Chemical Analysis of Acetylated Bovine Growth Hormone, a Growth-Hormone Inhibitor

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Purified bovine growth hormone was acetylated with acetic anhydride under various conditions. Acetylated bovine growth hormone prepared in anhydrous acetic anhydride at 43° for 5 min. inhibited the growth-promoting response of unmodified bovine growth hormone in hypophysectomized rats. This acetylated bovine growth hormone contained 18 total acetyl groups/mol., which included 12 N⁶-acetyl and 6–8 hydroxylamine-reactive acetyl groups (acetylated aliphatic hydroxy amino acids and 2–3 O-acetyltyrosine residues). Deacetylation of acetylated bovine growth hormone with hydroxylamine at pH 9.5 destroyed its ability to inhibit the growth-promoting activity of bovine growth hormone.

Acetyl-thyrotrophin has been demonstrated (Sonenberg & Money, 1957) to be a specific inhibitor for unmodified thyrotrophin in the chick (Sonenberg & Money, 1957, 1960) and human (Sonenberg, Money, Berman, Brener & Rawson, 1957; Sonenberg & Brener, 1964). Limited chemical analysis (Sonenberg & Money, 1957) of an essentially heterogeneous protein revealed complete blocking of amino groups with an equivalent number of acetyl groups. Acetyl-BGH‡ has been found (Sonenberg & Money, 1959, 1966) to inhibit unmodified BGH. In view of the high degree of purity of BGH (Dellacha & Sonenberg, 1964; Free & Sonenberg, 1966) available for these studies, an attempt was made to define precisely the groups substituted in acetyl-BGH. The present paper gives the results of such chemical analyses and the possible relation to the production of an inhibitor of BGH.

EXPERIMENTAL

Materials

BGH employed in these studies was prepared by the method of Dellacha & Sonenberg (1964) and was homogeneous by all criteria examined (Dellacha & Sonenberg, 1964) except starch-gel electrophoresis at pH 9.5 (Free & Sonenberg, 1966). All reagents employed were of analytical grade. In experiments where [¹⁴C]acetic anhydride was

employed, 1 mc of the reagent was mixed with acetic anhydride and distilled at 136 ± 1° to minimize contamination with radioactive acetic acid. The specific activity of [¹⁴C]acetic anhydride thus obtained was 5716 ± 120 (S.E.M.) counts/min./μmole of acetic anhydride (theoretically 4.3 mc/mole).

Acetohydroxamic acid, CH₃·CO·NH·OH, was synthesized by the method of Miolati (1892), and had m.p. 88.5–89.0° (literature m.p. 88°). (Found: C, 32.3; H, 6.6; N, 18.4. Calc. for C₂H₅O₂N: C, 32.0; H, 6.7; N, 18.7%).

Methods

Acetylation. (1) Acetyl-BGH (acetyl-BGH-1, acetyl-BGH-2 and acetyl-BGH-3) was prepared by suspending 1 g. of BGH in 100 ml. of acetic anhydride at 43.0° for 5–6 min. with vigorous stirring. To the suspension was added 100 ml. of distilled water, and the entire mixture was transferred to Visking cellophane tubing. The latter was dialysed against four to six changes of 6 l. of distilled water at 4°. The non-diffusible material was shell-frozen and freeze-dried.

(2) Acetyl-BGH (acetyl-BGH-5) was also prepared by adding 1.3 ml. of acetic anhydride to 1 g. of BGH dissolved in 15 ml. of acetic acid at 43.0°. The mixture was stirred vigorously for 7 min. and then evaporated to 3 ml. in a flash evaporator. After the addition of 10 ml. of distilled water, the mixture was dialysed and freeze-dried.

(3) Acetyl-BGH (acetyl-BGH-6) was also prepared by adding 1.3 ml. of acetic anhydride to 0.85 g. of BGH dissolved in 15 ml. of trifluoroacetic acid at 0–1°. The mixture was stirred vigorously for 20 min. and then 15 ml. of distilled water was added. The mixture was transferred to Visking dialysis tubing and dialysed against distilled water. The precipitate in the non-diffusible material was dissolved in acetic acid, dialysed again and freeze-dried.

(4) ¹⁴C-labelled acetyl-BGH (acetyl-BGH-7) was prepared by adding 120 mg. of BGH to 10 ml. of [¹⁴C]acetic

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‡ Abbreviation: BGH, bovine growth hormone.

anhydride (5716 counts/min./ μ mole of acetic anhydride, one half of which is available for acetylation) at 43.0°. The suspension was stirred vigorously for 6 min. Then 10 ml. of distilled water was added, and the mixture was dialysed and freeze-dried. The yield was 67.1 mg.

Deacetylation. To a suspension of 608 mg. of acetyl-BGH (acetyl-BGH-3) in 100 ml. of distilled water was added 1 ml. of 8M-hydroxylamine hydrochloride. The pH was adjusted to 9.5–9.6 with 4N-NaOH (the suspension cleared), and the mixture was maintained at 28–30° for 2 hr. with occasional addition of NaOH to keep the pH constant. Then 50 ml. of acetic acid was added to the mixture, and the latter was transferred to Visking tubing. The mixture was dialysed and freeze-dried to yield acetyl-BGH-4.

Chemical analysis. (1) Dinitrophenylation of BGH and acetyl-BGH. A sample (20 mg.) of protein (BGH or acetyl-BGH) was dissolved in 20 ml. of 0.1M-KCl in a water-jacketed reaction vessel in a Radiometer pH-stat at pH 8.5 at 40°. With constant stirring under N₂, 50 μ l. of 1-fluoro-2,4-dinitrobenzene was added to the mixture. When the rate of addition of standard 0.1N-KOH became constant (approx. 1.5–2 hr.) the mixture was acidified with 2–3 drops of 0.1N-HCl (with a fall in pH to approx. 3). The precipitate was washed twice with 50% (v/v) ethanol, once with ethanol and once with diethyl ether. The residue was dried *in vacuo* over P₂O₅.

(2) Acid hydrolysis. To 10 mg. of protein (BGH, acetyl-BGH, DNP-BGH or DNP-acetyl-BGH) in a tube was added 1 ml. of redistilled constant-boiling HCl. The tube was evacuated, sealed and maintained at 106 $\pm$ 1° for 24 hr. and 72 hr. The hydrolysate was dried in a flash evaporator and the residue dissolved in citrate buffer, pH 2.2, or 0.01N-HCl to give a concentration of approx. 2 mg./ml. The material was filtered through a Millipore filter to remove coloured insoluble material, and 0.5–1.0 ml. of the hydrolysates was applied to the column of the amino acid analyser.

(3) Amino acid analyses. The determinations of total amino acid composition were performed at the Institute for Organic Chemistry, University of Basle, Switzerland. Samples of hydrolysates were analysed on an amino acid analyser by the technique of Spackman, Stein & Moore (1958).

Amino acids that may possibly be acetylated were analysed mainly by using an accelerated system in a Beckman model 120B amino acid analyser. Long (60 cm. $\times$ 0.9 cm.) and short (10 cm. $\times$ 0.9 cm.) columns were employed with an elution rate of 68 ml./hr. and ninhydrin addition at 34 ml./hr. For the long column, only the second buffer was used for tyrosine and phenylalanine. The pH of the buffer of the short column was 5.22. The column temperature was maintained at 55° in both cases.

When ϵ -DNP-lysine was analysed with the other basic amino acids (Hirs, Halmann & Kyla, 1960), a standard short column (15 cm. $\times$ 0.9 cm.) was used under standard conditions except that the buffer pH and column temperature mentioned above were employed. Lysine, histidine, arginine and ϵ -DNP-lysine were eluted at or about 54, 66, 122 and 168 ml. respectively. Pure ϵ -DNP-lysine was employed to determine its position on the chromatogram as well as its colour yield.

To determine the number of acetylated amino acids the following assumptions were made: (a) the acetyl groups are completely released during acid hydrolysis so that the amino acid analysis of unmodified BGH and acetyl-BGH should

give comparable results (see Table 2); (b) the acetyl groups on lysine, tyrosine and histidine residues are stable during dinitrophenylation. For the acetyl group on the ϵ -amino group of lysine, the assumption was proved to be fairly valid.

For the calculation of basic amino acids and tyrosine, 12.8 and 12.4 respectively were used as the numbers of arginine and phenylalanine residues/mol. These were obtained from the results of the total amino acid analysis (Table 1) based on the assumed number of 24 for glutamic acid residues/BGH mol. of mol. wt. 22000 (Free & Sonenberg, 1966; Sonenberg, Kikutani, Free, Nadler & Dellacha, 1967). For some dinitrophenylated preparations, chromatographic analysis may have revealed fewer (4% or less) phenylalanine residues than the theoretical value. This would occur if there was an *N*-terminal phenylalanine that was dinitrophenylated if it had not been acetylated. Since one phenylalanine residue would introduce a negligible difference in the results, for simplicity it has not been considered in the calculations. The data are given as moles of amino acid/mole of BGH. The number of acetyl-amino acids is expressed as the number of the respective amino acid of the free form recovered from the dinitrophenylated acetyl-protein.

(4) Hydroxylamine-reactive acetyl groups. The method of Hestrin (1949) was employed. To a sample of the protein dissolved in 1.5 ml. of 0.1N-acetic acid were added 0.25 ml. of 8M-hydroxylamine hydrochloride and 0.5 ml. of 7N-NaOH. After 2 min. at room temperature, 0.5 ml. of 8N-HCl and then 0.25 ml. of 1.5M-FeCl₃ in 0.4N-HCl were added. Since acetyl-BGH was precipitated after the addition of HCl, the mixture was centrifuged, and the *E*₅₄₀ value of the supernatant was determined as aceto-hydroxamic acid-ferrous complex in a Beckman model DU spectrophotometer. The *E*₅₄₀ value was determined against a similarly developed colour with glucose penta-acetate as standard or directly with aceto-hydroxamic acid.

(5) Total acetyl groups. Total acetyl groups were determined from the radioactivity of the BGH preparation acetylated with [1-¹⁴C]acetic anhydride of known specific activity. Acetyl groups persistent after hydroxylamine treatment were determined for the washed precipitate of the radioactive acetyl-BGH after the treatment described in the preceding section. Radioactivity was determined in a liquid-scintillation counter (Packard Tri-Carb spectrometer).

(6) Protein concentration. Concentrations of BGH and acetyl-BGH were determined by the micro-Kjeldahl method and are expressed as molar concentrations, by using a reported N content (Dellacha & Sonenberg, 1964) and mol. wt. 22000 (Sonenberg *et al.* 1967). When the amount of sample was limited, the method of Lowry, Rosebrough, Farr & Randall (1951) was applied, with BGH or acetyl-BGH of known concentration as standards.

Assay of inhibition of bovine growth hormone. Female Sprague-Dawley rats were hypophysectomized at 3 weeks of age, rested for 2 weeks and then injected daily for 10 days. One group (ten rats) received only BGH (50 μ g./day/rat). Another group received BGH in the same dose and acetyl-BGH (2.5 mg./day/rat). A third group was injected with solvent (NaHCO₃-Na₂CO₃ buffer, pH 9.5). The inhibitory activity is expressed as the fraction of decrease in net weight gain (corrected for weight gain of solvent-injected group) of group 2 (acetyl-BGH and BGH) compared with group 1 (BGH alone).

RESULTS

Amino acid composition of bovine growth hormone. Total amino acid analyses were performed on two native BGH preparations, three acetyl-BGH preparations, acetylated in acetic anhydride (acetyl-BGH-3), in acetic acid (acetyl-BGH-5) and in trifluoroacetic acid (acetyl-BGH-6), and one hydroxylamine-treated BGH preparation. From these analyses (Table 1) it appeared that the amino acid composition was not appreciably altered by the above treatments.

Analyses of acetylated amino acid residues of acetylated bovine growth hormone. When BGH was acetylated for 5 or 6 min. at 43° in acetic anhydride (Table 2), all of the 12 lysine ϵ -amino groups/mol. were acetylated, i.e. were unavailable for dinitrophenylation (acetyl-BGH-1, acetyl-BGH-2 and acetyl-BGH-3). Less than 1 of the lysine ϵ -amino groups/mol. in unmodified BGH was unavailable for dinitrophenylation. On the other hand, all 6 tyrosine hydroxyl groups/mol. of BGH were dinitrophenylated, whereas in acetyl-BGH (acetyl-BGH-1, acetyl-BGH-2 and acetyl-BGH-3) approx. 2-3 tyrosine residues/mol. were unavailable for dinitrophenylation. Analysis of histidine before and after dinitrophenylation of BGH and acetyl-BGH suggested that 1 or less histidine imidazole group/mol. had been acetylated in acetyl-BGH.

When BGH was acetylated (acetyl-BGH-5) in acetic acid (Table 2) with acetic anhydride, 8 of 12

lysine ϵ -amino groups/mol. were acetylated, i.e. unavailable for dinitrophenylation. Acetylation of BGH (acetyl-BGH-6) in trifluoroacetic acid (Table 2) resulted in the acetylation of only 1 lysine ϵ -amino group/mol. After acetylation in acetic acid, only 1 tyrosine hydroxyl group/mol. and no histidine imidazole groups became unavailable for dinitrophenylation. Four tyrosine hydroxyl groups/mol. and no histidine became unavailable to dinitrophenylation after acetylation in trifluoroacetic acid.

Deacetylation of acetyl-BGH-3 prepared in acetic anhydride resulted in no loss of acetyl groups attached to lysine but essentially all (2/mol.) of the *O*-acetyl groups attached to tyrosine (acetyl-BGH-4; Table 2).

From the radioisotopic assay the total number of acetyl groups/mol. in acetyl-BGH prepared in acetic anhydride (acetyl-BGH-7) was 18 ± 1 , 12 ± 1 of which persisted after hydroxylamine treatment [see under 'Chemical analysis (4)' in the Methods section]. This is consistent with the number of hydroxylamine-reactive acetyl groups (6.1/mol.) of this preparation (acetyl-BGH-7; Table 3). Only 1 or 2 *O*-acetyl groups/mol. were introduced into BGH when the acetylation was performed in acetic acid (acetyl-BGH-5; Table 3), whereas 19 *O*-acetyl groups/mol. were introduced when trifluoroacetic acid was the solvent (acetyl-BGH-6; Table 3).

Inhibitory activity of preparations of acetylated bovine growth hormone. Only acetyl-BGH prepared

Table 1. *Amino acid compositions of native, acetylated and hydroxylamine-treated bovine growth hormone*

(BGH) refers to hydroxylamine-treated BGH. Ac_2O , AcOH , TFA and NH_2OH refer to acetic anhydride, acetic acid, trifluoroacetic acid and hydroxylamine employed in the treatment of BGH. The compositions are based on the assumed number of 24 glutamic acid residues/mol., as described in the text.

Amino acid Treatment ...	Amino acid composition (residues/mol. of mol.wt. 22000)					
	BGH —	BGH —	Acetyl-BGH-3 Ac_2O	Acetyl-BGH-5 AcOH	Acetyl-BGH-6 TFA	(BGH) NH_2OH
Lys	11.4	11.0	11.8	11.5	11.3	11.9
His	3.5	3.5	3.3	3.6	3.0	3.5
Arg	12.5	12.8	13.0	12.6	12.9	12.8
Asp	16.7	17.3	17.2	16.8	16.8	16.9
Thr	12.2	12.4	13.2	12.5	12.8	12.4
Ser	12.4	13.1	13.7	13.0	13.6	13.0
Glu	(24.0)	(24.0)	(24.0)	(24.0)	(24.0)	(24.0)
Pro	7.2	7.4	6.9	6.6	6.0	6.7
Gly	11.1	11.0	10.9	11.0	10.7	10.9
Ala	15.2	14.8	14.7	14.8	14.4	14.6
Cys	3.5	3.1	3.1	3.7	2.8	3.2
Val	7.3	7.2	7.2	7.2	6.9	7.2
Met	3.8	4.1	4.0	4.1	3.4	4.1
Ile	6.5	6.9	6.9	6.5	6.8	6.8
Leu	27.4	26.2	26.8	25.4	26.2	26.3
Tyr	6.2	6.0	6.2	5.8	5.5	5.8
Phe	12.5	12.4	12.8	12.0	12.4	12.5

Table 2. *Amino acid analyses of acetylated bovine growth hormones and their dinitrophenyl derivatives*

BGH was acetylated in acetic anhydride for 5 min. (acetyl-BGH-1 and acetyl-BGH-3) or 6 min. (acetyl-BGH-2). Acetyl-BGH-5 was prepared with acetic anhydride in acetic acid at 43° for 7 min. Acetyl-BGH-6 was prepared with acetic anhydride in trifluoroacetic acid at 0° for 20 min. Acetyl-BGH-4 was prepared by deacetylation of acetyl-BGH-3 as described in the text. The analysis of each lot of BGH from which the various derivatives were obtained is grouped with the respective acetyl-BGH preparations. For detailed conditions of amino acid analysis see the text. For abbreviations see Table 1. Underlined values correspond to the numbers of acetyl groups attached to the respective amino acid residues of acetyl-BGH preparations.

Sample	Prepared in	Dinitrophenylation	Amino acid analysis (residues/mol.)			
			Lys	DNP-Lys	His	Tyr
BGH*		—	11.9		3.7	6
BGH*		+	0.5	11.4	0.2	0
Acetyl-BGH-1*	Ac ₂ O	—	12.2		3.5	6
Acetyl-BGH-1*		+	11.9	0.0	0.8	2
Acetyl-BGH-2*	Ac ₂ O	+	11.8	0.0	0.4	3
BGH		—	12.4		3.2	6.0
BGH		+	0.7		0.0	0.0
Acetyl-BGH-3	Ac ₂ O	—	12.1		2.9	5.2
Acetyl-BGH-3		+	12.7		0.3	2.3
Acetyl-BGH-4†	NH ₂ OH	—	12.3			5.3
Acetyl-BGH-4†		+	12.2		0.1	0.2
BGH		—	12.8		3.3	5.8
BGH		+	0.5		0.1	0.2
Acetyl-BGH-5	AcOH	—	12.4		3.2	5.8
Acetyl-BGH-5		+	8.2		0.3	0.9
Acetyl-BGH-6	TFA	+	1.4		0.1	4.1
Assumed no. of reference amino acid‡			12.8 Arg		12.4 Phe	

* For these preparations, chromatography of the basic amino acids was performed under the standard (not accelerated) conditions.

† Acetyl-BGH-4 was prepared from acetyl-BGH-3 by deacetylation with hydroxylamine as described in the Methods section.

‡ See under 'Chemical analysis (3)' in the Methods section.

in acetic anhydride was able to offset the growth-promoting activity of unmodified BGH in hypophysectomized rats, e.g. acetyl-BGH-1 inhibited weight gain by 32% ($P < 0.01$) (Sonenberg & Money, 1966). Rats injected only with acetyl-BGH-1 showed neither growth nor toxicity. The weight gain of rats receiving unmodified BGH and acetyl-BGH prepared in acetic acid (acetyl-BGH-5) or in trifluoroacetic acid (acetyl-BGH-6) was 10% ($P > 0.9$) and 4% ($P > 0.9$) greater than that of animals injected with unmodified BGH alone. The weight gain of animals treated with unmodified BGH and acetyl-BGH deacetylated with hydroxylamine (acetyl-BGH-4) was 5% ($P > 0.9$) greater than that of animals receiving only native BGH.

DISCUSSION

Although other acetyl-proteins inhibited the growth-promoting response of BGH, only acetyl-BGH prepared in acetic anhydride under the conditions specified was an effective inhibitor of

BGH (Sonenberg & Money, 1966). Therefore it is of interest to compare an analysis of such an acetyl-BGH preparation (e.g. acetyl-BGH-1, acetyl-BGH-2, acetyl-BGH-3 or acetyl-BGH-7) with acetyl-BGH (acetyl-BGH-5 or acetyl-BGH-6) prepared under different conditions or acetyl-BGH treated with hydroxylamine (acetyl-BGH-4), which were without inhibitory activity. Acetyl-BGH prepared in aqueous media does not inhibit unmodified BGH (Sonenberg & Money, 1966) and indeed still retains growth-promoting activity (Reid, 1951; Collipp, Kaplan, Boyle & Shimizu, 1965). In such partially acetylated BGH preparations the lysine ϵ -amino groups are incompletely acetylated (Reid, 1951) and the α -amino groups are completely acetylated.

With regard to analytic methods it appeared that a small number of lysine ϵ -amino groups in unmodified BGH were unavailable for dinitrophenylation. However, it has been reported (Porter & Sanger, 1948) that there is only 95% recovery of ϵ -DNP-lysine after acid hydrolysis of a dinitro-

Table 3. *Hydroxylamine-reactive acetyl groups in bovine growth hormone and acetylated bovine growth hormone*

For detailed conditions of preparation of acetyl-BGH and assay method of hydroxylamine-reactive acetyl groups see the text.

Preparation	Hydroxylamine-reactive acetyl groups/mol.	Method of preparation of acetyl-BGH
BGH-27	0.06	—
BGH-28	0.05	—
Acetyl-BGH-1	6.1	Acetic anhydride, 43°, 5 min.
Acetyl-BGH-2	7.7	Acetic anhydride, 43°, 6 min.
Acetyl-BGH-3	8.0	Acetic anhydride, 43°, 5 min.
Acetyl-BGH-4	3.6	Acetyl-BGH-3 deacetylated at pH 9.5 with hydroxylamine, 30°, 120 min.
Acetyl-BGH-5	1.5	Acetic acid, acetic anhydride, 43°, 7 min.
Acetyl-BGH-6	19.0	Trifluoroacetic acid, acetic anhydride, 0°, 20 min.
Acetyl-BGH-7	6.1	[1- ¹⁴ C]Acetic anhydride, 43°, 5 min.

phenylated protein. In the determination of *O*-acetyl groups by the formation of an aceto-hydroxamic acid-ferrous complex, it was noted that both glucose penta-acetate and aceto-hydroxamic acid gave equivalent colours within experimental error. However, glucose penta-acetate proved to be a better standard because of its greater stability on storage.

Acetyl-BGH prepared in acetic anhydride (acetyl-BGH-1, acetyl-BGH-2 and acetyl-BGH-3) contained 11–12 *N*^ε-acetyl-lysine residues and 2–3 *O*-acetyltyrosine residues/mol. In contrast, acetyl-BGH prepared in acetic acid (acetyl-BGH-5) contained 8 *N*^ε-acetyl-lysine residues and 1 *O*-acetyltyrosine residue/mol. Acetyl-BGH prepared in trifluoroacetic acid (acetyl-BGH-6) contained only 1 *N*^ε-acetyl-lysine residue and 4 *O*-acetyltyrosine residues/mol. The numbers of *O*-acetyltyrosine residues/mol. cited above are probably correct, and at least represent a minimum number since it is possible that some *O*-acetyl groups are lost during dinitrophenylation. These data so far would suggest that incomplete acetylation of all the lysine residues and/or too few (1/mol. in acetyl-BGH-5) or too many (4/mol. in acetyl-BGH-6) *O*-acetyltyrosine residues are inimical to the production of a growth-hormone inhibitor. Since the numbers of hydroxylamine-reactive acetyl groups for these three types of acetyl-BGH preparations were 6–8, 1–2 and 19/mol. respectively, it would appear that too few or too many *O*-acetyl groups/mol. offset the production of a growth-hormone inhibitor. The importance of an optimum number of *O*-acetyl groups/mol. for growth-hormone-inhibitory activity is emphasized by the observation made in this study that a decrease of the number of these groups by hydroxylamine treatment from 8/mol. (acetyl-BGH-3) to 4/mol. (acetyl-BGH-4) led to a complete

loss of inhibitory activity. This occurred with complete retention of all acetyl substitutions on lysine residues.

Of the 6–8 (Table 3) hydroxylamine-reactive acetyl groups/mol. in acetyl-BGH prepared in acetic anhydride (acetyl-BGH-1, acetyl-BGH-2 and acetyl-BGH-3), 2–3 are *O*-tyrosyl-substituted (Table 2), and the balance are substitutions on the aliphatic hydroxy amino acids, presumably serine and possibly threonine residues. With regard to *O*-acetyl substitutions, with the data available from this study it is not possible to decide whether both phenolic and aliphatic *O*-acetylation are necessary for inhibitory activity. The loss of inhibitory activity of acetyl-BGH by deacetylation treatment is unlikely to be the result of oxidation of thiol groups, for BGH contains no cysteine residues (Free & Sonenberg, 1966; Li & Chung, 1956; Parcells, 1961; Wolfenstein, Santomé & Paladini, 1966).

There is consistency in the data for the presence of 18 (acetyl-BGH-7) total acetyl groups/mol. by radiochemical analysis, the persistence of 12 acetyl groups/mol. after hydroxylamine treatment, the presence of 12 *N*^ε-acetyl-lysine residues/mol. and 6 (acetyl-BGH-7) to 8 hydroxylamine-reactive *O*-acetyl groups/mol.

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