Since all natural (−)-flavanones generally have S-chirality at C-2,[2b] the structure VII could be given to sophoranochromene.

Acknowledgement We are deeply indebted to Dr. T. Namba, Osaka University, for his advice in determining the material. We also express our deep gratitude to Mr. S. Uehara, Managing Director of this company, for his permission to publish this paper, to Dr. S. Ikawa, Managing Director of this company, and to Dr. I. Tanaka, Director of this laboratory, for their encouragement.

Research Laboratory,
Taisho Pharmaceutical Co., Ltd.,
Takata 3-chome, Toshima-ku, Tokyo

MANKI KOMATSU
TSUYOSHI TOMIMORI
KATSUO HATAYAMA
YUKIKO MAKIGUCHI
NAOKO MIKURIYA

Received March 28, 1969

[Chem. Pharm. Bull. 17(6)1304—1305(1969)]

UDC 543.854.73 : 547.457.1.08

Microdetermination of Glucose using 3-Methyl-2-benzothiazolone Hydrazone Hydrochloride and Glucose Oxidase-Catalase Enzyme System

Glucose oxidase–catalase enzyme system has recently been introduced into the assay of glucose in biological fluids.[1,2] This system is claimed to be more sensitive and reproducible than glucose oxidase-peroxidase system when chromotropic acid–sulfuric acid reagent is used for color development.[1] However, this method requires heating the sample with concentrated sulfuric acid which may react with other substances than glucose to give color and, in addition, the concentrated acid may be troublesome to handle in a clinical laboratory. The method described in this paper utilizes 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH), a highly sensitive reagent for aliphatic aldehydes,[3] in place of chromotropic acid–sulfuric acid reagent. MBTH was found to react with formaldehyde generated by the action of catalase on methanol at pH 5.6 which is at the same time optimal hydrogen ion concentration for glucose oxidase. This method also proved to be more sensitive and simple than the chromotropic acid method.

Reagents——1) Enzyme Reagent: 0.4 g of glucose oxidase (GOD-III, Boeringer, Mannheim) is dissolved in 20% methanol to make 100 ml. 2) Phosphate buffer, pH 5.6. 3) 0.5% MBTH aqueous solution. 4) Ferric Chloride Reagent: 0.83 g of $FeCl_3.6H_2O$ is dissolved in 10% $KHSO_4$ aqueous solution to make 100 ml.

Procedure——To 1 ml of sample solution containing 1 to 20 μg of glucose is added 1 ml of enzyme reagent and 2 ml of phosphate buffer and the mixture is incubated at 37°—38° for 90 min. Then 0.5% MBTH is added and the resulting mixture is allowed to stand at room temperature for 60 min. Finally, ferric chloride reagent is added and the absorbance is read after 60 min at 620 mμ against the reagent blank. Linear relationship is observed in the range of 1 μg to 20 μg per ml of sample solution.

When applied to human serum, to which is added 100 mg and 200 mg of glucose per ml, 100.0 and 102.5% recovery was observed, respectively. Somogyi protein precipitation meth-

1) F.W. Sunderman, Jr. and F.W. Sunderman, *Amer. J. Clin. Path.*, **36**, 75 (1961).
2) S. Ikawa and T. Obara, *Jap. J. Clin. Path.*, **13**, 197 (1965).
3) E. Sawicki, T.R. Hauser, T.M. Stanley and W. Elbert, *Anal. Chem.*, **33**, 93 (1961); This reagent was recently applied by the authors to microdetermination of amino sugars: A. Tsuji, T. Kinoshita, and M. Hoshino, *Chem. Pharm. Bull.* (Tokyo), **17**, 217 (1969); *idem*, *ibid.*, in press.

od was found to be appropriate for the present method, and 20 μl of serum was shown to be sufficient for the glucose estimation.

Details of the experiment will be reported in the near future.

School of Pharmaceutical Sciences,
Showa University,
Shinagawa-ku, Tokyo

Akio Tsuji
Toshio Kinoshita
Akiko Sakai

Squibb Japan Inc.,
Akasaka, Minato-ku, Tokyo

Masanori Hoshino

Received April 5, 1969

[Chem. Pharm. Bull. 17(6)1305—1306(1969)]

UDC 581.19 : 582.29

Formation of Lichen Substances by Mycobionts of Lichens. Isolation of (+)Usnic Acid and Salazinic Acid from Mycobionts of *Ramalina* spp.

The formations of usnic acid, didymic acid, and rhodocladonic acid by mycobiont of lichen, *Cladonia cristatella*, were reported once by Castle and Kubsch.[1] However Ahmadjan[2] and Fox[3] denied the results of the earlier investigation by reexamination using the isolated fungi from the same lichen.

Recently Mosbach[4] revealed the formation of pulvic acid derivatives by the lichen fungus of *Candelariella vitellina*. Hess[5] suggested the contribution of algal symbiont of lichens to the formation of depsides and depsidones, while Mosbach[6] proved the presence of esterase in the algal symbiont of *Umbilicaria pustulata* suggesting its role in the depside biosynthesis in lichens.

We have found recently that (+)usnic acid and a depsidone, salazinic acid, are formed by the isolated mycobiont of lichen,[7] *Ramalina crassa* (Del.) Mot. which was cultivated for 5 months at 8—20°C on Hamada's No.117 medium (glucose 20 g, dried yeast 5 g, agar 20 g, water 1 liter, pH 5.1—5.6). The former compound has also been obtained from the mycobiont of *Ramalina yasudae* Räs. which was cultivated on the malt-yeast extract medium (malt extract 20 g, yeast extract 2 g, agar 20 g, dist. water 1 liter, pH 5.5). (+)Usnic acid, mp 201—202°, $[\alpha]_D$+480—519° (in $CHCl_3$), was isolated in a crystalline form from the above lichen mycobiont colonies in a yield of 0.022% and 1.8%, respectively, and identified by a mixed fusion as well as by IR and mass spectral comparisons with the authentic sample.

Salazinic acid was proved in the extract of mycobiont of *R. crassa* by thin-layer chromatography on silica gel G impregnated with 0.5N oxalic acid developed with benzene-acetone

1) H. Castle and F. Kubsch, *Arch. Biochem.*, **23**, 158 (1949).
2) V. Ahmadjian, *Bryologist*, **67**, 87 (1964).
3) C.H. Fox, personal communication (July 3, 1967).
4) K. Mosbach, *Acta Chem. Scand.*, **21**, 2331 (1967).
5) D. Hess, *Z. Naturforsch.*, **14b**, 345 (1959).
6) K. Mosbach, *Biochem. Biophys. Res. Comm.*, **22**, 145 (1966).
7) The mycobionts were isolated either by the micromanipulator or the test tube methods. (R.W. Davidson and T.E. Hinds, *Phytopathology*, **48**, 216 (1958)). Polyspore cultures were used for chemical analysis. All the lichen specimens used for the isolation of mycobionts are preserved in the Herbarium of the National Science Museum, Tokyo (TNS).