Scheme 4.

was continued for a further 24 hr. The cells were harvested by centrifugation at 3600 rev./min. in an MSE 18 centrifuge, refluxed with ethanolic 5% (w/v) KOH (50 ml.) for 1 hr. and the non-saponifiable material was extracted with ether. The ether extract, which contained 28% (2.5×10^6 counts/100 sec.) of the original radioactivity, was fractionated by preparative thin-layer chromatography into two bands, A and B. The less polar band A contained 11.6% (1.05×10^6 counts/100 sec.) of the original radioactivity and was not characterized. The more polar band B contained 17% (1.5×10^6 counts/100 sec.) of the original radioactivity and was extracted with ether, diluted with ergosterol (1 g.) and crystallized to constant radioactivity (563 counts/100 sec./mg.; total activity $563 \times 1000 = 5.6 \times 10^5$ counts/100 sec.). This represents 6.2% incorporation of 24-methylenelanosterol into ergosterol (7 μ g. of 24-methylenelanosterol converted/g. dry wt. of yeast cells). Confirmatory evidence for the incorporation of 24-methylenelanosterol (XII) into ergosterol (IV) was provided by ozonolysis to yield 2,3-dimethylbutyraldehyde, which was isolated as the dimer derivative. This derivative retained quantitatively the radioactivity of the original ergosterol.

In conclusion we suggest the pathway shown in Scheme 4 for the biosynthesis of ergosterol side chain.

We are most grateful to Dr J. F. McGhie for giving us the experimental details for the large-scale preparation of lanosteryl acetate, and to Professor K. A. Munday for his help and encouragement throughout this investigation. The purchase of radioactive materials was made possible by a grant from the Medical Research Council.

Akhtar, M., Hunt, P. F. & Parvey, M. A. (1966). *Chem. Comm.* (in the Press).

Alexander, G. J., Gold, A. M. & Schwenk, E. (1957). *J. Amer. chem. Soc.* **79**, 2967.

Badar, S., Guglielmetti, L. & Arigoni, D. (1964). *Proc. Chem. Soc., Lond.*, p. 16.

Castle, M., Blondin, G. & Nes, W. R. (1963). *J. Amer. chem. Soc.* **85**, 3306.

Corwin, L. M., Schroeder, L. J. & McCullough, W. G. (1956). *J. Amer. chem. Soc.* **78**, 1372.

Klein, H. P., Eaton, N. R. & Murphy, J. C. (1954). *Biochim. biophys. Acta*, **13**, 591.

Lederer, E. (1964). *Biochem. J.* **93**, 449.

Lewis, D. A. & McGhie, J. F. (1956). *Chem. & Ind.* p. 550.

Parks, L. W. (1958). *J. Amer. chem. Soc.* **80**, 2023.

Schwenk, E. & Alexander, G. J. (1957). *Arch. Biochem. Biophys.* **76**, 65.

Wilton, D. C., Akhtar, M. & Munday, K. A. (1966). *Biochem. J.* **98**, 29c.

Biochem. J. (1966) **100**, 40c

Amino Acid Sequence in the Active Site of Phosphoglucomutase from Rabbit Muscle

By CELIA P. DE MILSTEIN

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(Received 21 June 1966)

Phosphoglucomutase (α -D-glucose 1,6-diphosphate) is an enzyme widely distributed in Nature that catalyses the interconversion of glucose 1-phosphate and glucose 6-phosphate. The presence of phosphate in this enzyme was demonstrated by Jagannathan & Luck (1949), who also showed that it may readily be exchanged with phosphate in the

substrates and thus that it is probably involved in the active centre of the enzyme. Later this phosphate group was shown to be covalently bound to a serine residue (Anderson & Jollès, 1957). Milstein & Sanger (1961), using ^{32}P -labelled enzyme, showed that a unique sequence around the serine phosphate residue could be isolated from phospho-

glucomutase and reported it as: Thr-Ala-SerP-His-Asx (where SerP represents serine phosphate and Asx means either aspartic acid or asparagine). This sequence was later confirmed by two other Laboratories (Joshi, Hashimoto, Hanabusa, Dougherty & Handler, 1965; Harshman & Najjar, 1965). However, Harshman & Najjar (1962) reported the sequence around the serine phosphate as: Asx-Gly-Glu-SerP-Ala-Gly; and Harshman & Najjar (1965) reported that in fact the catalytic site contains two serine residues on one peptide chain separated by four amino acid residues. This result is not in agreement with the earlier results referred to. It is known that the sequence Glu-SerP-Ala is found in liver aliesterase (Jansz, Posthumus & Cohen, 1959), but, when radio-autographs of electrophoretograms of partial acid hydrolysates of labelled liver aliesterase and ^{32}P -labelled phosphoglucomutase were compared (Sanger, 1963), no common bands were apparent.

The possibility that one of the serine phosphate residues was not observed by Milstein & Sanger (1961) because of differences in labelling or in the stability of one of them was investigated in the present study by trying different conditions of isolating the labelled enzyme. Three methods of labelling the phosphoglucomutase were tried, the two used by Milstein & Sanger (1961) and a third in which glucose 6- ^{32}P -phosphate was similarly prepared by the hexokinase reaction but in which the ^{32}P -labelled ATP was produced from $\text{H}_3^{32}\text{PO}_4$ by the method of Glynn & Chappell (1964). In preparations used by previous workers it was necessary to dialyse the labelled enzyme against several changes of water, over long periods of time (usually several days), to remove completely the excess of ^{32}P phosphoesters and inorganic phosphate. In the present experiments it was found that the time of dialysis could be shortened. The labelled phosphoglucomutase was dialysed for 1 day and then run through Sephadex G-25 in 0.1M-ammonium acetate buffer, pH 6.0. This procedure was just as effective as the usual much longer period of dialysis.

In all cases, when the major radioactive products of a tryptic digest were subjected to partial acid hydrolysis and run by electrophoresis at pH 3.5, no pattern similar to that shown by liver aliesterase was obtained. This is further confirmation of the work by Milstein & Sanger (1961). However, it was considered necessary to examine the sequence reported by Harshman & Najjar (1965) by more direct methods. The results reported here do not confirm their proposed sequence including two SerP in one peptide, but are in close agreement with those obtained on flounder phosphoglucomutase (Hashimoto, Del Rio & Handler, 1966).

Rabbit-muscle enzyme (Najjar, 1962) was

labelled by means of substrate made by enzymic phosphorolysis of starch by using potato juice in the presence of $\text{H}_3^{32}\text{PO}_4$ (Milstein & Sanger, 1961). This method proved to be the best one when modified by substituting tris-HCl for phosphate buffer, pH 7.0, and by eliminating histidine and Mg^{2+} from the incubation mixture. The labelled enzyme was purified by dialysis and gel filtration as described above. It was denatured either by heating at 100° for 30 min. or with 10 vol. of acid acetone (2 vol. of 0.5N-HCl with 8 vol. of acetone) at room temperature for 15 min. and then subjected to tryptic hydrolysis for 17 hr.

The hydrolysate was first purified by running it through a Sephadex G-25 column in N-acetic acid, after which it was run by paper electrophoresis at pH 6.5. Two main bands, 1 and 2, were obtained (these correspond to bands B and A respectively in the work of Milstein & Sanger, 1961); they were further purified by paper electrophoresis at pH 3.5. Band 2 separated into two main bands, which after further purification at pH 2.1 and 4.7 were completely hydrolysed by acid and analysed in a Spinco amino acid analyser. Their compositions were very similar. The difference in mobility that they exhibited may be due to a change in charge during handling. The normal α -aspartyl residues present in the peptides may be converted into the $\alpha\beta$ -ring form, which then gives rise to the β -form, as has been described for other systems (Sanger, 1963).

The tryptic peptides, however, were not sufficiently pure for the purpose of this study and the removal of impurities proved to be difficult. As a first step they were further digested with subtilisin, with 60 μg . for 6 hr. (yielding mainly peptide TS2; Table 1) or with 500 μg . for 4 hr. (yielding mainly peptide TS6; Table 1) in 1% (w/v) ammonium carbonate buffer, pH 9.0, at 37° . This gave smaller and purer radioactive peptides than those prepared with trypsin. The subtilisin peptides were still further purified by paper electrophoresis at pH 6.5 followed by electrophoresis at pH 3.5. The amino acid analyses of two major radioactive peptides

Table 1. *Amino acid composition of subtilisin peptides*

Residues below 0.15 are not included.		
Peptide.....	TS2	TS6
His	1.06	Not done
Asp	2.88	2.90
Thr	0.93	—
Ser	0.89	0.95
Pro	1.66	2.14
Gly	3.00	3.27
Ala	1.09	—
Phe	1.00	0.94

Thr-Ala-SerP-His-Asx-(Asx,Asx,Pro,Pro,Gly,Gly,Gly)-Phe

Fig. 1. Proposed partial sequence of peptides TS1, TS2 and TS6.

(TS2 and TS6) so obtained are shown in Table 1. Another peptide (TS1) that was obtained from subtilisin digestion showed an amino acid composition practically identical with that of peptide TS2 except that phenylalanine was absent, indicating that this residue is either the *N*- or the *C*-terminus. However, it could be shown by the 'dansyl' (1 - dimethylaminonaphthalene - 5 - sulphonyl chloride) method (Gray & Hartley, 1963) that threonine was the *N*-terminal residue in this peptide and therefore phenylalanine should be the *C*-terminus. These results, taken with those of Milstein & Sanger (1961), suggest the partial sequence shown in Fig. 1, and which contrasts with that suggested by Harshman & Najjar (1965), namely: Thr-Ala-SerP-His-Asx-Gly-Glx-Ser-Ala-Gly-Leu-Asx-Leu.

Only one serine is present in the peptides isolated

in the present work and no glutamic acid, serine, alanine or leucine residues are among the other eight amino acids that must follow the aspartic acid. On the other hand, proline and phenylalanine, found to be present in these peptides, are absent from the sequence reported by Harshman & Najjar (1965).

The reasons for the discrepancies remain obscure.

In conclusion, the results presented in this paper do not confirm the presence of the two phosphorylated serine residues in digests of rabbit-muscle phosphoglucomutase.

- Anderson, L. & Jollès, G. R. (1957). *Arch. Biochem. Biophys.* **70**, 121.
- Glynn, I. M. & Chappell, J. B. (1964). *Biochem. J.* **90**, 147.
- Gray, W. R. & Hartley, B. S. (1963). *Biochem. J.* **89**, 379.
- Harshman, S. & Najjar, V. A. (1962). *Fed. Proc.* **21**, 233.
- Harshman, S. & Najjar, V. A. (1965). *Biochemistry*, **4**, 2526.
- Hashimoto, T., Del Rio, C. & Handler, P. (1966). *Fed. Proc.* **25**, 408.
- Jagannathan, V. & Luck, J. M. (1949). *J. biol. Chem.* **179**, 569.
- Jansz, H. S., Posthumus, C. H. & Cohen, J. A. (1959). *Biochim. biophys. Acta*, **33**, 396.
- Joshi, J. G., Hashimoto, T., Hanabusa, K., Dougherty, H. W. & Handler, P. (1965). In *Evolving Genes and Proteins*, p. 207. Ed. by Bryson, V. & Vogel, H. J. New York and London: Academic Press Inc.
- Milstein, C. & Sanger, F. (1961). *Biochem. J.* **79**, 456.
- Najjar, V. A. (1962). In *The Enzymes*, 2nd ed., vol. 6, p. 161. Ed. by Boyer, P. D., Lardy, H. A. & Myrback, K. New York: Academic Press Inc.
- Sanger, F. (1963). *Proc. chem. Soc., Lond.*, p. 76.