formed from sedoheptulose.[13] Gas-liquid chromatography revealed peaks corresponding to sedoheptulosan as well as to sedoheptulose.

D-manno-*Heptulose*, (10 mg), $[\alpha]_D$ +17° (*c* 1.75). Lead tetraacetate oxidation of the sugar (40 μmoles) under the conditions stated above gave erythrose as the main product, as confirmed by paper chromatography and electrophoresis. D-*manno*-heptulose 1-(*N'*-benzyl-*N'*-phenyl)-2-(*N'*-phenyl)-osazone was prepared according to White and Secor,[14] m.p. and mixed m.p. 192–196°.

Acknowledgement. The authors are indebted to Dr. N. K. Richtmyer for a reference sample of D-*glycero*-D-*manno*-octulose.

1. Ottestad, E., Brochmann-Hanssen, E., Øiseth, D. and Nordal, A. *J. Pharm. Pharmacol.* **11** (1959) 689.
2. Begbie, R. and Richtmyer, N. K. *Carbohyd. Res.* **2** (1966) 272.
3. Sephton, H. H. and Richtmyer, N. K. *Carbohyd. Res.* **2** (1966) 289.
4. Jones, J. K. N. and Sephton, H. H. *Can. J. Chem.* **38** (1960) 753.
5. Racker, E. and Schroeder, E. *Arch. Biochem. Biophys.* **66** (1957) 241.
6. Klevstrand, R. and Nordal, A. *Acta Chem. Scand.* **4** (1950) 1320.
7. Charlson, A. J. and Richtmyer, N. K. *J. Am. Chem. Soc.* **82** (1960) 3428.
8. Perlin, A. S. and Brice, C. *Can. J. Chem.* **34** (1956) 541.
9. Trevelyan, W. E., Procter, D. P. and Harrison, J. S. *Nature* **166** (1950) 444.
10. Sweeley, C. C., Bentley, R., Makita, M. and Wells, W. W. *J. Am. Chem. Soc.* **85** (1963) 2497.
11. Sephton, H. H. and Richtmyer, N. K. *J. Org. Chem.* **28** (1963) 2388.
12. Richtmyer, N. K. and Pratt, J. W. *J. Am. Chem. Soc.* **78** (1956) 4717.
13. Haskins, W. T., Hann, R. M. and Hudson, C. S. *J. Am. Chem. Soc.* **74** (1952) 2198.
14. White, L. M. and Secor, G. E. *J. Org. Chem.* **27** (1962) 2240.

Received September 17, 1970.

Biosynthesis of Spermidine and Spermine in Regenerating Rat Liver: Some Properties of the Enzyme Systems Involved

AARNE RAINA and PEKKA HANNONEN

Department of Medical Chemistry, University of Helsinki, Helsinki, Finland

In previous reports from this laboratory it was shown that the enzyme activities catalysing the synthesis of spermidine and spermine are increased during liver regeneration.[1,2] In these studies the enzymatic synthesis of spermine was demonstrated for the first time with liver preparations. Putrescine was also shown to influence the rate of spermine synthesis, stimulating it at low concentrations, whereas higher concentrations were inhibitory.[2] A report has lately appeared, showing that putrescine is a competitive inhibitor of spermine synthesis by prostatic enzyme.[3] Evidence has also been presented which suggests that a single prostatic enzyme may catalyse the production of both spermidine and spermine.[3] Therefore, our observation on the stimulation of spermine synthesis by low concentrations of putrescine seemed to be of considerable interest for understanding the mechanisms possibly regulating liver polyamine synthesis and prompted a closer study with partially purified enzyme preparations from regenerating liver. In the present study some properties of the crude enzyme preparations are described.

Material and methods. Putrescine-1,4-^{14}C dihydrochloride (specific activity 11.27 mC/mmole), DL-methionine-2-^{14}C (sp. act. 4.08 mC/mmole) and DL-methionine-1-^{14}C (sp. act. 3.54 mC/mmole) were purchased from the New England Nuclear Corporation. Unlabelled putrescine, spermidine, and spermine were supplied by Calbiochem. Before use, the radioactive putrescine and unlabelled spermidine were purified by chromatography with Dowex 50-H^+. Yeast inorganic pyrophosphatase was obtained from Sigma.

Labelled *S*-adenosylmethionine (SAM) was synthesized from ATP and either DL-methionine-2-^{14}C or DL-methionine-1-^{14}C, as described earlier,[2] and purified by chromatography with Dowex 50-H^+. Labelled 5'-deoxyadenosyl-(5'),3-aminopropyl-(1), methyl-

Table 1. Synthesis of polyamines by preparations from 35-h regenerating liver and ventral prostate. Isoelectric precipitation and ammonium sulphate fractionation were carried out as described in Methods. With SAM as the substrate, spermine synthesis was assayed in both the absence (−Pu) and presence of 0.02 mM putrescine (+Pu). n.d. = not determined; Spd = spermidine; Sp = spermine.

Enzyme fraction	Total protein mg	Polyamine synthesis (pmoles/mg protein/30 min) from SAM-2-^{14}C					from decarboxylated SAM-^{14}C		
		Spermidine	Spermine		Spd/Sp		Spermidine	Spermine	Spd/Sp
			−Pu	+Pu	−Pu	+Pu			
A. Liver									
Dial. supernatant	3300	242	19.7	34.4	12.3	7.0	8 600	101	85
$(NH_4)_2SO_4$-fractions									
II (33−45 %)	220	340	n.d.	35.7	n.d.	9.5	7 480	81	92
III (45−57 %)	1000	408	40.7	58.1	10.0	7.0	14 400	122	118
IV (57−65 %)	550	100	12.9	57.0	7.8	1.8	750	150	5.0
B. Ventral prostate									
Dial. supernatant	n.d	2390	390	511	6.1	4.7	7 660	976	7.8

sulphonium salt (decarboxylated SAM) was prepared from radioactive SAM labelled at the C-2 position of the methionine moiety, with the aid of crude SAM decarboxylase (ammonium sulphate fraction prepared from *E. coli* as described by Tabor [4]), and purified on a Dowex 50-H^+ column.[5] Paper chromatography and paper electrophoresis showed that the final product was 85−90 % pure, containing about 10 % of SAM as an impurity (*cf.* Ref. 5).

Ventral prostates were obtained from male Wistar rats aged 12 months. The animals to be partially hepatectomized were female rats weighing 110−150 g. Partial hepatectomy was performed according to Higgins and Anderson.[6] The livers were homogenized with 3 volumes of cold 0.25 M sucrose containing 1 mM mercaptoethanol and 0.3 mM EDTA, pH 7.0, in a homogenizer of Potter-Elvehjem type. All subsequent operations were performed at temperatures below 4°C. The homogenate was centrifuged at 100 000 *g* for 60 min. The supernatant was passed through a glass wool plug to remove lipids. A sample was set aside and dialysed as described below. The rest of the supernatant fraction was adjusted to pH 5.0 by careful addition of 0.2 M sodium acetate, pH 4.0.[5] The resulting precipitate was removed by immediate centrifugation and the pH of the supernatant adjusted to pH 6.5 by addition of 1 M sodium phosphate, pH 7.4. Ammonium sulphate solution (pH 6.5, saturated at 0°C) was added slowly with stirring to give the relative saturation concentrations indicated in Table 1. Stirring was continued for 30 min at 0°C, after which the precipitates were collected by centrifugation, dissolved in a small volume of 0.005 M sodium phosphate-1 mM mercaptoethanol-0.3 mM EDTA, pH 7.0, and dialysed against the same solution for 16 h at 4°C. The dialysed samples could be stored at −70°C for several months without loss of enzyme activity.

The methods for the assay of spermidine and spermine synthesis were slightly modified from those described by Pegg and Williams-Ashman.[3,5] With ammonium sulphate fractions as the enzyme source, dithiothreitol was found to stimulate the syntheses of both spermidine and spermine by 10−30 % and was therefore added routinely to the incubation mixture. Spermine synthesis was assayed in the presence of 0.02 mM putrescine. The pH in all incubations was 7.5. The medium for the assay of spermidine synthesis contained, in a total volume of 0.5 ml, 50 μmoles of sodium phosphate pH 7.5 (final), 0.5 μmole of dithiothreitol, 1 μmole of putrescine, 0.09 μmole of SAM-2-^{14}C and 1 to 8 mg of protein. For determination of spermine synthesis, the amount of putrescine was reduced to 0.01 μmole and 1 μmole of spermidine was added. After incubation for 30 min at 37°C, the formation of radioactive polyamines was determined as described previously.[2] Under these conditions the rate of synthesis of both spermidine and spermine was linear for at least 30 min and proportional to the amount of added protein in the range mentioned above. The systems were saturated with respect to putrescine and spermidine and close to saturated with SAM (*cf.* Ref. 2).

When labelled decarboxylated SAM (0.18 mM) was used as the substrate instead of

SAM, the incubation volume was reduced to 0.2 ml and the protein content was 0.3 to 3 mg. Putrescine was omitted from the spermine-synthesizing reaction mixture. The reaction was terminated by adding 0.1 ml of 1 N NaOH, and alkaline digestion was carried out in a boiling water bath for 30 min to degrade the decarboxylated SAM.[3] The fractions were acidified and applied to Dowex 50-H^+ columns and washed with 2 N HCl. Radioactive polyamines were eluted with a small amount of 6 N HCl and separated by paper electrophoresis after concentration in a vacuum evaporator.[2] Column chromatography (but not alkaline digestion) was also necessary when ^{14}C-putrescine was used as the labelled precursor.[2]

Results. Table 1 summarizes the results of fractionation of the 100 000 *g* supernatant derived from 35-h regenerating liver. Isoelectric precipitation resulted in a loss of one-third of the total protein and a proportional loss of the spermidine- and spermine-synthesizing enzyme activities (not shown). Practically all the enzyme activity left after this stage was precipitated by ammonium sulphate between 33—65 % saturation. Table 1 also shows that with SAM as the labelled precursor, putrescine stimulated spermine synthesis with both dialysed supernatant and ammonium sulphate fractions as the enzyme source. This stimulation was about four-fold with fraction IV. No stimulation was obtained with cadaverine or 1,3-diaminopropane. Ammonium sulphate fractionation resulted in partial separation of the spermidine- and spermine-synthesizing enzyme activities. This is clearly seen from the ratio of spermidine/spermine synthesized, especially when the latter was assayed in the presence of putrescine. However, this would not necessarily mean that separate enzymes catalyse the synthesis of spermidine and spermine.

The mechanism by which small amounts of putrescine stimulate spermine synthesis is not obvious. It probably involves the decarboxylation step of SAM (allosteric effect?), because no stimulation was obtained with decarboxylated SAM as substrate (not shown). With the use of radioactive putrescine, it was demonstrated that the diamine was not directly incorporated into spermine. The concentration of putrescine capable of stimulating spermine synthesis is close to that found in normal liver.[2] Therefore we propose that putrescine should be included routinely in the assay system of spermine synthesis.

With labelled SAM as precursor, the dialysed prostatic supernatant was 10—15 times as active in spermidine synthesis as the supernatant from regenerating liver. The use of decarboxylated SAM instead of SAM caused increases in spermidine synthesis of up to 35-fold (Table 1), *i.e.* to the level obtained with the prostatic preparation. On the other hand, in spermine synthesis the increase was only two- to threefold (assayed in the presence of putrescine). The difference observed between the liver and prostatic preparations in ability to utilize SAM and decarboxylated SAM as the substrate for spermidine synthesis is of considerable interest, especially in view of the suggestion that a single enzyme system may catalyse both the decarboxylation of SAM and the transfer of the propylamine group from decarboxylated SAM to putrescine.[5] Several possible explanations can be advanced for the above difference, but they must be regarded as purely speculative until the enzymes have been purified extensively. When prostatic and liver supernatant fractions were mixed, the resulting enzyme activities were practically additive, which speaks against but by no means excludes the presence of activator(s) or inhibitor(s) of SAM decarboxylase activity in these preparations. On the other hand, SAM decarboxylase and propylamine transferase may be separate enzymes or these two may form complexes in different proportions in different tissues. These problems can only be solved by extensive purification of the enzyme activities in question. This is a difficult task, especially in the case of spermine, because the enzyme activity synthesizing spermine is low in most tissues and the assay method tedious and expensive. Testis and brain seem to contain relatively high spermine synthetase activity (unpublished results) and may be useful for further characterization of this enzyme. Further work in this direction is in progress in our laboratory.

Acknowledgement. This work was supported by grants from the *Sigrid Jusélius Foundation* and from the *National Research Council for Medical Sciences*, Finland.

1. Hannonen, P., Raina, A. and Hölttä, E. *Scand. J. Clin. Lab. Invest.* **25** *Suppl.* **113** (1970) 99.

2. Raina, A., Jänne, J., Hannonen, P. and Hölttä, E. *Ann. N.Y. Acad. Sci. In press.*
3. Pegg, A. E. and Williams-Ashman, H. G. *Arch. Biochem. Biophys.* **137** (1970) 156.
4. Tabor, C. W. In Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology*, Academic, New York 1962, Vol. 5, p. 756.
5. Pegg, A. E. and Williams-Ashman, H. G. *J. Biol. Chem.* **244** (1969) 682.
6. Higgins, G. H. and Anderson, R. M. *Arch. Pathol.* **12** (1931) 186.

Received September 28, 1970.

Addition of Cyclopentadiene and Indene to 1,3,5-Trinitrobenzene

ROBERT WAHREN and OLOF WENNERSTRÖM

Department of Organic Chemistry, Royal Institute of Technology, S-100 44 Stockholm 70, Sweden

The formation of stable σ-complexes — Meisenheimer complexes — in the nucleophilic substitution of aromatic polynitro compounds is well documented.[1,2] Such complexes can be general intermediates in aromatic S_N2 reactions, although the role of σ- and π-complexes is still under discussion.[3]

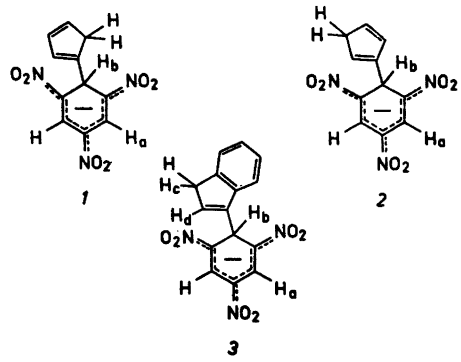
Meisenheimer compounds can also be regarded as salts of nitronic acids and, if reasonably stable, can be expected to react analogously to other salts of nitronic acids.[4]

We have recently found new methods for preparing Meisenheimer compounds from phenylethynylcopper[5] or 2,6-dimethoxyphenylcopper[6] and 1,3,5-trinitrobenzene in pyridine.

Cyclopentadienylcopper has been prepared from cyclopentadiene and copper(I) oxide in the presence of suitable phosphines.[7] Organosilver compounds are less reactive (although thermally more labile) than the corresponding copper compounds[8] and react more slowly with 1,3,5-trinitrobenzene.[5,9]

Cyclopentadiene (or indene), silver oxide [but not copper(I) oxide] and 1,3,5-trinitrobenzene reacted in pyridine to give a beautiful red solution from which a Meisenheimer compound was isolated. Cyclopentadiene and trinitrobenzene also reacted in pyridine in the absence of silver compounds, but more slowly.

Silver oxide possibly facilitates the formation of a cyclopentadienyl anion, a reasonable intermediate in the reaction, besides increasing the electrophilicity of 1,3,5-trinitrobenzene by co-ordination with a nitro group.



Cyclopentadiene (15 mmol), 1,3,5-trinitrobenzene (10 mmol) and silver oxide (5 mmol) were stirred for 24 h in pyridine (100 ml). A red colour developed slowly. Ice and hydrochloric acid (2 M) in slight excess wre added. A dark red precipitate formed. A red compound was separated from silver chloride by acetone extraction to give dark red crystals, recrystallised from ethanol (2.8 g, 78 %, m.p. 128–33° decomp.). (Found: C 53.5; H 4.0; N 15.5. Calc. for $C_{16}H_{14}N_4O_6$: C 53.6; H 3.9; N 15.6.)

The visible spectrum of the product (Fig. 1) is typical for Meisenheimer compounds from 1,3,5-trinitrobenzene.[1]

Monosubstituted cyclopentadienes rearrange under mild conditions to mixtures of the 1- and 2-substituted isomers.[10] The NMR spectra (at 60 MHz and 100 MHz) of the isolated product in pyridine-d_5 can best be rationalised on the assumption that the two isomers *1* and *2* are present in approximately equal amounts. The spectrum showed the following charac-