Isolation of 6,7-Dimethyl-8-ribityl Lumazine from a Riboflavineless Mutant of *Aspergillus nidulans*

Since the production of riboflavine in mammals is dependent on the intestinal microflora, the biosynthesis of riboflavine has been studied mostly in microorganisms. The successful isolation of a pair of pteridine derivatives, 6,7-dimethyl-8-ribityl lumazine and 6-methyl, 7-hydroxy-8-ribityl lumazine, in the flavinogenic microorganisms *Eremothecium ashbyii*[1], *A. gossypii*[2], and in others, have been reported and confirmed by isotopic labelling studies. However, the exact mechanism of conversion of these compounds to riboflavine remains to be established. PLAUT[3] has postulated that the additional four carbon unit needed in the riboflavine molecule comes from the additional molecule of 6,7-dimethyl-8-ribityl lumazine. PANICKER and SHANMUGASUNDARAM[4] have shown, using *Aspergillus nidulans* mutants deficient in riboflavine, that this lumazine compound first decomposes to give 4-ribityl-amino, 5-amino uracil and is the more immediate precursor of the vitamin, and that the two compounds are interconvertible, being in equilibrium with each other in the system.

The present note deals with the identification of the more stable 6,7-dimethyl-8-ribityl lumazine in the cultures of a riboflavine-requiring mutant of *Aspergillus nidulans*. The compound 6,7-dimethyl-8-ribityl lumazine isolated from the cultures of *E. ashbyii* and *A. gossypii* fluoresces green under UV-light and its chemical structure has been tentatively established by comparison with a pure synthetic compound.

One of the 5 non-allelic riboflavineless mutants of *Aspergillus nidulans*, designated as $ribo_1$, with green spore colour was grown in riboflavine supplemented minimal medium which consisted of the following composition: $NaNO_3$ 6.0 g, KH_2PO_4 1.52 g, KCl 0.52 g, $MgSO_4\,7H_2O$ 0.52 g, glucose 20.0 g, with traces of iron and zinc and 1.0 mg riboflavine/l. The pH was adjusted to 6.4. 2 l of this medium were sterilized and inoculated with the spore suspension of the mutant and incubated at 37°C for ten days. At the end of the incubation period, the cultures were concentrated by vacuum distillation to about 5 ml and chromatographed on acid alumina containing N-butanol and absolute ethanol using the method of PLAUT[5]. The sample was made up in a solvent composed of 1 ml water, 9 ml ethanol and 10 ml N-butanol, then allowed to pass through the adsorbent and the column was washed by an additional 10 ml of the solvent. Riboflavine was eluted from the column with 50 ml of the solvent system containing 80 ml of N-butanol and 28 ml each of ethanol and water, and 6,7-dimethyl-8-ribityl lumazine was eluted using the solvent containing 80 ml N-butanol, 28 ml ethanol and 56 ml water. The column was then washed with 50 ml of 50% (v/v) ethanol and 50 ml of 0.03 *M* ammonium hydroxide to elute 6-methyl, 7-hydroxy-8-ribityl lumazine. The eluates were concentrated to about 1 ml by per-evaporation at 3° to 5°C in a cold room. They were spotted on Whatman No. 3 filter paper along with authentic samples and chromatographed, using the solvent butanol-ethanol-water (50:15:35), and were examined under UV-light. The eluted spot corresponding to the green fluorescing authentic sample of 6,7-dimethyl-8-ribityl lumazine, having an Rf value of 0.26, was found to have an identical absorption spectrum in the UV-region in a Unicam SP-700 spectrophotometer. Violet spot for the eluted 6-methyl, 7-hydroxy-8-ribityl lumazine could not be detected. This is the first observation wherein 6,7-dimethyl-8-ribityl lumazine has been isolated from a riboflavine-requiring mutant[6].

Zusammenfassung. Die Gegenwart von 6,7-Dimethyl-8-ribityl-Lumazin, einem Vorläufer des Riboflavins in seiner Biosynthese, ist im Kulturfiltrat einer riboflavinlosen Mutante von *Aspergillus nidulans* festgestellt worden.

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[1] T. MASUDA, Pharmacol. Bull. (Tokyo) *5*, 28, 136 (1956).
[2] G. F. MALEY and G. W. E. PLAUT, Fed. Proc. *17*, 268 (1958).
[3] G. W. E. PLAUT, J. biol. Chem. *235*, PC-41 (1960).
[4] R. K. PANICKER and E. R. B. SHANMUGASUNDARAM, Bull. nat. Inst. Sci. (India) *25*, 22 (1964).
[5] G. W. E. PLAUT, J. biol. Chem. *238*, 2225 (1963).
[6] Acknowledgments: The authors thank Dr. MASUDA (Japan) for supplying the authentic samples of lumazines.

Cation Transport in Normal Human Red Cells Treated with Sulfhydryl Compounds

Some of us have recently observed[1] that treatment of normal human red cells with AET (2-amino-ethylisothiouronium bromide) or cysteine, under suitable experimental conditions, modifies them in such a way that their behaviour in some in vitro hemolysis tests becomes similar to that of paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes. The effect is supposedly due to the –SH groups that both substances possess. So far, a few enzymatic properties of these treated red cells have been investigated and related to those of PNH erythrocytes[2]. Since in vitro studies with K^{42} have recently shown[3] that PNH red cells have normal K^+ influx and efflux rates, we have investigated whether the treatment with AET or cysteine modifies the cation transport of normal human erythrocytes. The blood of seven healthy adults was drawn with heparin; the red cells were separated by centrifugation and washed thrice with saline. An aliquot was treated with the above-mentioned sulfhydryl compounds as previously described[1]. (The Ham's test was performed on each sample to ascertain that the PNH-like behaviour had been achieved.) Another aliquot was similarly

[1] G. SIRCHIA, S. FERRONE, and F. MERCURIALI, Blood *25*, 502 (1965).
[2] G. SIRCHIA, S. FERRONE, R. MILANI, and F. MERCURIALI, to be published.
[3] J. V. AUDITORE, R. C. HARTMAN, and E. F. COLE, J. clin. Invest. *38*, 702 (1959).

handled, but no altering substance was added; it was used as control. All these red cell aliquots were successively resuspended in autologous plasma and stored at 4°C for 5 days, as described elsewhere[4]. Each sample was then divided into 6 separate aliquots of 5 ml: 3 of these were immediately centrifuged and the remaining 3 were incubated at 37°C for 3 h in a Dubnoff shaker moving at a rate of 50 oscillations/min. K^+ concentration was measured in the plasma of all the aliquots, before and after incubation of the blood, with a Beckman DU flame photometer. K^+ uptake by the cells was calculated by difference, according to the method of KAHN and ACHESON[5], determining the hematocrit value before and after incubation.

Results are summarized in the Table. It is seen that during the incubation both the control and treated red cells removed measurable quantities of K^+ from the plasma against a concentration gradient: hence by an active mechanism. This phenomenon was demonstrable in spite of the concomitant increase of hemolysis produced – especially in treated cell samples – by warming and shaking, which caused a passive shift of K^+ from the lysed cells to the plasma.

K^+ uptake by untreated and AET- or cysteine-treated red cells incubated at 37°C

Sample No.	K^+ uptake (mEq/l of cells ± S.D.[a])		
	Control	AET[b]	Cysteine[b]
1	+ 5.0 (± 0.08)	+ 4.8 (± 0.11)	+ 5.3 (± 0.03)
2	+ 6.7 (± 0.03)	+ 6.0 (± 0.07)	+ 5.6 (± 0.12)
3	+ 3.3 (± 0.08)	+ 5.2 (± 0.07)	+ 4.3 (± 0.23)
4	+ 5.2 (± 0.10)	+ 3.5 (± 0.00)	+ 3.0 (± 0.00)
5	+ 5.4 (± 0.12)	+ 2.5 (± 0.05)	+ 3.8 (± 0.00)
6	+ 3.4 (± 0.00)	+ 4.4 (± 0.11)	+ 7.0 (± 0.00)
7	+ 2.5 (± 0.04)	+ 2.3 (± 0.00)	+ 3.0 (± 0.03)

[a] S.D. = standard deviation of the mean of 3 determinations.
[b] Red cells treated with the altering substance.

The analysis of variance showed that the differences of K^+ uptake between the three groups (control and treated red cells) were not significant ($P > 0.2$).

The results of our experiments show that the enzymatic abnormalities occurring in normal erythrocytes treated with AET or cysteine do not impair the cation transport mechanism that acts at the cell surface. Apparently the two altering substances are not specific inhibitors of the membrane sulfhydryl groups, as are mercury, *n*-ethylmaleimide and *p*-mercuribenzoate, whose inhibitory effect on the cation transport mechanism of the red cell has been demonstrated[6–8].

Riassunto. È stato studiato il meccanismo di scambio cationico di membrana di emazie umane normali trattate con composti sulfidrilici (AET e cisteina). Si è osservato che, durante l'incubazione di 3 h a 37°C, effettuata dopo 5 giorni di soggiorno a 4°C, le emazie normali trattate con i suddetti composti rimuovono attivamente dal plasma normali quantitativi di K^+. I risultati vengono brevemente discussi.

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Istituto di Clinica Medica dell'Università di Milano (Italy), June 21, 1965.

[4] G. D'AMICO and P. P. FOÀ, Exper. *15*, 144 (1959).
[5] J. B. KAHN JR. and G. H. ACHESON, J. Pharmacol. exp. Therap. *115*, 305 (1955).
[6] R. WEED, J. EBER, and A. ROTHSTEIN, J. gen. Physiol. *45*, 395 (1962).
[7] R. F. SHEETS and H. E. HAMILTON, J. lab. clin. Med. *52*, 138 (1958).
[8] H. S. JACOB and J. H. JANDL, J. clin. Invest. *41*, 779 (1962).

A Preliminary Study of the Immunoelectrophoretic Properties of Pregnant Mares Serum (PMS) Together with its Application to the Diagnosis of Pregnancy in the Mare

In this study the specificity of the reactions between pregnant mare serum (PMS) and a developed antisera have been examined by electrophoretic and immunoelectrophoretic techniques. The knowledge gained has been applied to determine the presence or otherwise of a pregnancy in a small group of 8 mares.

On clinical examination a skilled practitioner can usually diagnose pregnancy in the mare between the 38th and 44th day after coitus. ELMENDORFF, LORAINE, and WALLEY[1] and ANTONIADES[2], by the use of various biological pregnancy tests, were able to verify the presence of pregnancy at approximately 40 days, while WIDE and WIDE[3], who used a hemagglutination inhibition technique (HAI), were able to demonstrate that the diagnosis of pregnancy was possible using this technique. They did not, however, test the sera before the 44th day, hence their report is not helpful in determining how early an immunological test will detect the presence of chorionic gonadotrophin in the serum of the mare.

Materials and methods. Electrophoretic and immunoelectrophoretic studies: Agar-gel electrophoretic and immunoelectrophoretic studies were carried out using the techniques as described by MCCARTHY, PENNINGTON, and CRAWFORD[4].

Hemagglutination inhibition assay design: These were identical with those described by MCCARTHY et al.[4] using PMS for the control solutions.

Preparation of antisera: Rabbits were immunized with a solution containing PMS and Bentonite in a dose level

[1] H. V. ELMENDORFF, J. H. LORAINE, and J. K. WALLEY, J. Endocr. *25*, 107 (1962).
[2] H. U. ANTONIADES, *Hormones in Human Plasma* (J. and A. Churchill, London 1960).
[3] M. WIDE and L. WIDE, Nature *198*, 1017 (1963).
[4] C. MCCARTHY, G. W. PENNINGTON, and W. S. CRAWFORD, J. Obstet. Gynaec. Brit. Commw. *71*, 1 (1964).