Reports and Letters

Occurrence of D-Glyceric Acid in Tobacco Leaves

The recent discovery (*1–3*) in leaves of enzymes that catalyze the dehydrogenation of the D-enantiomorph of glyceric acid again raised the question of the occurrence of free glyceric acid in the leaves of higher plants. D-Glyceric acid has been isolated from cress seedlings (*4*), but the numerous reports of glyceric acid in leaves of other plants have been based almost entirely on chromatographic evidence. The present report describes the isolation of D-glyceric acid from mature green leaves of *Nicotiana tabacum* var. Connecticut shade-grown and gives chemical proof of its identity and configuration.

The preliminary separation of glyceric acid was carried out by a modification of previously described techniques (*5*). A water extract of 15 g of dried green tobacco leaves was added to a column of Dowex 1-X10 (200 to 400 mesh, acetate form) (*6*) 12 cm by 3.5 cm². The column was washed with water, and the organic acids were eluted with 1*N* acetic acid. Fractions of 5-ml volume were collected, their composition being established by paper chromatography of small aliquots (*5*). Glyceric acid was eluted in the region of fraction 60. Appropriate fractions were pooled to give a solution that contained glyceric acid, acetic acid, and only one other contaminating acid. This solution was evaporated to dryness at 45°C in a stream of air (*5*), and the residue was dissolved in 1 ml of water.

Further purification of the glyceric acid was accomplished by partition chromatography (*7*) of this fraction. The solution was dried in small portions on a blotter disk (*7*), and the disk was placed on the surface of a column 2.1 cm in diameter that contained 25 g of silica gel. Glyceric acid was eluted with a mixture of equal volumes of chloroform and *n*-butanol equilibrated with 0.5*N* H_2SO_4. Fractions of about 10-ml volume were collected. The contaminating acid was not eluted under these conditions. The glyceric acid peak was located in the region of fraction 30 by titration of small aliquots of the effluent, and the appropriate fractions were pooled. These fractions contained chloroform, butanol, and a small quantity of sulfuric acid in addition to glyceric acid. The organic solvents were removed by distillation to a small volume in a vacuum, water was added, and the solution was neutralized with barium hydroxide. The barium sulfate was removed, and barium glycerate was then precipitated by the addition of 3 to 4 vol of alcohol. The white precipitate was collected by centrifugation and dried in a vacuum.

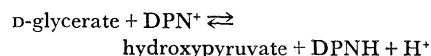
The yield at this point, based on titration of the fractions from the silica gel column, was approximately 72 percent. It is difficult to assess the over-all yield, since a reliable technique for determining glyceric acid in water extracts of plant tissue is not available. However, a reasonable estimate is about 50 percent.

The barium glycerate exhibited the same qualitative chromatographic behavior on ion-exchange columns (*5*), on filter paper (*5*), and on partition columns (*7*) as authentic calcium glycerate. A sample of the isolated material yielded the characteristic deep blue color when it was treated with the naphthoresorcinol-H_2SO_4 reagent of Feigl (*8*).

A portion of the barium glycerate from tobacco leaves was converted to sodium glycerate by passage of a solution through Dowex 50-X8 (*6*) in the sodium form, and the *p*-phenylphenacyl ester was prepared (*1*). It melted at 145° to 146°C. The derivative of a known sample of D-glycerate melted at 144° to 145°C, and a mixture showed no depression of the melting point.

The specific rotation of barium glycerate isolated from tobacco, measured in 6-percent ammonium molybdate (*9*), was $[\alpha]_D^{20} = +94°$ (concentration, 0.16 g/100 ml). A value of +104° has been reported for authentic barium D-glycerate (*9*).

As final evidence of the identity and configuration of the acid isolated from tobacco leaves, a sample was incubated with crystalline glyoxylic acid reductase (*3*) and DPN (oxidized diphosphopyridine nucleotide). This enzyme has specific D-glyceric acid dehydrogenase activity associated with it and catalyzes the following reaction:



$$\text{D-glycerate} + \text{DPN}^+ \rightleftarrows \text{hydroxypyruvate} + \text{DPNH} + \text{H}^+$$

A 6.67 m*M* solution of the sodium glycerate isolated from tobacco leaves was incubated with the enzyme and DPN. After the reaction had proceeded to equilibrium, the concentration of the added glycerate was calculated from the quantity of DPNH (reduced diphosphopyridine nucleotide) formed and was found to be 6.25 m*M* (*10*).

These data provide proof of the presence of D-glyceric acid in the tobacco leaf. The quantity present is of the order of 5 to 15 milliequivalents/kg (fresh weight) or 0.5 to 1.5 percent of the dry weight.

Leaf tissue that was plunged within a few seconds of being picked into boiling 70-percent alcohol or into boiling water also contained approximately the same quantity of glyceric acid. Thus, it seems unlikely that more than a small fraction of the free glyceric acid present in the dried leaf tissue arises from cleavage of 3-phospho-D-glyceric acid by phosphatases during the sampling procedure.

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Small Polyribonucleotides with 5′-Phosphomonoester End-Groups

Study of the products of alkaline digestion of ribonucleic acid (RNA) from certain viruses has shown that in some cases the polynucleotide chain is terminated by a phosphate group esterified at carbon 5′ of the nucleoside moiety (*1*). This is also the case (*2*) with the highly polymerized polyribonucleotides synthesized by polynucleotide phosphorylase,

Table 1. Nucleotides derived from the AMP polymer. The R_f values and the electrophoretic mobilities show a regular progression with increasing length of chain. Further, the parent compounds and the products after treatment with phosphomonoesterase fall in the same order when listed according to extent of migration. This is supplementary evidence for a family of compounds belonging to a homologous series.

Ultraviolet absorbing band	Yield, expressed as micromoles of adenine*	R_f in isopropanol-NH_3†		Electrophoretic mobility‡	
		Before monoesterase§	After monoesterase	Before monoesterase	After monoesterase
I. Adenosine	0.1	0.43	0.43	3.9‖	3.9‖
II. Mononucleotide	0.4	0.14	0.43	9.0	3.9‖
III. Dinucleotide	0.25	0.07	0.23	12.0	3.0
IV. Trinucleotide	0.7	0.03	0.15	14.2	6.5
V. Tetranucleotide	0.6	0.01	0.05	14.2	7.8

* The incubation mixture contained 2.5 μmole (as adenine) of the AMP polymer. 0.5 μmole of ultraviolet absorbing material (expressed as adenine equivalents) appeared at the origin of the descending chromatogram, presumably long polynucleotides.
† See reference *10*.
‡ Paper electrophoresis in 0.05*M* formate, *p*H 3.5, was carried out exactly as described by Markham and Smith (*10*). Movement is expressed as centimeters per 2 hours, at 20 V/cm.
§ This was a purified seminal phosphomonoesterase (*4*), which removed the 5′-phosphomonoester group but did not split the 3′–5′ internucleotidic phosphodiester bond.
‖ This is extent of movement toward cathode. All other compounds migrated toward the anode.

an enzyme recently isolated from *Azotobacter vinelandii* (*3*). However, any small polynucleotides hitherto obtained by enzymatic or chemical degradation of RNA were shown to possess C3′ or C2′ phosphomonoester end-groups.

The only thoroughly studied enzyme known to liberate small polynucleotides from RNA is pancreatic ribonuclease. These oligoribonucleotides possess in all cases a terminal phosphate group esterified at C3′ (*4, 5*). The 5′-phosphate ended polyribonucleotides synthesized by polynucleotide phosphorylase (*6*) are no exception to this rule, since they yield 3′-phosphate ended oligonucleotides on digestion with pancreatic ribonuclease (*7*). Acid hydrolysis of RNA can also yield small polynucleotides but with the phosphomonoester end-group at C3′ or C2′, not at C5′ (*8*).

We have now found that small polynucleotides with C5′ phosphate end-groups are liberated on incubation of the biosynthetic adenosine-5′-phosphate (AMP) polymer (*6*) with an enzyme preparation from liver nuclei. Pancreatic ribonuclease has no action on the AMP polymer (*6*). A brief report of the isolation and identification of ribodinucleotides, trinucleotides, and tetranucleotides, consisting of adenosine-5′-phosphate residues linked to one another through 5′, 3′-phosphoribose diester bonds and therefore possessing C5′ phosphomonoester end-groups, is the object of this communication.

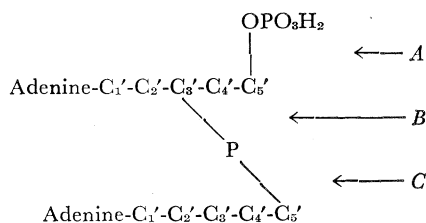

Fig. 1. 5′-Phosphoadenosine-3′ : adenosine-5′-phosphate. Arrows *A, B,* and *C* represent points at which dinucleotide can be hydrolyzed.

Extracts obtained from guinea pig liver nuclei, by treatment with alkali and potassium chloride as described by Mills *et al.* (*9*), were found to have nuclease activity toward the biosynthetic AMP polymer, producing small polynucleotides. This activity was partially purified by fractionation, first with neutral, then with alkaline ammonium sulfate.

The enzyme (0.26 mg protein) was incubated in 0.01*M* $MgCl_2$ and 0.02*M* phosphate buffer, *p*H 7.2, with 1.4 mg of the AMP polymer. After 20 hours at 37°C the incubation mixture (1.0 ml) was evaporated to 0.2 ml and directly chromatographed on Whatman No. 3MM paper in the isopropanol-ammonia system of Markham and Smith (*10*). Six bands were visible in ultraviolet light. Table 1 shows the amount of material in each band, expressed as adenine, as well as R_f values and electrophoretic mobilities.

Band I was found to be adenosine. Band II was shown to be adenosine-5′-phosphate, from its behavior on paper chromatography and paper electrophoresis, and by its conversion to adenosine with bull semen 5′-nucleotidase free of diesterase activity (*11*).

Band III was found to be the dinucleotide, 5′-phosphoadenosine-3′: adenosine-5′-phosphate, the structure of which is shown diagrammatically in Fig. 1. Identification of this dinucleotide is based on the following evidence. (i) It behaved chromatographically as an adenine containing dinucleotide. (ii) On treatment with purified human semen phosphomonoesterase (*4*) hydrolysis occurred at *A* (Fig. 1), and the compound was quantitatively converted to adenosine-3′: adenosine-5′-phosphate, a dinucleoside monophosphate. This derivative was identified by its R_f in two solvent systems and its electrophoretic mobility as compared with a previously studied reference compound derived from RNA (*12*). Furthermore, it yielded equimolar (1.0:1.1) amounts of adenosine and adenylic acid by treatment with either alkali (*10*) or spleen phosphodiesterase (*13*). (iii) The dinucleotide was quantitatively converted to adenosine-5′-phosphate by purified snake venom phosphodiesterase (*14*), by hydrolysis at *B* (Fig. 1). (iv) Hydrolysis for 18 hours at 24°C with 0.5*N* NaOH converted the dinucleotide to an equimolar (1.0:0.9) mixture of adenosine and adenosine 3′(2′):5′ diphosphate through splitting at *C* (Fig. 1). The diphosphates were identified by chromatographic and electrophoretic comparison (*12*) with adenosine 2′:5′ diphosphate derived from triphosphopyridine nucleotide (*15*). (v) Finally, oxidation with periodate followed by incubation at *p*H 10.5 (*16, 17*) led to cleavage at *C* (Fig. 1) giving, along with other oxidation products, adenine and adenosine-3′:5′ diphosphate. The latter was differentiated from adenosine 2′:5′ diphosphate, as is described elsewhere (*12*).

The compounds corresponding to bands IV and V were identified by similar methods as the corresponding trinucleotide and tetranucleotide, respectively. Both compounds were oxidized by periodate and were split to adenosine-5′-phosphate by snake venom phosphodiesterase, indicating the position of the phosphomonoester group at C5′. Small polyribonucleotides with 3′-phosphomonoester end-groups are not attacked by the venom preparation, but the venom does act on deoxyribopolynucleotides having 5′-phosphomonoester groups (*18*). Conversely, it is of interest that spleen phosphodiesterase, which rapidly hydrolyzes small polyribonucleotides with 3′-phosphomonoester end-groups, was unable to split the oligonucleotides of Table 1.

Recent work (*3, 6, 19*) has emphasized the significance of nucleoside-5′-polyphosphates in nucleic acid metabolism. Studies are under way to determine whether small polyribonucleotides with 5′-phosphomonoester end-groups may be intermediates in the synthesis as well as in the degradation of nucleic acids.

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Effect of Cortisone on 17-Ketosteroid Excretion in Patients with Diabetes Mellitus

It has been fairly well established by several investigators (*1–6*) that the administration of cortisone to normal healthy adults as well as to patients with various nonmalignant diseases is associated with little if any rise in the excretion of urinary 17-ketosteroids. Hence our unexpected finding of a substantial rise in urinary 17-ketosteroids following the oral administration of cortisone to patients with diabetes mellitus was regarded with considerable interest, especially in view of the current interest in the possible role of the pituitary-adrenal axis in the development of vascular complications in diabetes mellitus (*7, 8*). The studies being reported were performed as part of a systematic exploration of the metabolic interrelationships between the adrenal cortex and diabetes mellitus (*9*). The effects of cortisone on the urinary excretion of 17-ketosteroids and also of reducing corticosteroids forms the basis of this preliminary report.

Each of six patients (four with unstable and two with stable diabetes, *10*), was studied on the metabolism ward for a prolonged period. Each patient received a chemically constant diet of identical foods and food values throughout the period of hospitalization. An initial stabilization period of at least 2 to 3 weeks, during which insulin type and dose were adjusted to achieve optimal regulation, was allowed before each cortisone experiment was begun. Patients received cortisone daily in equally divided doses around the clock (Table 1). Total urinary 17-ketosteroids were determined in duplicate by the modification of the method of Talbot *et al.* (*11*), including a correction for nonketonic chromogens. The reducing corticosteroids in urine were extracted with chloroform at *p*H 1.0 after hydrolysis for 48 hours with beta-glucuronidase at 47°C. The corticosteroids in the neutral extract were determined by a modification of a colorimetric method using blue tetrazolium (*12*).

The results are listed in Table 1. As a whole, the base-line excretion of 17-ketosteroids tended to be in the low-normal or slightly below-normal range. In the four patients who received both the low and moderately high doses of cortisone, a significant rise in the excretion of 17-ketosteroids was observed when small daily doses of cortisone were administered. An example is shown in Fig. 1. When the dose of cortisone was increased to 150 mg daily, a prompt sharp additional rise occurred in all four patients. The highest 24-hour increment above the average base-line value for these four patients was 40.1, 30.8, 34.3, and 23.9 mg, respectively. In the two patients who received the 150-mg doses only, the increments were less pronounced, being 7.1 and 9.1 mg, respectively.

In contrast to the control values for 17-ketosteroids, the control values (average) for total reducing corticosteroids were in the normal range. However, individual values both above and below the normal range were noted. The rise in reducing corticosteroids in response to low and moderately high doses of cortisone paralleled the rise in 17-ketosteroids, although in most cases it was not as great. When the cortisone was discontinued, both the urinary 17-ketosteroid and reducing-corticosteroid levels reverted promptly to the control values or below.

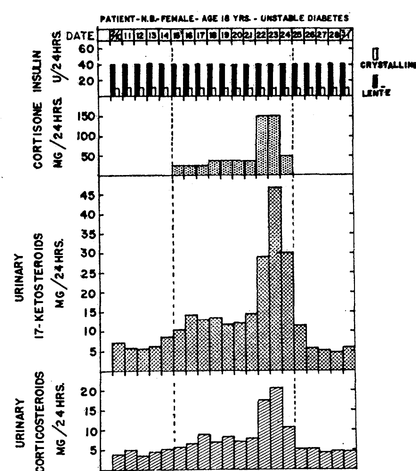

Fig. 1. An example of the marked response in the excretion of 17-ketosteroids and reducing corticosteroids to low and moderately high doses of cortisone.

Marked rises in the urinary excretion of 17-ketosteroids following cortisone administration have also been reported in patients with prostatic carcinoma and other malignancies (*4, 13, 14*). These patients received larger doses of cortisone (300 mg daily) than those used in the present study. Although cortisone has induced sharp rises in the excretion of 17-ketosteroids in both diseases, it remains for future studies to determine whether or not the mechanisms are similar.

It is noteworthy that a diabetic may

Table 1. Excretion of 17-ketosteroids and reducing corticosteroids in response to oral administration of cortisone. The range of values of the amounts excreted is given in milligrams per 24 hours.

Patient	Control		Cortisone: Dose of 25–37.5 mg/24 hr		Cortisone: Dose of 150 mg/24 hr		Recovery	
	Days	Amount	Days	Amount	Days	Amount	Days	Amount
17-Ketosteroids								
N.B.	5	5.7– 8.7	7	10.3 –14.6	2	29.0–46.8	5	4.9– 5.8
S.S.	5	4.1– 7.5	6	4.9 –12.1	2	17.0–37.1	4	5.9–10.3
R.E.	5	1.7– 4.8	7	0.66– 9.3	2	10.8–37.1		4.89
J.P.	5	4.8– 8.7	7	8.7 –20.0	2	27.1–31.0	5	3.5– 8.3
G.W.	5	10.1–10.2			3	9.0–17.2	4	3.3–10.3
L.V.	5	2.3– 4.3			3	4.0–12.4	5	2.5– 5.3
Reducing corticosteroids								
N.B.	5	3.5– 5.1	7	5.7 – 8.9	2	17.6–20.5	5	4.2– 5.2
S.S.	5	2.3– 6.4	6	6.5 – 8.6	2	8.0–14.0	5	3.2– 7.9
R.E.	5	4.8– 5.8	7	6.5 – 9.5	2	10.6–21.7	4	4.3– 6.3
J.P.	5	6.9–15.5	7	3.7 –16.8	2	22.2–33.6	5	5.5–10.4
G.W.	5	5.5– 7.9			3	22.0–28.0	4	4.4– 5.5
L.V.	5	1.3– 7.7			3	6.2– 9.0	5	2.4– 5.9