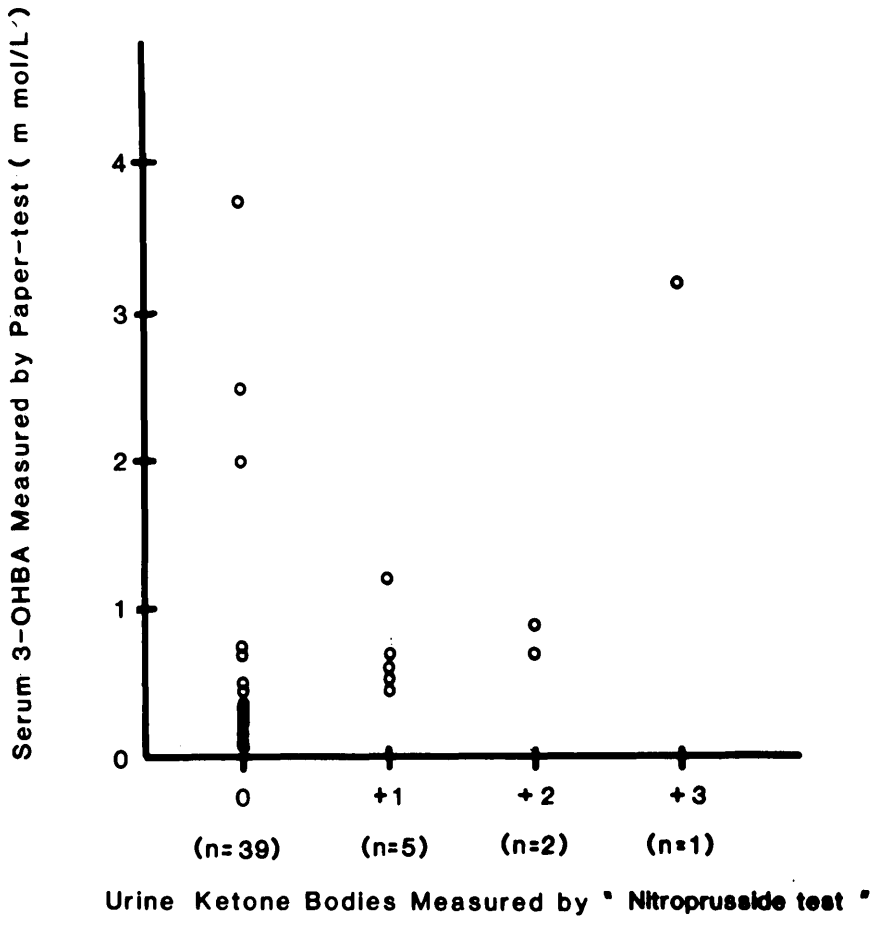

FIG. 5. Relationship between degree of ketonuria detected by nitroprusside test and serum 3-OHBA levels determined by paper-strip test before breakfast in a summer camp. The subjects were the same as those in Figure 4.

the main clues for the present development of a paper-strip test for 3-OHBA. Lactate did not exhibit any interference. At present, the paper strip is stable for 3 mo in the refrigerator. For generalized clinical application, further improvement is necessary. An effort is now being made to develop rapid reactive (color develops with 2–3 min) and more stable (can be kept for 1 yr at room temperature) paper-strip tests for 3-OHBA.

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