manganese dioxide when shaken with 2% potassium permanganate solution, indicative of unsaturation.

About 1.5 g. of VI was shaken with 10 ml. of 5% sulfuric acid. A slight rise in temperature was noted. The aqueous phase was separated and exhibited a strong positive iodoform test. The organic phase was refluxed for two hours with 20 ml. of a 30% potassium hydroxide solution, cooled and acidified with 6 *N* sulfuric acid, extracted with ether and dried over anhydrous sodium sulfate. The ether was evaporated and the residue recrystallized from acetone and identified as succinic acid by a mixed melting point of 184–185° with an authentic sample. These reactions together with the evidence of the infrared spectrum indicate VI to be carbethoxymethylketene diethylacetal.

The Reaction of Bromoform and Potassium *t*-Butoxide with Ketene Diethylacetal.—Into a 250-ml. three-neck round-bottom flask fitted with a reflux condenser protected by a calcium chloride drying tube, a mechanical stirrer, and a constant pressure dropping funnel, were placed 16.7 g. (0.15 mole) of powdered potassium *t* butoxide, 11.6 g. (0.1 mole) of III, 25 ml. of anhydrous *t*-butyl alcohol and 10 ml. of *n*-octane. The flask was immersed in a water-bath maintained at 10°. A solution of 25.2 g. (0.1 mole) of bromoform in 10 ml. of *n*-octane was added dropwise with stirring which was continued for one-half hour after addition was complete. To the reaction mixture was then added 100 ml. of ice-water, the whole shaken and transferred to a separatory funnel. An additional 30 ml. of *n*-octane was added and the phases separated. The organic layer was washed with three 100-ml. portions of ice-water, dried over 10 g. of anhydrous sodium sulfate, and the solvent removed by distillation from a water-bath at 35°. The water-bath was replaced by an oil-bath and the residue distilled *in vacuo* to give 9.2 g. (51%) of ethyl α-bromoacrylate, boiling at 72.0–72.5° at 31 mm. and identified by comparison of its infrared spectrum with that of an authentic sample prepared by the method of Marvel, Dec, Cooke and Cowan.[15] When the crude reaction mixture was heated at higher pressures, *i.e.*, 155 mm. and above, ethyl bromide was condensed in the Dry Ice–acetone trap in the line leading to the pump.

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PITTSBURGH 13, PENNA.

COMMUNICATIONS TO THE EDITOR

STUDIES ON NATURALLY OCCURRING PHOSPHOINOSITIDES[1]

Sir:

The exact structure of the monophosphoinositides (diacyl glycerylphosphorylinositols) has not been established completely. In the present study evidence is presented as to the possible structure and configuration of the glycerylphosphorylinositol core.

Beef liver phosphoinositide[2] (1.02 g.) (P, 3.34; N, <0.08; N/P ~0.05; Inositol/P, 1.05) was dissolved in 70 ml. of water and, after the addition of 18 ml. of 1 *M* methanolic NaOH, hydrolyzed for 1 hr. at 37°.[3] After removal of sodium with resin (IR-120 (H^+)), the mixture was extracted with CCl_4, $CHCl_3$ and petroleum ether. The aqueous layer was neutralized with cyclohexylamine and the water removed by lyophilization. The residue was dissolved in 10 ml. of water and an equal volume of acetone added. After 2 hr. at 0°, the solution was centrifuged at 0° and the precipitate (I) and soluble fraction (II) separated. I represented 7% of the total phosphorus and by paper chromatography contained glycerylphosphoryl inositol (GPI), free inositol and inositol 1- and 2- phosphates.[4] II was concentrated at 10° *in vacuo* to 5 to 7 ml. and then lyophilized. The residue was dissolved in 1.0 ml. of water, 30 ml. of absolute ethanol added and the mixture stored overnight at −30°. The white crystals were collected by filtration and washed with a small volume of cold absolute ethanol. After drying *in vacuo* over P_2O_5 overnight the yield was 302 mg. (63.4%); m.p., 124–126° (uncorr.). Calculated for cyclohexylamine salt of GPI ($C_{15}H_{32}O_{11}NP$) (433.42): P, 7.16; N, 3.23; inositol/P, 1.00; α-substituted glycerol, 21.3; found: P 7.09; N, 3.28; inositol/P, 0.93, 1.1; α-substituted glycerol, 21.1. Optical rotation values (*C*, 2 in water): salt, $[\alpha]^{25}D$ −13.5° ± 0.3; $[M]^{25}D$ −58.5° ± 1.3°; free acid[5], $[\alpha]^{25}D$ −18.7° ± 0.4°; $[M]^{25}D$ −62.5 ± 1.4°.

Through use of periodic acid (in slight excess of stoichiometric amount) in neutral solution, the glycerol portion of GPI is oxidized quantitatively while the inositol moiety is not attacked. Thus, any contribution by the glycerol to optical activity can be abolished. In a typical experiment: 40.62 mg. cyclohexylamine GPI (94.0 μmoles) was dissolved in 1 ml. of water and over a 15-minute period 0.96 ml. of 0.1 *M* aqueous $NaIO_4$ was added with continuous stirring. After an additional 10 min. the solution was diluted with water to 50 ml., and titrated with thiosulfate. Theoretical

(1) Supported by funds from the National Science Foundation.

(2) D. J. Hanahan and J. N. Olley, *J. Biol. Chem.*, **231**, 813 (1959).

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(4) F. L. Pizer and C. E. Ballou, THIS JOURNAL, **81**, 915 (1959). Dr. Ballou very kindly made this manuscript available to us in advance of its publication.

(5) J. N. Hawthorne and G. Hübscher, *Biochem. J.*, **71**, 195 (1959). These authors report an $[\alpha]^{24}D$ value of −18 ± 2° for GPI isolated by ion exchange chromatography.

for 96 μmoles periodate → iodate is 1.92 ml. of 0.1 *M* thiosulfate; found, 93 μmoles $NaIO_4$ (1.86 ml. 0.1 *M* thiosulfate) had been consumed and 93.5 μmoles of formaldehyde produced. This showed that attack was centered only on the glycerol portion of the GPI molecule.

Subsequently, 98.5 μmoles of GPI-cyclohexylamine was oxidized, as above, with 1.01 ml. of 0.1 *M* $NaIO_4$. The optical rotation of the glycolaldehydeinositol phosphate was $[\alpha]^{25}D$ −13.2 ± 0.3°; $[M]^{25}D$ −53.0 ± 1.2°.

These results strongly support the proposal that the phosphate is esterified to the 1 or 4 position on the myo-inositol and this asymmetric unit accounts for the major optical activity of the GPI. The above findings are in agreement with the recent excellent observations of Pizer and Ballou[4] on the nature of the inositol phosphates of soybean inositol lipides. Further, the data here show that the attachment of the phosphate to the glycerol is at the 3-position.

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RECEIVED MARCH 30, 1959

ADENOSINE-5′-TRIPHOSPHATE REQUIREMENT FOR LUMINESCENCE IN CELL-FREE EXTRACTS OF RENILLA RENIFORMIS[1]

Sir:

A survey of the ATP[2] requirement for luminescence in extracts of a variety of luminous organisms was reported[3] recently in which a negative ATP response was obtained for the sea pansy, *Renilla reniformis*. Using similar techniques, negative results also were obtained in this laboratory with the same organism, but by varying the extraction procedure very active extracts that require ATP have been obtained from *R. reniformis*.

Using crude extracts, a 5–10-fold increase in light intensity was obtained by adding ATP. With ammonium sulfate fractions, 50–100 fold stimulations by ATP have been observed (Table 1). The system is specific for adenine-containing nucleotides since other nucleoside-5′-triphosphates such as GTP, CTP, and UTP are inactive. In addition to ATP, AMP and ADP also show activity, but the type of response to these compounds is different from that with ATP. Thus at concentrations corresponding to near saturating levels of ATP, AMP and ADP are only one-third as effective. At saturating levels, AMP and ADP are about 80% as effective as ATP in supporting luminescence. Adenosine is inactive in this system.

Since the extracts contain a phosphatase that rapidly converts ADP to AMP, the ADP effect can be explained in terms of its conversion to AMP. Adenylate kinase activity cannot be demonstrated. The AMP used in these experiments did not contain ATP or ADP as determined by ATP analysis using the firefly technique.[4,5] ADP was measured by coupling adenylate kinase to the firefly system. The AMP effect is not due to oxidative phosphorylation since AMP is effective in a phosphate-free system as well as in the presence of 2,4-dinitrophenol.

TABLE I

EFFECT OF ATP ON THE LUMINESCENCE OF *R. Reniformis* EXTRACTS

Conditions: 0.05 *M* potassium phosphate, *p*H 7.5 (1.1 ml.); reduced glutathione (2 μmoles); nucleoside-5′-triphosphate (0.5 μmole); enzyme (0.5 mg. protein); total volume, 1.4 ml. Divalent cations such as Mg^{++} or Mn^{++} have no effect on the complete system.

Additions	Relative light intensity
None	2
ATP	101
GTP, CTP, or UTP	2

In the presence of ATP, a relatively stable intermediate accumulates under anaerobic conditions. It does not appear to be an adenylate derivative since inorganic pyrophosphate ($10^{-3}M$, final concentration) or purified inorganic pyrophosphatase has no effect on the luminescence system. These observations are in direct contrast to the firefly system[6] which requires the intermediate formation of an enzyme bound adenylate derivative.

The effect of AMP on this system may be interpreted as a cyclic phenomenon involving either ATP or ADP formation, although the mechanism of action of these adenine-containing nucleotides is not understood.

In addition to ATP and the enzyme fraction, the requirements for *Renilla* luminescence include oxygen and a dialyzable heat-stable factor obtained from boiled *Renilla* extracts. Flushing with nitrogen abolishes the light, which is returned by flushing with oxygen. Using a resolved enzyme, 80-fold stimulations of luminescence can be observed upon the addition of boiled extract. Attempts to demonstrate a specific metal requirement have not succeeded.

The author is indebted to Dr. John M. Teal, University of Georgia Marine Institute, Sapelo Island, for supplying the animals used in these experiments.

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(2) Abbreviations: AMP, adenosine-5′-monophosphate; ADP, adenosine-5′-diphosphate; ATP. GTP, CTP, and UTP, the nucleoside-5′-triphosphates of adenosine, guanosine, cytidine, and uridine, respectively.

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(4) B. L. Strehler and J. R. Totter, *Arch. Biochem. and Biophys.*, **40**, 28 (1952).

(5) W. D. McElroy and B. L. Strehler, *Bacteriol. Revs.*, **18**, 177 (1954).

(6) W. D. McElroy, *Proc. Natl. Acad. Sci., U.S.*, **33**, 342 (1947).

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ON THE MOLECULAR BONDING OF LYSINE VASOPRESSIN AT ITS RENAL RECEPTOR SITE[1]

Sir:

Although the elucidation of the structure and synthesis of vasopressin has been accomplished elegantly by du Vigneaud and collaborators the mechanism of the antidiuretic action of the hormone remains obscure. We have evidence that the

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