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Steroid 21-hydroxylase is a major autoantigen involved in adult onset autoimmune Addison's disease

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An adrenal-specific protein reacting with autoantibodies in the sera of patients with adult onset Addison's disease has been purified from human adrenal glands. The protein, mol.wt. 55K, has the biochemical characteristics of steroid 21-hydroxylase and reacts on Western blots with rabbit antibodies to recombinant 21-hydroxylase. Absorption of the native human 55K adrenal protein with human adrenal autoantibodies prevented the subsequent reaction of the 55K protein with rabbit antibodies to 21-hydroxylase in Western blot analysis. In addition, human adrenal autoantibodies reacted with recombinant 21-hydroxylase expressed in yeast. These data indicate that the adrenal specific enzyme steroid 21-hydroxylase is a major autoantigen involved in adult onset autoimmune Addison's disease.

Autoimmunity; 21-Hydroxylase; Addison's disease; Adrenal autoantibody

1. INTRODUCTION

Adult onset autoimmune Addison's disease is characterised by adrenal autoantibodies [1,2] which react with a 55K adrenal-specific protein [3]. We reasoned that this adrenal-specific antigen might be the adrenal-specific enzyme steroid 21-hydroxylase. Consequently we purified the enzyme and studied its interaction with adrenal autoantibodies.

2. MATERIALS AND METHODS

2.1. *Patients' sera, tissue samples and autoantibody measurements*

Sera from 36 patients with adult onset Addison's disease (diagnosed on the basis of clinical and biochemical adrenal insufficiency) were studied. In addition, sera from patients with a variety of autoimmune diseases were analysed (10 Hashimoto's disease, 10 Graves' disease, 5 rheumatoid arthritis, 4 primary biliary cirrhosis and 5 lupus erythematoses). Also, sera from 10 individual healthy normal subjects and a pool of sera from 9 normal healthy subjects were included. Autoantibodies reacting with adrenal, thyroid, smooth muscle, gastric parietal cells and pancreas were assessed by immunofluorescence using unfixed tissue sections in 2 separate laboratories. In addition, adrenal and thyroid autoantibodies in some sera were studied by immunoprecipitation of ^{125}I-labelled adrenal or thyroid microsomal preparations as described previously [3]. Other autoantibodies were measured using kits obtained from RSR Ltd., Cardiff, UK. Human adrenal glands were recovered from redundant tissue surrounding the kidneys removed from cadaveric donors, human placenta was obtained at delivery, and human thyroid tissue was obtained at thyroidectomy for Graves' disease.

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2.2. *Isolation, solubilisation and purification of tissue microsomal fractions*

Microsomal pellets were isolated from tissue homogenates as described previously [3] and stored at −70°C. When required, the pellets were solubilised using 3% sodium cholate in 100 mM phosphate buffer pH 7.0 containing 20% glycerol, 0.1 mM EDTA and 0.1 mM DTT (basal buffer) as described previously [4,5]. Solubilised microsomes (6 ml), were run on a 1 × 10 cm column of Octyl-Sepharose (Pharmacia) and eluted with basal buffer (25 ml). Fractions (3 ml) were monitored at OD_{280nm} (to detect total protein) and at OD_{418nm} (to detect cytochrome p450 proteins [5]). Further elution was carried out using increasing concentrations of Emulgen 913 (Kayo-Atlas) in the basal buffer. Aliquots from groups of fractions were then pooled, concentrated (Centricon 10 microconcentrators) and analysed for the presence of adrenal antigen by a variety of procedures.

2.3. *Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots*

Samples (crude solubilised microsomes or fractions from the Octyl-Sepharose column) were analysed on polyacrylamide gels (9%) under reducing conditions [6]. After electrophoresis, gels were either stained with Coomassie blue, fixed and dried or blotted onto nitrocellulose membranes. In some experiments, the protein band of interest was cut out from the stained gel and the protein electroeluted. Western blot analysis was carried out using the 'renaturing' modification of Birk and Koepsell [7]. After renaturation, the membranes were reacted with Addison or control sera (1:400 dilution) followed by anti-human immunoglobulin–horseradish peroxidase conjugate (Amersham). After reaction with a chemiluminescence generating substrate (ECL system from Amersham) the nitrocellulose membrane strips were exposed to photographic film for between 10–60 s and then developed. In some experiments, rabbit antibody to recombinant steroid 21-hydroxylase [8] and rabbit antibody to microsomal epoxide hydrolase [9] were used in combination with anti-rabbit IgG horseradish peroxidase conjugate.

2.4. *Analysis by dot blot*

In the case of dot blot analysis, adrenal antigen was applied to

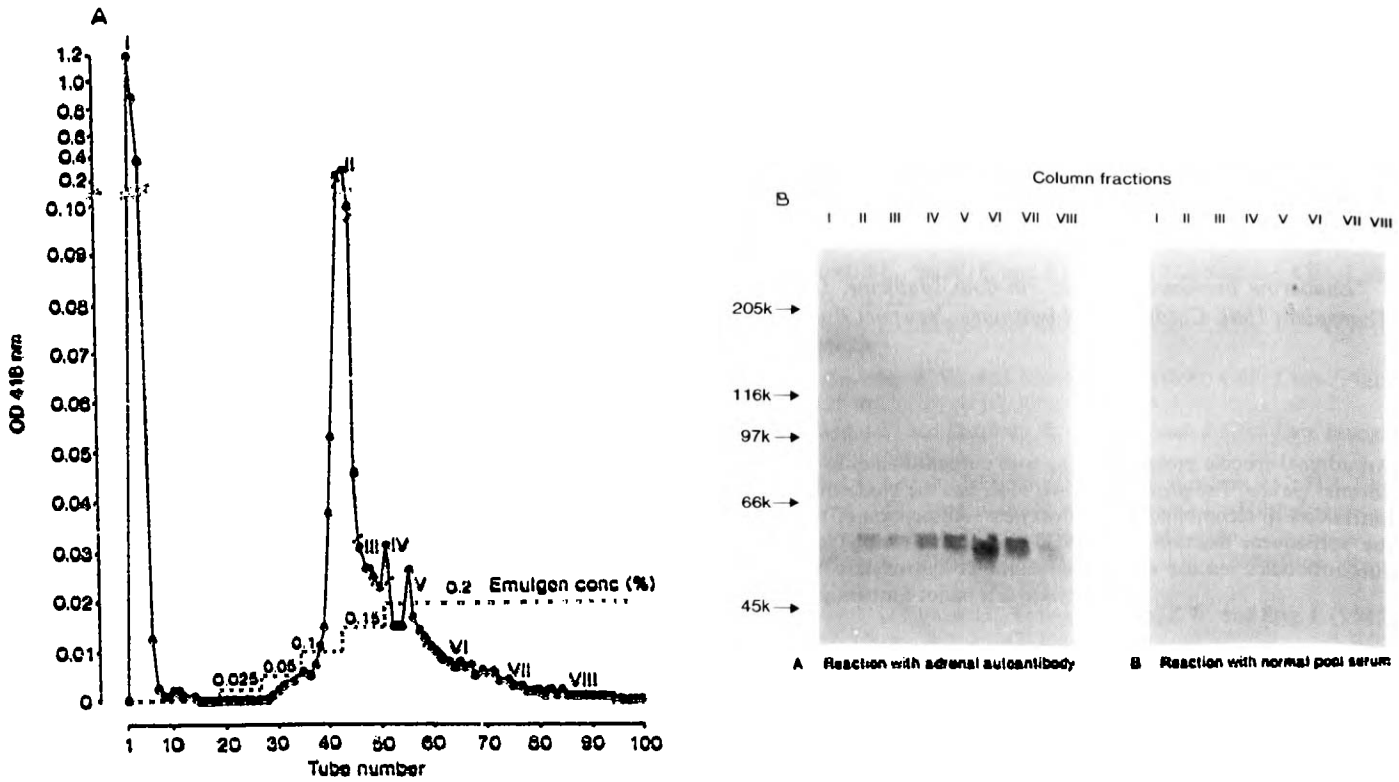

Fig. 1. (A) Elution of adrenal microsomal proteins run on Octyl-Sepharose. (B) Western blot analysis of adrenal microsomal proteins eluted from Octyl-Sepharose.

nitrocellulose membranes and allowed to dry. The procedure then followed the protocol for Western blot analysis as described above.

2.5. *Expression of 21-hydroxylase in* Saccharomyces cerevisiae

The 21-hydroxylase gene in pCD//pC21/3c [10] (ATCC) was modified by replacing the first 13 amino acids with the first 14 amino acids of *STE2* gene and placed under the control of the *GAL1* promoter in pYES 2.0 (Invitrogen). The 21-hydroxylase gene was subcloned from pCD//pC21/3c as a *Bam*HI fragment into the vector pTZ18 (Pharmacia). Digestion with *Sph*I followed by partial digestion with *Nar*I yielded a large fragment comprising the entire coding region of 21-hydroxylase apart from 41 base pairs at the 5′ end. This was then ligated into pYES cut with *Bam*HI and *Sph*I using a *Bam*HI–*Nar*I linker comprising 11 base pairs of non-coding and 42 base pairs of *STE2* coding sequence [11]. Transformants of *Saccharomyces cerevisiae* (C13 ABYS 8B) were grown in selective media and used to inoculate expression cultures in YEP-glucose (2%) or YEP-galactose (2%). After 48 h at 30°C, the cells were harvested, broken by vortexing with glass beads in 1% sodium deoxycholate and analysed on SDS-PAGE followed by Western blotting.

2.6. *Absorption of adrenal antigen with adrenal antibodies*

IgG was purified from normal pool sera and individual patient sera using chromatography on protein A-glass bead columns (Bioprocessing Consett, UK). Aliquots of purified adrenal protein were incubated with the IgGs (2–5 mg·ml^{-1}) for 1 h at 37°C. A protein A-glass bead suspension in PBS was then added to bind free IgG and IgG complexed to adrenal antigen(s). After 1 h at room temperature, the mixture was centrifuged (12,000 × *g*, 10 min, 4°C) and the supernatants analysed by SDS-PAGE and Western blotting using Addison sera and rabbit antibodies to recombinant 21-hydroxylase.

3. RESULTS

3.1. *Solubilisation and purification of adrenal microsomes*

A typical elution profile of adrenal microsomal proteins run on Octyl-Sepharose is shown in Fig. 1A. Fractions eluting from the column were pooled, concentrated and designated I–VIII as shown in Fig. 1A. Analysis on SDS-PAGE showed that most of the protein was present in fractions I, II and III. Fractions IV to VIII contained much less protein and this was resolved principally into 3 Coomassie-stained bands with M_r = 51K, 55K and 60K (data not shown). The highest concentration of the 55K band was present in fraction VI and Western blot analysis using adrenal autoantibody positive sera indicated that the autoantibodies recognised a 55K protein most of which was present in fraction VI (Fig. 1B). Parallel studies were carried out in which solubilised placental microsomal proteins were separated on an Octyl-Sepharose column and analysed by Western blotting. None of the column fractions contained material which reacted specifically with adrenal autoantibodies (data not shown). Western blot analysis of fraction VI showed that rabbit antibody to 21-hydroxylase reacted specifically with the 55K protein (Fig. 2, lanes 2 and 3) whereas an antibody to epoxide hydrolase reacted with a protein of mol.wt. 49K (Fig. 2, lane

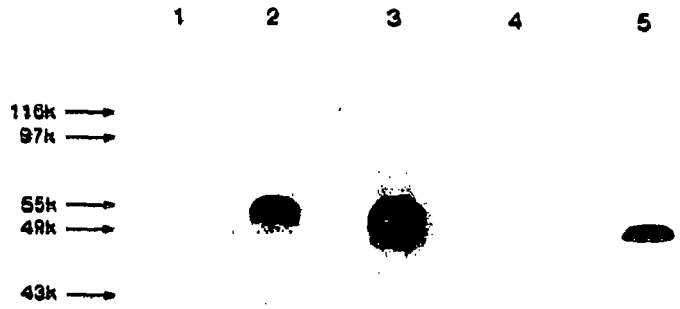

Fig. 2. Western blot analysis of fraction VI of Octyl-Sepharose column. Lane 1, reaction with normal pool serum. Lane 2, reaction with adrenal autoantibody positive serum. Lane 3, reaction with rabbit 21-hydroxylase antibody. Lane 4, reaction with normal rabbit serum. Lane 5, reaction with rabbit epoxide hydrolase antibody.

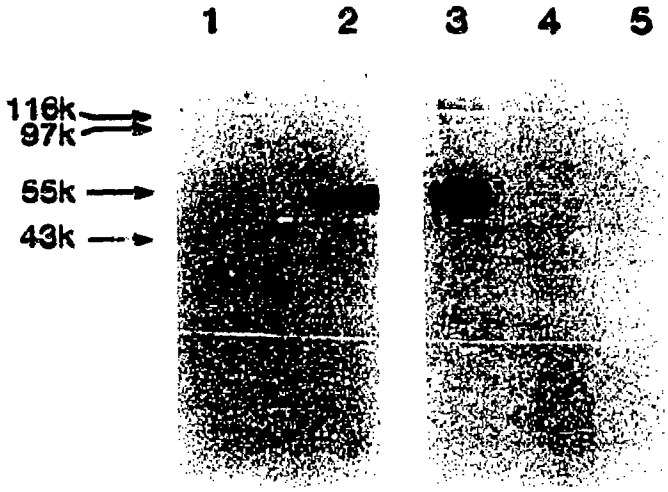

Fig. 3. Western blot analysis of 55K adrenal protein electroeluted from an SDS-PAGE run. Lane 1, reaction with normal pool serum. Lane 2, reaction with adrenal autoantibody positive serum. Lane 3, reaction with rabbit 21-hydroxylase antibody. Lane 4, reaction with rabbit epoxide hydrolase antibody. Lane 5, reaction with normal rabbit serum.

5). In some experiments, the 55K band from SDS-PAGE analysis of fraction VI was cut out of the gel, electroeluted, run on PAGE to confirm homogeneity (data not shown) and analysed by Western blot. The eluted band reacted strongly with adrenal autoantibody positive serum and with rabbit antibody to 21-hydroxylase but not with epoxide hydrolase antibody nor with normal pool human serum, nor with normal rabbit serum (Fig. 3).

3.2. *Expression of 21-hydroxylase in* Saccharomyces cerevisiae

Adrenal autoantibodies and rabbit antibodies to 21-hydroxylase reacted strongly and specifically with a 55K protein expressed in transformed yeast (Fig. 4, lanes 5 and 6). Normal pool human serum and normal rabbit serum did not react with this 55K protein band. In addition, adrenal autoantibodies and rabbit antibodies to recombinant 21-hydroxylase did not react specifically with any proteins expressed in non-transformed yeast or yeast expressing human thyroid peroxidase (Fig. 4, lanes 9,2 and 8,3 respectively). Furthermore, expression of the 55K protein band recognised by au-

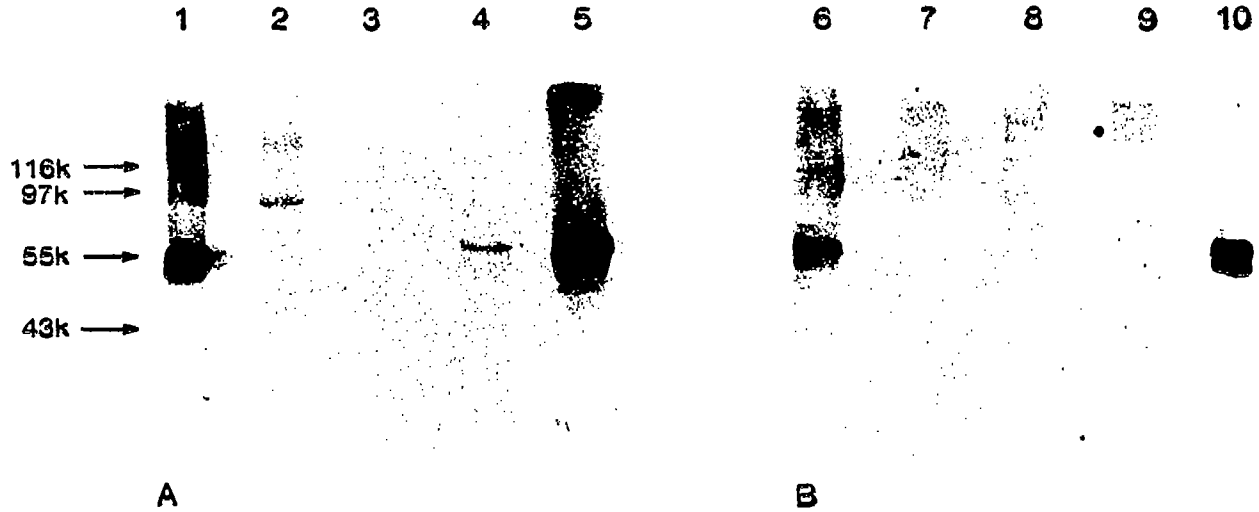

Fig. 4. Analysis of yeast extracts by SDS-PAGE and Western blotting. Lanes 1 and 10, native 21-hydroxylase. Lanes 2 and 9, extract from non-transformed yeast. Lanes 3 and 8, extract from yeast expressing human thyroid peroxidase. Lanes 4 and 7, extract from yeast transformed with 21-hydroxylase gene grown in medium containing glucose. Lanes 5 and 6, extract from yeast transformed with 21-hydroxylase gene grown in medium containing galactose. (A) Reaction with rabbit antibody to 21-hydroxylase. (B) Reaction with adrenal autoantibody positive serum.

toantibodies and rabbit antibodies was clearly increased in transformed yeast grown in galactose expression medium compared to glucose expression medium (Fig. 4, lanes 6,7 and 4,5 respectively).

3.3. *Absorption studies*

Western blot analysis indicated that rabbit antibody to recombinant 21-hydroxylase did not react with native adrenal 55K protein which had been pre-absorbed with IgG from human adrenal autoantibody positive Addison sera (data not shown). Pre-absorption with normal pool serum IgG had no effect on reactivity with 21-hydroxylase antibody.

3.4. *Analysis of serum samples by dot blot assay*

Out of 36 Addison sera studied, 26 (72%) showed the presence of adrenal autoantibodies by immunofluorescence, immunoprecipitation, or Western blotting (Table I). All 26 of these sera were also positive by dot blot assay using purified native human adrenal antigen (Table I). Ten Addison sera were negative for the presence of adrenal autoantibodies by immunofluorescence, immunoprecipitation or Western blotting and all of these 10 were negative by dot blot assay. Only one of the 44 control sera studied was positive for adrenal autoantibodies in the dot blot assay. This serum was from a patient with Graves' disease and type II diabetes mellitus, and the presence of autoantibodies reactive with the 55K adrenal protein was confirmed by Western blotting.

Table I

Autoantibody assays in patients with adult onset Addison's disease

	Adrenal Autoantibody assays				Thyroid Autoantibodies			
Addison patient No.	Dot Blot	Western Blot	Immuno-fluorescence	Immunoprecip	TgAb U/ml	TPOAb U/ml	TRAb	Other Autoantibodies
1	+	++++	nt	nt	0.6	30	neg	
2	+	++++	nt	nt	16	30	neg	
3	+	+++	nt	nt	14.5	30	45.5	
4	+	+++	nt	nt	6	30	neg	
5	+	++	nt	nt	neg	2.7	neg	
6	+	+	+	+	30	30	91.9	a,b,c
7	+	++	nt	nt	1.2	21	neg	
8	+	+	nt	nt	neg	2.0	neg	
9	+	+	nt	nt	neg	26	neg	
10	+	+	nt	nt	12.5	17	neg	
11	+	+++	nt	nt	7.5	1.9	neg	
12	+	++	+	+nt	20	8.9	neg	a,c,d
13	+	++	nt	nt	neg	neg	neg	
14	+	+	nt	+	0.5	21	neg	
15	+	nt	+	+	neg	neg	neg	c,e
16	+	nt	+	+	neg	neg	neg	
17	+	nt	++	+	2.2	30	neg	b,c,g
18	+	+	+	+	neg	neg	neg	c
19	+	+	+	+	1.7	21	neg	c
20	+	+	+	+	neg	1.7	neg	c
21	+	+	+	+	neg	25	neg	c
22	+	+	+	+	16	34	18.3	
23	+	+	+	+	30	30	neg	d,e,f
24	+	+	+	+	8.4	12	6.8	
25	+	+	+	nt	30	30	47.6	c
26	+	+	+	nt	8.2	30	neg	c
27	neg	nt	neg	neg	neg	neg	neg	
28	neg	nt	neg	neg	neg	neg	neg	
29	neg	nt	neg	neg	neg	neg	neg	
30	neg	nt	neg	neg	0.4	8.6	neg	d
31	neg	nt	neg	neg	neg	neg	neg	c,d
32	neg	nt	neg	neg	neg	5.0	neg	
33	neg	nt	neg	neg	neg	neg	neg	
34	neg	neg	nt	nt	neg	neg	neg	
35	neg	neg	nt	nt	neg	neg	neg	
36	neg	neg	neg	neg	neg	neg	neg	

a = antitubulin antibodies; b = anti-islet cell antibodies; c = anti-parietal cell antibodies; d = anti-nuclear antibodies; e = anti-mitochondrial antibodies; f = anti-desmin antibodies; g = anti-steroid producing cell antibodies; nt = not tested.

4. DISCUSSION

Chromatography of solubilised adrenal microsomes on Octyl-Sepharose indicated that a protein band of 55K was eluted principally in fraction VI with smaller amounts of 55K protein in fractions IV, V and VII (Fig. 1A). Previous studies with Octyl-Sepharose chromatography have shown that 21-hydroxylase is eluted in similar column fractions [5]. Western blotting showed that adrenal autoantibodies recognised the adrenal 55K protein in the column fractions and this reaction was by far the strongest in fraction VI (Fig. 1B). Tissue specificity of the autoantibody reaction was confirmed using placental material purified in the same way. Western blot analysis indicated that adrenal autoantibodies and antibodies to recombinant human 21-hydroxylase reacted with the adrenal 55K protein present in column fraction VI (Fig. 2). In addition, both types of antibody reacted with the 55K adrenal protein after electroelution from SDS-PAGE (Fig. 3). Furthermore, absorption studies confirmed that the 55K adrenal protein recognised by adrenal autoantibodies and rabbit 21-hydroxylase antibodies was indeed the same protein. Finally, when 21-hydroxylase was expressed in yeast, it was recognised by adrenal autoantibody positive Addison sera and rabbit antibody to recombinant human 21-hydroxylase. Duan Wu et al. [12] used a similar system to express enzymatically active human 21-hydroxylase in yeast. Consequently, 4 different approaches involving (i) purification of native human adrenal proteins; (ii) Western blotting with Addison sera and rabbit antibody to recombinant 21-hydroxylase; (iii) antibody absorption studies and (iv) expression of human 21-hydroxylase in yeast, indicate that 21-hydroxylase is a major autoantigen in adult type Addison's disease. In order to develop an assay for 21-hydroxylase autoantibodies, we evaluated the use of the Octyl-Sepharose column fraction VI (Fig. 1) in a dot-blot system. Out of 36 Addison sera analysed for adrenal autoantibodies by dot-blot assay, 26 (72%) were positive. Adalysis of the sera by immunofluorescence and/or immunoprecipitation and/or Western blot, confirmed the presence of adrenal autoantibodies in all 26 dot-blot positive sera (Table I). The 10 Addison sera negative by dot-blot assay were also negative in the three other systems used to detect adrenal autoantibodies (Table I). In dot-blot assays of control sera, only one serum (from a patient with Graves' disease) was found to be positive. This positive Graves' serum was confirmed to have antibodies reactive with the 55K band on Western blot. Our observations with the dot blot assay are in good agreement with previous studies of large numbers of sera using immunofluorescence [13–15] with most studies reporting adrenal autoantibodies in a few patients with non-adrenal autoimmune disease including Graves' disease [13–15]. Our data indicate therefore that the dot-blot system based on partially purified 21-hydroxylase provides a sensitive, specific method for measuring 21-hydroxylase autoantibodies. In view of our results and the recent findings by Krohn et al. [15], it would appear that two distinct enzymes, i.e. 17-alpha-hydroxylase and 21-hydroxylase are the autoantigens involved in two clearly-distinct forms of Addison's disease [2,17]. In *early onset* Addison's disease which is most commonly associated with hypoparathyroidism and mucocutaneous candidosis (type I polyendocrine autoimmune syndrome) autoantibodies seem to be directed against 17-α-hydroxylase [16] whereas in *adult onset* Addison's disease, autoantibodies are directed against 21-hydroxylase.

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