Short Communications and Preliminary Notes

The D-glucosamine-6-phosphate N-acetylase of yeast

LELOIR AND CARDINI have found[1] that crude extracts of *Neurospora crassa* are able to catalyze the N-acetylation of D-glucosamine and of D-glucosamine-6-phosphate (Gm-6-P). The reaction requires coenzyme A (coA), adenosinetriphosphate (ATP) and acetate ion, and presumably, goes by way of acetyl-coA formed by the acetate activating enzyme of LIPMANN *et al.*[2]. Because of the presence of hexokinase which catalyzed the phosphorylation of glucosamine to Gm-6-P by ATP[3] and of various phosphatases, it was not possible to decide whether the *Neurospora* enzyme acetylates only the free amino sugar, only Gm-6-P, or both substances. The N-acetylation of added glucosamine had already been found to occur as a slow reaction in a crude enzyme system isolated by CHOU AND SOODAK from pigeon liver[4]. KATZ *et al.*[5] have described a similar reaction in *Clostridium kluyveri*. A preliminary report has been published by ROSEMAN[6] of the preparation of N-acetyl-glucosamine-6-phosphate (Ac-gm-6-P) by chemical acetylation of Gm-6-P.

An enzyme has now been obtained from baker's yeast which catalyzes the N-acetylation of Gm-6-P. Neither glucosamine-1-phosphate (Gm-1-P) nor glucosamine is acetylated at a measurable rate. The product of the reaction with Gm-6-P has been shown to be Ac-gm-6-P by isolation of the latter ester through barium salt precipitation by ethanol followed by chromatography on Dowex-1 (formate). In one experiment in which 11.6 μmoles of Ac-gm-6-P (together with adenosine-5'-phosphate) were in the barium-free solution subjected to chromatography, 5.3 μmoles were recovered as a dry powder following lyophilization of the 1 *N* HCOOH eluates which included the peak. When this material was dissolved in 5.0 ml of water, the solution contained 1.07 μmoles/ml of Ac-gm-6-P as determined by a modification of the MORGAN AND ELSON method[7] with N-acetylglucosamine as a reference standard. The solution contained no inorganic phosphorus and 1.11 μmoles/ml of total organically bound phosphorus (FISKE-SUBBAROW determination[8]). These analyses indicate that the enzymically prepared Ac-gm-6-P is substantially pure. The yeast enzyme which has been studied here transfers acetyl groups from acetyl-coA to Gm-6-P as shown by its requiring either chemically synthesized acetyl-coA (prepared according to KAUFMAN *et al.*[9]) or the acetate-activating enzyme system of LIPMANN[2] (see Table I).

TABLE I

ENZYMIC FORMATION OF N-ACETYL-GLUCOSAMINE-6-PHOSPHATE

Reaction mixtures contained: glutathione, $7\cdot10^{-3}$ *M*; potassium phosphate, $3\cdot10^{-2}$ *M*; $MgCl_2$, $7\cdot10^{-3}$ *M*; KF, $3.3\cdot10^{-2}$ *M*; Gm-6-P, $1.5\cdot10^{-3}$ *M*; vol. 3 ml; 37°. In A, B, C, D, E, F, G, potassium acetate, $6.7\cdot10^{-3}$ *M*; ATP, $1.3\cdot10^{-2}$ *M*; incubated 30 min. G and H also contained *tris*-(hydroxymethyl)-amino-methane buffer (THM), $2.9\cdot10^{-2}$ *M*. Expt. H, incubation time, 20 min.

Expt. No.	*Gm-6-P Acetylating enzyme (mg protein)*	*Additional acetate-activating enzyme (mg protein)*	*Pabst CoA (M)*	*Synthetic acetyl-coA (M)*	*pH*	*Ac-g-6-P Formed (μmoles)*
A	1.0	0	0	0	7.2	0
B	1.0	0	$8\cdot10^{-5}$	0	7.2	1.41
C	0	0.9	$8\cdot10^{-5}$	0	7.2	0.02
D	1.0	0.9	$8\cdot10^{-5}$	0	7.2	1.63
E	1.0	0.9	$8\cdot10^{-4}$	0	7.2	1.78
F	1.0	0.9	$8\cdot10^{-5}$	0	6.7	1.23
G	1.0	0.9	$8\cdot10^{-5}$	0	8.2	2.18
H	2.3	0	0	$3.6\cdot10^{-3}$	8.1	1.14

The acetylating enzyme has been obtained by sonic disintegration* of yeast (Fleischmann) in potassium phosphate solution according to the procedure described for the acetate-activating

* The author wishes to thank Prof. ARTHUR KORNBERG for making available to him a 10 kc Raytheon magnetostrictive oscillator.

enzyme[2]. Precipitation of nucleic acid with protamine sulfate, followed by dialysis of the supernatant fluid against 0.002 M ethylenediaminetetraacetic acid (EDTA), pH 6.7, gave a solution of the crude enzyme which could be frozen and thawed repeatedly without change in activity. It could be purified further by $(NH_4)_2SO_4$ fractionation and treatment with calcium phosphate gel. Fig. 1 shows the formation of Ac-gm-6-P as a function of time and of enzyme concentration. When glucosamine was added as the only substrate to a system similar to that of Fig. 1, acetylated amino sugar appeared at the same rate. This was due to the presence of hexokinase which rapidly converted the added glucosamine to Gm-6-P, the true substrate of the acetylating enzyme. This fact was demonstrated by testing the activity of the enzyme toward glucosamine in the presence of preformed acetyl-coA and in the absence of ATP. As shown in Fig. 2, no acetylated product was formed, whether the acetyl-coA was synthesized chemically or by the action of the acetate-activating enzyme (in this case, ATP was removed subsequent to acetyl-coA formation by incubating with excess glucose and a large amount of yeast hexokinase).

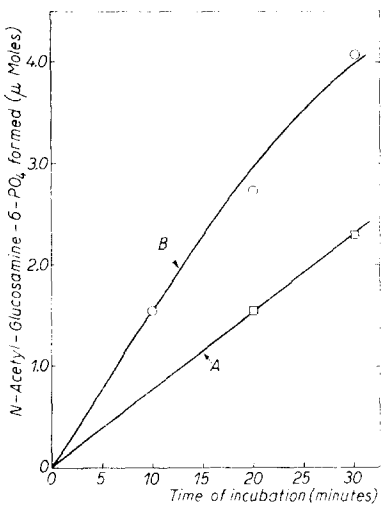

Fig. 1. Enzymic synthesis of N-acetyl-glucosamine-6-phosphate by a specific N-acetylase from yeast. The yeast fraction also contained the acetate-activating enzyme. Composition of reaction mixtures: Gm-6-P, $2.48 \cdot 10^{-3}$ M; glutathione, $7 \cdot 10^{-3}$ M; KF, $3.3 \cdot 10^{-2}$ M; K acetate, $6.7 \cdot 10^{-3}$ M; potassium phosphate, $3.3 \cdot 10^{-2}$ M; $MgCl_2$, $7 \cdot 10^{-3}$ M; Pabst coA, $8 \cdot 10^{-5}$ M; ATP, $1.3 \cdot 10^{-2}$ M; vol. 3 ml; pH 7.5; 37°. Curve A, 3.0 mg protein; curve B, 6.1 mg protein.

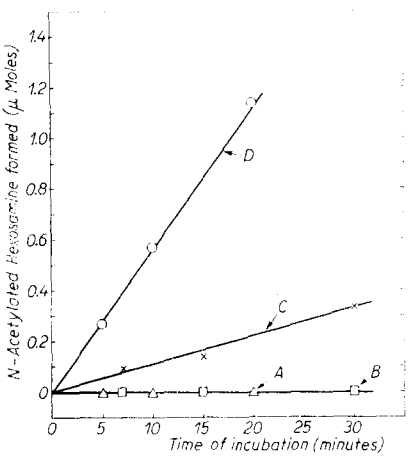

Fig. 2. Non-acetylation of glucosamine by glucosamine-6-phosphate N-acetylase in presence of added acetyl-coA. Curves A and D with synthetic acetyl-coA, $3.6 \cdot 10^{-3}$ M and 2.3 mg protein. In A the substrate was $1.55 \cdot 10^{-3}$ M glucosamine; in D the substrate was $1.43 \cdot 10^{-3}$ M Gm-6-P. Curves B and C with acetyl-coA preformed by acetate-activating enzyme and present at $4.1 \cdot 10^{-4}$ M; 0.78 mg protein containing the acetylating enzyme. In B the substrate was $1.45 \cdot 10^{-3}$ M glucosamine; in C the substrate was $1.39 \cdot 10^{-3}$ M Gm-6-P. Additional components of all reaction mixtures: glutathione, $6 \cdot 10^{-3}$ M; potassium phosphate, $2.5 \cdot 10^{-2}$ M; KF, $3 \cdot 10^{-2}$ M; $MgCl_2$, $5 \cdot 10^{-3}$ M; THM, $3 \cdot 10^{-2}$ M; vol. 3 ml; pH 8.1; 37°. In B and C, adenosine-5′-phosphate, $3 \cdot 10^{-3}$ M; glucose, $1.2 \cdot 10^{-2}$ M.

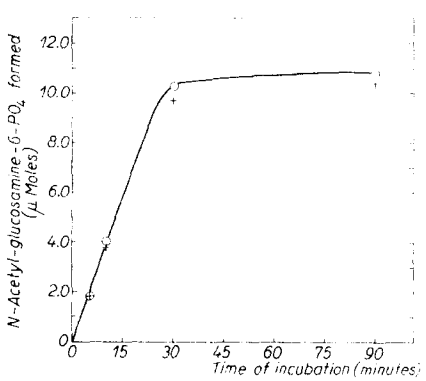

Fig. 3. Non-acetylation of glucosamine-1-phosphate by glucosamine-6-phosphate N-acetylase in presence of acetate-activating enzyme. Composition of reaction mixture: Gm-1-P, $4.10 \cdot 10^{-4}$ M; Gm-6-P, $1.27 \cdot 10^{-3}$ M; ATP, $1.3 \cdot 10^{-2}$ M; $MgCl_2$, $6.7 \cdot 10^{-3}$ M; K acetate, $6.4 \cdot 10^{-3}$ M; coA, $8.3 \cdot 10^{-4}$ M; potassium phosphate, $2.1 \cdot 10^{-2}$ M; glutathione, $6.5 \cdot 10^{-3}$ M; KF, $3.1 \cdot 10^{-2}$ M; THM, $2.9 \cdot 10^{-2}$ M; EDTA, $1.7 \cdot 10^{-4}$ M; vol. 9.6 ml; 10.4 mg protein; pH 8.2; 37°. ○, Analysis of filtrate prior to acid hydrolysis; +, analysis of filtrate after hydrolysis for 4 min at 100° in 0.58 N HCl. 12.2 μmoles Gm-6-P and 3.9 μmoles Gm-1-P present initially.

The possibility of activity of the enzyme with Gm-1-P as substrate was investigated by preparing an equilibrium mixture of Gm-6-P and Gm-1-P with rabbit muscle phosphoglucomutase[10]. After heating to destroy the mutase, the solution was added to an acetylating system (Fig. 3). The reaction was then allowed to proceed to completion, and the amount of acetylated amino sugar at various times was determined in aliquots of the reaction mixture before acid hydrolysis (analysis for Ac-gm-6-P), and after acid hydrolysis (analysis for Ac-gm-6-P plus any Ac-gm-1-P which may have been present). Inasmuch as there was no increase produced by acid hydrolysis in the amount of acetylated product, and since the acetylating reaction went at least 90% of the way to completion calculated from the Gm-6-P added, there was no evidence for any acetylation of Gm-1-P. Since the analytical method used to determine Ac-gm-6-P is specific for acetylhexosamines in which carbon atom 1 is not combined in glycosidic linkage, Ac-gm-1-P would be expected to give a positive test only after acid hydrolysis to N-acetyl-glucosamine.

The enzyme which acetylates Gm-6-P does not require Mg^{++} or inorganic phosphate ion. It is not inhibited by fluoride ion. The Michaelis constant for Gm-6-P appears to be about $5 \cdot 10^{-4}$ *M*. The enzyme appears to be saturated at rather low concentrations of acetyl-CoA. The further purification of this enzyme, its properties, and its role in glucosamine metabolism are being studied.

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1 L. F. LELOIR AND C. E. CARDINI, *Biochim. Biophys. Acta*, 12 (1953) 15.
2 M. E. JONES, S. BLACK, R. M. FLYNN AND F. LIPMANN, *Biochim. Biophys. Acta*, 12 (1953) 141.
3 D. H. BROWN, *Biochim. Biophys. Acta*, 7 (1951) 487.
4 T. C. CHOU AND M. SOODAK, *J. Biol. Chem.*, 196 (1952) 105.
5 J. KATZ, I. LIEBERMAN AND H. A. BARKER, *J. Biol. Chem.*, 200 (1953) 417.
6 S. ROSEMAN, *Federation Proc.*, 13 (1954) 283.
7 W. T. J. MORGAN AND L. A. ELSON, *Biochem. J.*, 28 (1934) 988.
8 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
9 S. KAUFMAN, C. GILVARG, O. CORI AND S. OCHOA, *J. Biol. Chem.*, 203 (1953) 869.
10 D. H. BROWN, *J. Biol. Chem.*, 204 (1953) 877.

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Fractionation of deoxyribonucleoprotein

CHARGAFF[1] *et al.* recently achieved a fractionation of DNA into fractions of differing base composition by extracting denatured nucleoprotein with sodium chloride solutions of increasing concentration. They suggested that the basis of this fractionation might be the existence of bonds of varying stability between the protein and the DNA which were broken progressively with increasing salt concentration. In a previous communication[2], we showed that nucleoprotein fractions containing nucleic acids having differing base compositions may be obtained by successive extractions with salt solutions of constant strength. It was shown also that the proportions of protein to DNA varied in the nucleoprotein fractions obtained in this way.

A partial analysis has now been made of the proteins present in the calf thymus nucleoprotein fractions obtained by successively extracting chloroform-denatured nucleoprotein with 0.6 *M* sodium chloride solutions. It has been found that the ratios of the basic amino-acids, arginine and lysine, differ considerably in the different fractions (Table I).

In these experiments the saline extracts of chloroform-treated nucleoprotein were obtained as previously described[2]. Normal hydrochloric acid was then added to each extract to a final concentration of *N*/5 HCl. The solutions were allowed to stand several hours and then centrifuged. The resulting supernatant solutions were adjusted to pH 11 with ammonium hydroxide and the histones precipitated by addition of two volumes of acetone. The histones were redissolved in water, dialysed and finally freeze dried. All operations were carried out below 5° C.

It has been shown that relatively small amounts of protein remain with the nucleic acid after one extraction with a strong acid[3]. However, there is no reason to suppose that the strong acid preferentially extracts one histone component (although weak acids do)[3]. The amino-acid compositions of these residual amounts have not so far been determined, but they are unlikely to change the general character of the results given here, which are thought to be of sufficient interest to justify publication.