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Mammalian Glycinamide Ribonucleotide Transformylase: Purification and Some Properties†

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ABSTRACT: Glycinamide ribonucleotide transformylase, the first of the two formyl group transferases of de novo purine biosynthesis requiring 10-formyltetrahydrofolate, has been purified 1500-fold, nearly to homogeneity, from the murine lymphoma cell line L5178Y. Purification of the enzyme was facilitated by the use of a gelatin protease "affinity" resin. This mammalian enzyme is a monomer of approximate M_r 110000. The kinetic studies are consistent with a sequential reaction mechanism and yield Michaelis constants of 0.4 mM for the substrate, glycinamide ribonucleotide, and 0.25 μM for the cofactor analogue 10-formyl-5,8-dideazafolate. A minimum V_{max} of 2 μmol/(min·mg) was obtained for the purified enzyme, from which a turnover number of 4 s^{-1} was calculated.

The pathway of de novo purine biosynthesis was first elucidated by Buchanan and Greenberg and their co-workers in the early 1950s (Buchanan, 1960). Their work led to the identification of the intermediates of this pathway and the enzymic activities responsible for their interconversion. Glycinamide ribonucleotide (GAR)[1] was identified as one of these intermediates and a preliminary characterization of the GAR TFase reaction was reported (Hartman & Buchanan, 1959). These experiments, as well as subsequent enzymological studies

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[1] Abbreviations: GAR, glycinamide ribonucleotide; GAR TFase, glycinamide ribonucleotide transformylase; 2-ME, 2-mercaptoethanol; Me_2SO, dimethyl sulfoxide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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(Caperelli et al., 1980), were performed with avian liver. However, in the liver of uricotelic organisms such as birds, the major role of this active pathway is elimination of waste nitrogen. In contrast, in ureotelic animals such as mammals, the role of the far less active pathway is to provide purine nucleotides for further anabolic sequences. This important difference in activity and purpose of the pathway suggests that fundamental differences in regulation or perhaps even among the respective enzymes could exist between the two pathways. I now report the first purification of GAR TFase from a mammalian source as a first step toward examining this possibility.

The enzyme GAR TFase, which utilizes 10-formyltetrahydrofolate as the source of the formyl group, is of interest as a potential target for cancer chemotherapy because of its involvement in folate cofactor metabolism. In the work reported here, I have used a murine lymphocytic leukemia cell line as the source for mammalian GAR TFase and have achieved a 1500-fold purification of the enzyme, producing nearly homogeneous GAR TFase. The enzyme has been subjected to preliminary kinetic and structural studies.

Experimental Procedures

Materials

Sepharose 4B was purchased from Pharmacia and cross-linked and reduced as described by Porath et al. (1971). McCoy's 5a (modified) medium, sodium pyruvate, calf serum, and penicillin (10 000 units/mL)–streptomycin (10 mg/mL) solution were from GIBCO Laboratories. The stock culture of L5178Y was generously provided by Dr. John Biaglow, Department of Radiology, Case Western Reserve University. Pluronic F-68 was the generous gift of BASF Wyandotte, Wyandotte, MI. α,β-GAR was synthesized according to Chettur & Benkovic (1977). Gelatin (Fisher granular, 100 Bloom) gels at 10% (w/v) were cross-linked with formaldehyde as described by Polson et al. (1976). Rabbit muscle phosphorylase *a*, bovine albumin, fumarase, yeast alcohol dehydrogenase, α_1-antitrypsin, aprotinin, type II-O ovomucoid, and 1-[3-(dimethylamino)propyl]-3-ethyl-carbodiimide hydrochloride were purchased from Sigma. 5,8-Dideazafolate was prepared by the method of Achayra & Hynes (1975). L(+)-5,10-Methenyltetrahydrofolate was generously provided by Dr. Carroll Temple, Jr., Southern Research Institute.

10-Formyl-5,8-dideazafolate–Sepharose was prepared from 10-formyl-5,8-dideazafolate (Hynes et al., 1977). Cross-linked, reduced Sepharose 4B (10 g, wet wt) suspended in 15 mL of water at 0–5 °C was activated with 2 g of CNBr at pH 10.5–11.0 following the procedure of Nishikawa & Bailon (1975). After washing, the gel was suspended in 5 mL of 0.2 M $NaHCO_3$, pH 10.0, containing 1.5 mL of 3,3′-iminobis(propylamine) and shaken for 16 h at 25 °C. The gel was washed sequentially with 500 mL of H_2O, 100 mL of 0.2 N acetic acid, 500 mL of H_2O, 100 mL of 0.5 N NaOH, 500 mL of H_2O, 100 mL of 0.2 N acetic acid, and 1 L of H_2O. The washed gel was shaken in 10 mL of 1 M ethanolamine, pH 8.3, for 3 h at 25 °C and washed with 1 L of H_2O. The gel was suspended in 10 mL of 20 mM 10-formyl-5,8-dideazafolate and treated with 1-[3-(dimethylamino)propyl]-3-ethyl-carbodiimide hydrochloride (1.92 g, 10 mmol) in small portions while maintaining the pH at pH 6.5 with 1 N NaOH. After proton release diminished (1 h), the gel was shaken at 25 °C for 36 h, followed by washing with 500 mL of H_2O, 250 mL of 0.2 N acetic acid, and 1 L of H_2O. The A_{255} of the initial filtrate and washes was recorded to determine the amount of unbound ligand. The analysis indicated a bound ligand concentration of 7 μmol/mL of settled gel.

The 10-acetyl-5,8-dideazafolate–Sepharose was prepared from 10-acetyl-5,8-dideazafolate[2] as described for the preparation of 10-formyl-5,8-dideazafolate–Sepharose. UV analysis of the filtrate and washes indicated a bound ligand concentration of 2.7 μmol/mL of settled gel.

Methods

Cell Culture. The cells were grown in suspension culture at 37 °C in a humidified 5% CO_2 atmosphere to late log phase. The growth medium employed was McCoy's 5a (modified) supplemented with calf serum (5% v/v), sodium pyruvate (0.01% w/v), penicillin G (100 units/mL), and streptomycin (100 mg/mL) and modified for suspension culture by the addition of Pluronic F-68 (0.1% w/v) (Clive & Spector, 1975). The doubling time was 10–12 h under these growth conditions. Cells from 3 L of media were collected by centrifugation at 500*g* for 10 min, washed by suspending the pellet in phosphate-buffered saline and centrifuging at 500*g* for 10 min, and stored as the pellet at −85 °C.

Gel Electrophoresis. SDS–PAGE was performed with 7% separating and 3% stacking gels in the buffer system described by Laemmli (1970) with the following proteins (subunit molecular weight) as molecular weight markers: rabbit muscle phosphorylase *a* (92 500), bovine albumin (66 000), fumarase (48 500), and yeast alcohol dehydrogenase (35 000).

Sucrose Gradient Ultracentrifugation. Linear sucrose density gradients, 5–20% (w/v), prepared in 50 mM Tris-HCl, pH 7.5–10 mM 2-ME were poured at 25 °C and allowed to equilibrate at 4 °C for 24 h. The sample, 0.45 mL, containing 10 μg of GAR TFase and 0.5 mg of yeast alcohol dehydrogenase, M_r 141 000, was layered onto the gradient (4.5 mL) and centrifuged at $192000g_{r,max}$ for 13 h at 4 °C in an AH-650 rotor with a Sorvall OTD 75B centrifuge. Immediately following centrifugation, the gradient was fractionated from the top into fractions of 6 drops with a Buchler Auto Densi-Flow IIC connected to a fraction collector in the drop-collecting mode. Analysis of the molecular weight was according to the method of Martin & Ames (1961).

Protein Concentration. The protein concentration was determined by the method of Sedmak & Grossberg (1977) with BSA, dissolved in 10 mM potassium phosphate, pH 7.5, to establish the standard curve. Samples were dialyzed against 10 mM potassium phosphate, pH 7.5, prior to analysis to avoid possible interference by the "stabilizers" in the assay.

Enzyme Assays. GAR TFase was assayed by monitoring the production of 5,8-dideazafolate at 295 nm with $\epsilon = 18.9$ mM^{-1} (Smith et al., 1981b). The assays were performed at 35 °C and pH 6.8 with the solution containing 100 mM potassium phosphate, pH 6.8, 1 mM α,β-GAR, 100 μM 10-formyl-5,8-dideazafolate, and enzyme in 1 mL. All components except cofactor were incubated for 5 min at 35 °C, and the reaction was initiated by the addition of cofactor. The assays were dependent on enzyme concentration and linear with time during the initial part of the reaction. No turnover of cofactor was observed in the absence of either enzyme or GAR.

Serine hydroxymethyl transferase was assayed by following the production of benzaldehyde from β-phenylserine at 279 nm as described by Schirch & Diller (1971). The assay for 5,10-methenyltetrahydrofolate cyclohydrolase activity was performed according to Caperelli et al. (1980). Alcohol de-

[2] The synthesis of 10-acetyl-5,8-dideazafolate and its biological activity will be reported elsewhere. Details of the synthesis, prior to publication, are available from the author.

hydrogenase was assayed at 35 °C and pH 8.8 by monitoring the production of NADH at 340 nm (Vallee & Hoch, 1955).

Kinetics. The initial velocity studies of the GAR TFase reaction were performed in the assay system described above with the exception that the concentration of α,β-GAR was varied from 0.2 to 2.0 mM, while 10-formyl-5,8-dideazafolate was maintained at a fixed concentration that was varied from 0.2 to 1.0 μM. The initial linear reaction rate was used for the velocity calculation.

The initial velocity studies of the GAR TFase reaction employing L(−)-10-formyltetrahydrofolate as cofactor were performed by monitoring the production of tetrahydrofolate at 298 nm as described by Smith et al. (1981a) with the following modifications: (1) the concentration of α,β-GAR in the assay was 1 mM; (2) the buffer used was 50 mM potassium phosphate, pH 6.8.

Enzyme Purification. All steps were performed at 4 °C, except where noted. Stabilizers refer to the following additions to buffers: Me_2SO (10% v/v) and ethylene glycol (25% v/v).

Extraction. The cells (4.2 g) were thawed rapidly at 37 °C and swollen for 20 min in 2 volumes of homogenization buffer, which consisted of 10 mM potassium phosphate, pH 7.5, 10 mM 2-ME, 1 mM EDTA, 0.25 M sucrose, α_1-antitrypsin (25 μg/mL), type II-O ovomucoid (0.25 mg/mL), and aprotinin (2.5 μL/mL). The cells were lyzed by utilization of a Tissumizer (Tekmar) set at "50" for six 10-s pulses with a 10-s pause between each pulse, followed by centrifugation at $164000g_{r,max}$ for 45 min in an AH-650 rotor with a Sorvall OTD 75B centrifuge.

Protamine Sulfate Precipitation. The homogenate supernatant was adjusted to pH 7.5 with 3 N NH_4OH and brought to 0.1% protamine sulfate by the addition of 2% protamine sulfate in 10 mM potassium phosphate, pH 7.5. After stirring for 20 min, the suspension was centrifuged at 39000g for 10 min in an SS-34 rotor with a Sorvall RC 5B centrifuge.

Ammonium Sulfate Fractionation. The protamine sulfate supernatant was brought to 35% saturation by addition of solid $(NH_4)_2SO_4$ while the pH was maintained at pH 7.5 with 3 N NH_4OH. After being stirred for 1 h, the suspension was centrifuged at 39000g for 10 min, the pellet discarded, and the supernatant brought to 55% saturation. The 35–55% precipitate was collected by centrifugation at 39000g for 10 min and dissolved in homogenization buffer.

Gelatin Chromatography. The redissolved 35–55% $(NH_4)_2SO_4$ pellet was applied to a column (1.25 × 6.85 cm) of formaldehyde-cross-linked gelatin that had been equilibrated with 50 mM Tris-HCl, pH 7.5–10 mM 2-ME. The column was eluted with this buffer in 1-mL fractions. The fractions that contained GAR TFase were combined and dialyzed against 10 mM Tris-HCl, pH 7.5–10 mM 2-ME plus stabilizers.

10-Acetyl-5,8-dideazafolate–Sepharose Chromatography. The dialyzed gelatin elution was applied to a column of 10-acetyl-5,8-dideazafolate–Sepharose (1.25 × 6.2 cm) that had been washed with 0.1 M Tris-HCl, pH 7.5, and equilibrated with 10 mM Tris-HCl, pH 7.5–10 mM 2-ME plus stabilizers. The column was washed with 0.175 M Tris-HCl, pH 7.5–10 mM 2-ME plus stabilizers until the A_{280} was low and constant (approximately 10 column volumes) and eluted with 0.25 M Tris-HCl, pH 7.5–10 mM 2-ME and stabilizers in 2-mL fractions. Fractions containing GAR TFase were combined and dialyzed against 10 volumes of 10 mM Tris-HCl, pH 7.5–10 mM 2-ME plus stabilizers.

10-Formyl-5,8-dideazafolate–Sepharose Chromatography. The dialyzed elution was applied to a column (1.25 × 4.5 cm) of 10-formyl-5,8-dideazafolate–Sepharose that had been washed with 0.1 M Tris-HCl, pH 7.5, and equilibrated with 10 mM Tris-HCl, pH 7.5–10 mM 2-ME plus stabilizers. The column was washed with 0.15 M Tris-HCl, pH 7.5–10 mM 2-ME plus stabilizers until the A_{280} was low and constant (approximately 20 column volumes) and eluted with 0.15 M Tris-HCl, pH 7.5–10 mM 2-ME plus stabilizers containing 0.5 mM 10-formyl-5,8-dideazafolate in 1-mL fractions. After 1 column volume of elution buffer had passed through the column, elution was discontinued for 3 h and then resumed. Activity-containing fractions were combined and concentrated approximately 15-fold with a Bio-Molecular Dynamics MCF-20 concentrator utilizing a M_r 15000 cut-off membrane.

Table I: Purification of GAR Transformylase

fraction	vol (mL)	protein (mg)	sp act.[a]	tot act.[b]	x-fold purification (% yield)
(1) homogenate supernatant	9.5	157	0.01	1.0	1 (100)
(2) protamine sulfate supernatant	9.6	142	0.01	1.1	1.2 (108)
(3) ammonium sulfate, 35–55%	6.7	81	0.01	0.9	1.6 (83)
(4) gelatin	15	95	0.01	0.9	1.5 (89)
(5) 10-acetyl-5,8-dideaza-folate–Sepharose	78	3	0.17	0.5	25 (48)
(6) 10-formyl-5,8-dideazafolate–Sepharose	16.7	0.02	9.9	0.2	1478 (22)
(7) concentrate	1.1	0.02	3.3	0.1	470 (8)

[a] Specific activity is reported as micromoles per minute per milligram of protein at 35 °C. [b] Total activity is micromoles per minute at 35 °C.

Results

Enzyme Purification. The successful scheme devised for the purification of mammalian GAR TFase is outlined in Table I. The method results in a 1500-fold purification to yield nearly homogeneous enzyme. The inclusion of protein protease inhibitors during the first three steps and, in particular, the use of the gelatin chromatography step resulted in stabilization of the crude protein mixture. The efficacy of the gelatin column was demonstrated by comparing the stability of pre- and post-gelatin fractions after storage at 4 °C for 12 h. There was quantitative recovery of GAR TFase activity in material eluted from the gelatin column, while protein that had not been processed through the gelatin column retained only 20% of its initial activity after 12 h at 4 °C. Although the first four steps of the schemes result in negligible increase in specific activity, they yield protein that is stabilized, relative to the crude extract, for further manipulation.

GAR TFase activity was eluted from the 10-acetyl-5,8-dideazafolate–Sepharose column, the first of the two folate-analogue affinity resins used in the purification, with 0.25 M Tris-HCl, pH 7.5–10 mM 2-ME plus stabilizers. The inclusion of 0.5 mM 10-formyl-5,8-dideazafolate neither in elution buffers of lower Tris concentration nor in the 0.25 M Tris buffer resulted in either increased purification or yield of GAR TFase. In contrast to this result, the inclusion of 0.5 mM 10-formyl-5,8-dideazafolate in the 0.15 M Tris elution buffer of the 10-formyl-5,8-dideazafolate–Sepharose column resulted in a higher purification of GAR TFase than that achieved with either a higher concentration of Tris alone or a combination of Tris and potassium phosphate buffer. By halting the elution of the 10-formyl-5,8-dideazafolate–Sepharose column for several hours after 1 column volume of elution buffer had passed through, we were able to obtain the GAR TFase ac-

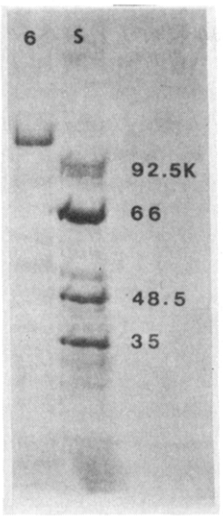

FIGURE 1: Coomassie Blue stained SDS–PAGE of the purified protein. Lane 6 depicts the protein profile obtained with protein from fraction 6 (Table I). Twenty micrograms of total protein was applied. The electrophoretic mobilities of phosphorylase *a* (M_r 92 500), bovine albumin (M_r 66 000), fumarase (M_r 48 500), and yeast alcohol dehydrogenase (M_r 35 000) standards are indicated (lane S).

tivity in an approximately 3-fold more concentrated solution than that obtained by continuous elution of the column. Presumably, this reflects the time required to establish the equilibrium between free ligand and bound enzyme. Longer equilibration times, up to 12 h, did not lead to significant improvement over the 3-h interval.

An examination of the SDS–PAGE (data not shown) of protein eluted from each of these columns showed that both affinity resins resulted in enrichment of the same high molecular weight protein but that the remainder of the protein pattern was not identical. It was reasoned that sequential elution of GAR TFase from both of these affinity columns would further enrich GAR TFase while minimizing the number of protein contaminants and their amounts. Indeed, this combination of affinity columns resulted in a purification of GAR TFase of approximately 1000-fold. Protein eluted for the 10-acetyl column contained neither the trifunctional protein of folate cofactor metabolism as measured by 5,10-methenyltetrahydrofolate cyclohydrolase activity nor serine hydroxymethyl transferase. The order in which the affinity columns were employed was chosen because of the apparent tight binding of 10-formyl-5,8-dideazafolate to the enzyme (see below). Residual cofactor bound to the enzyme after biospecific cofactor elution from the 10-formyl column might prevent absorption of the enzyme by the 10-acetyl column. Utilization of these columns in the order described obviated this concern. Sodium dodecyl sulfate gel electrophoresis of the purified protein is depicted in Figure 1. Protein eluted from the second affinity resin shows one major Coomassie Blue stained band. The protein is quite dilute and therefore unstable, at this stage. Upon concentration of the protein sample, some activity is lost, despite the fact that the stabilizers are still present.

Native and Subunit Molecular Weight. The subunit M_r of GAR TFase, estimated from its mobility relative to protein standards in SDS gel electrophoresis, is 102 000. Sucrose density gradient ultracentrifugation indicated a native M_r of 125 000 for GAR TFase. These data suggest that GAR TFase isolated from the murine lymphomia cell line L5178Y is a monomer of M_r 100 000–125 000 in contrast to the chicken liver enzyme which is a dimer with a subunit M_r of 61 000 (Caperelli et al., 1980).

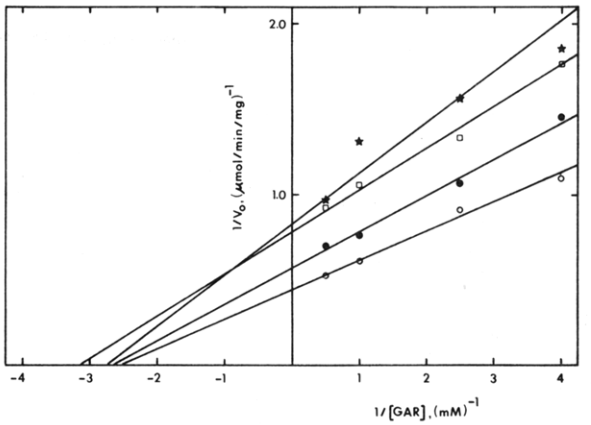

FIGURE 2: Lineweaver–Burk plot of initial velocity data for GAR TFase as a function of α,β-GAR concentration. The concentration of 10-formyl-5,8-dideazafolate was 1 (○), 0.5 (●), 0.25 (□), and 0.2 μM (solid star).

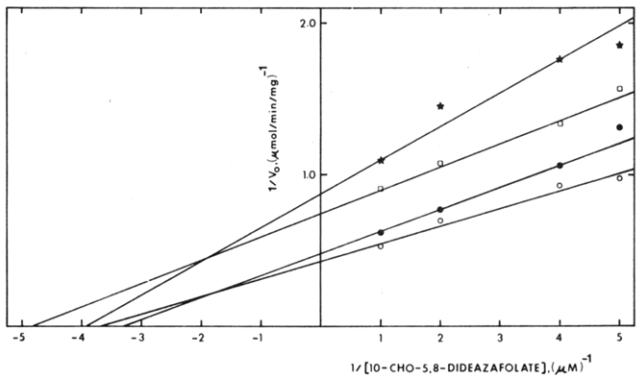

FIGURE 3: Lineweaver–Burk plot of initial velocity data for GAR TFase as a function of 10-formyl-5,8-dideazafolate concentration. The concentration of α,β-GAR was 2 (○), 1 (●), 0.4 (□), and 0.25 mM (solid star).

Kinetics. Initial velocity studies of the GAR TFase reaction employing α,β-GAR and 10-formyl-5,8-dideazafolate as cofactor revealed a sequential kinetic pattern as depicted in Figures 2 and 3. The kinetic constants derived from these plots were $K_{m,app}$ = 0.25 μM for 10-formyl-5,8-dideazafolate, $K_{m,app}$ = 0.4 mM for α,β-GAR, and $V_{max,app}$ = 2 μmol/(min·mg). Since these studies used the protein after concentration, which has lost some activity, the maximum velocity obtained is underestimated by at least a factor of approximately 3, which corresponds to the activity retained after protein concentration. The turnover number calculated from $V_{max,app}$ is 4 s^{-1}. The initial velocity studies with L(−)-10-formyltetrahydrofolate as cofactor yielded a $K_{m,app}$ = 0.8 μM and V_{max} = 1.3 μmol/(min·mg).

Discussion

GAR TFase has been purified approximately 1500-fold, nearly to homogeneity, from the murine lymphoma cell line L5178Y, constituting the first reported purification of this important folate-dependent, de novo purine biosynthetic enzyme activity from a mammalian source. This has been achieved through the use of two folate-analogue affinity resins, one of which proved most effective under conditions of biospecific elution. A key strategy of this successful scheme was the stabilization of the desired activity by rapid removal of protease from the crude preparation. This was accomplished by using a protease "affinity" gel composed of cross-linked gelatin. The inexpensive, easily prepared gel was highly effective in this particular instance and may of the more general

use as a mild, high-yield procedure for protein purification. Polson (1977) had demonstrated that formaldehyde-cross-linked gelatin efficiently separated chymotrypsin and chymotrypsinogen A.

The mammalian enzyme appears to differ from that isolated from chicken liver in several respects, both structurally and kinetically. This enzyme appears to copurify not with the trifunctional folate metabolizing enzyme nor with serine hydroxymethyl transferase as had been observed with the chicken liver enzyme (Caperelli et al., 1980; Smith et al. 1981b). The affinities of the mammalian and avian enzyme for their substrate, GAR, are comparable, while the mammalian enzyme has a higher apparent affinity for the cofactor analogue, $K_{m,app}$ = 0.25 μM, than does the avian enzyme, $K_{m,app}$ = 1.9 μM (Smith et al., 1981b). However, the efficient utilization of the folate analogue 10-formyl-5,8-dideazafolate, by both the mammalian and avian enzymes suggests a similar cofactor binding site for both transformylases. Both enzymes appear to have slightly higher affinities for the deazafolate cofactor analogue than for the natural folate cofactor.

The isolation of mammalian GAR TFase should provide a basis for studying the role of this enzyme in de novo purine biosynthesis, with particular reference to its relationship to folate cofactor metabolism. We are continuing our studies on mammalian GAR transformylase, including its substrate and cofactor specificity.

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Registry No. GAR TFase, 9032-02-4; GAR, 10074-18-7; 10-acetyl-5,8-dideazafolic acid, 94843-77-3.

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