inhibition of the enzyme in the granule fraction and little inhibition in the nuclear and supernatant fractions. This result together with higher stability and lower pH optimum of acid phosphatase in granule fraction clearly indicates that the properties of the enzyme in this fraction are distinctly different from those of the enzyme in the nuclear and soluble fractions.

The morphological heterogeneity of PMN leucocyte granules has been shown by electron microscopy(9,10). The decision as to whether heterogeneous granules contain acid phosphatase of identical properties cannot be made prior to the development of a method for differential fractionation of the granules.

Summary. The present study has revealed that 45% of acid phosphatase in rat polymorphonuclear leucocytes can be recovered from the granular fraction, 21% from the nuclear fraction, and 34% from the supernatant fraction. Acid phosphatase in the granule fraction appeared to have properties distinctly different from those of the enzyme in the nuclear and soluble fractions. The enzyme in the granule fraction was more stable, possessed a lower pH optimum, and was preferentially inhibited by sodium fluoride.

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Electrical Stimulation and Metabolism of a Phosphoinositide Complex in the Salivary Gland.* (31322)

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Burford and Huggins(3,4) reported that supersensitive submaxillary glands of cats secreted more water, sodium, and mucin than did normal glands when both were stimulated with doses of either acetylcholine or epinephrine found to be the threshold for the normal gland. Subcutaneous injection of radiophosphorus followed by a 2-hour experimental period of secretory stimulation resulted in an increased specific activity of the phosphorus in the phosphoinositide complex (polyphosphoinositides) and in phospholipid fractions obtained from supersensitive glands when compared with normal glands. When atropine or dihydroergotamine was used to inhibit salivary secretion, the specific activities of the phosphoinositide complex and phospholipid fractions did not increase in the supersensitive gland.

Many theories concerning the basis for developing supersensitivity in submaxillary glands have been reported(5,6,9,25). To an-

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swer questions concerning the possibility that an increased blood flow to the supersensitive gland was responsible for the increased metabolic activity, secretion in the submaxillary gland was induced by electrical stimulation of its preganglionic parasympathetic and sympathetic innervation. Under these conditions, an increased blood flow on the stimulated side occurs, and, in the presence of atropine, secretion is inhibited whereas the increased blood flow is not affected(13). The data to be presented will show that there is a relationship between secretion induced by electrical stimulation and phospholipid and phosphoinositide complex metabolism and that this relationship is altered in the presence of atropine. The observations reported below indicate that it is largely the monophosphoinositide component of both fractions which is responsible for the increased metabolic activity. More specifically, these data indicate that the phosphorus atom most affected is the phosphodiester bridge between glycerol and inositol.

Methods. Cats, male and female, weighing between 2.0-4.0 kg, were used in this study. Pentobarbital sodium (30 mg/kg), was used (intraperitoneally) as the anaesthetic for all surgical procedures. The *chorda lingual* nerve or the sympathetic trunk were unilaterally exposed by standard surgical procedures. They were then electrically stimulated at 10 cps, supramaximally, for 1 hour using a Lab-Tronic Physio-Stimulator, Model D-103-B. In some experiments, atropine (0.5 mg/kg or dihydroergotamine (50 μg/kg) were given intraperitoneally to block the secretory effect of electrical stimulation. Radiophosphorus $Na_2HP^{32}O_4$ in a dose of 0.5 mc/kg was administered subcutaneously on the outer aspect of the right thigh 15 minutes prior to initiation of electrical stimulation. Procedures for collection of saliva and for separation and analysis of the different phosphorus-containing fractions have been described by Burford and Huggins(4).

The phosphoinositide complex and phospholipid fractions, both labeled with radiophosphorus, were separated by thin-layer chromatography (TLC) according to procedures described by Mangold(20) and Skidmore and Entenman(23). The glass plates were specifically cut with dimensions of 3¾″ × 19″ × ⅛″ and were chemical and heat resistant. Silica gel containing 5% $CaSO_4$ as a binder was used as the absorbent and was applied to the plates with a Camag apparatus from a slurry in deionized water. The freshly prepared plates were dried for 1 hour at 120°C, cooled to room temperature, and used immediately. Separation was accomplished in a solvent system of Chloroform:Methanol: *7N* Ammonia (60:35:5, v/v) at approximately 23°C for 6 hours. In all cases, the amount of material applied to the plates represented equal weights of phosphorus from control and stimulated glands. Radioautography was carried out by exposing the Saran wrapped TLC plates to Kodak No-screen-X-ray film. The separated components were visualized by iodine vapor, ninhydrin, Dragendorf reagent, or radioautography and identified by comparison with purified standards. Phosphatidylcholine was isolated by the method of Pangborn(21), phosphatidyl serine and phosphatidyl ethanolamine by the method of Rouser(22), monophosphoinositide by the method of Hannahan and Olley(12) and triphosphoinositide by the method of Dittmer and Dawson(8). The counting from TLC plates was done in the presence of silica gel scraped from the plate with the sample. Corrections were made from the absorption of radioactivity by silica gel in which a known amount of radioactivity was plated in the presence and absence of this material.

The several components which were obtained by TLC of the phospholipid fraction and of the phosphoinositide complex were eluted from the silica gel plates. These components as well as the phosphoinositide complex were hydrolyzed and separated into the various water-soluble phosphate esters by descending paper chromatography according to the procedure described by Andrade and Huggins(1,2). Radioactivity and phosphate analysis were obtained after digestion of the paper in perchloric acid and the subsequent counting of an aliquot of the digest.

The *t* test was used to assess the level of significance in the control and experimental organs. In those experiments dealing with stimulated and nonstimulated glands from the same animal, the *t* test was used to determine

TABLE I. Effect of Unilateral Electrical Stimulation of the *Chorda Lingual* Nerve of the Cat on Metabolism of the Phosphoinositide Complex and Other Phosphorus-Containing Fractions from the Submaxillary Gland of the Cat.

Treatment	No. of animals	Specific activity (counts/min μg P) Total acid-soluble *P*	7-Min hydrolyzable *P*	Phospholipid *P*	Phosphoinositide complex *P*
Non-stimulated (N)	5	2216 ±522	1895 ±403	53 ± 34	470 ±245
Stimulated (S)	5	3817 ±110	2610 ±651	193 ±123	1143 ±573
Ratio S/N		1.7 ± .3	1.37± .05	3.9 ± 1.2	2.5 ± .106
				P .001	P .001
Pretreatment with atropine (0.5 mg/kg)					
Non-stimulated (N)	4	2165 ±622	1780	30 ± 16	347 ±135
Stimulated (S)	4	2653 ±546	2110	36 ± 13	451 ±111
Ratio S/N		1.3 ± .22	1.2	1.3 ± .2	1.4 ± .2
				P .3-.2	P .2-.1

Fifteen minutes after subcutaneous injection of $NaH_2P^{32}O_4$ (0.5 mC/kg) electrical stimulation, supramaximally, of the *chorda lingual* nerve was initiated, and continued for one hour. Animal was then sacrificed, submaxillary glands were removed and partitioned into the different phosphorus-containing fractions according to Burford and Huggins(4).

the difference between two correlated means by the paired comparison method. A *P* value of 0.05 is interpreted as indicating a statistical difference in the variables examined(24).

Results. Effect of electrical stimulation of the chorda lingual *nerve on the phosphorus metabolism of submaxillary gland.* Table I presents data which show the effect of the electrical stimulation of the *chorda lingual* nerve on the phosphorus metabolism of the submaxillary gland. Although not shown in this table, phosphorus concentrations were invariant in nonstimulated *vs* stimulated glands, regardless of experimental conditions. Because of variations in biological activity between experimental animals, the ratio "stimulated-to-nonstimulated" (S/N) was used as an indication of increased metabolic activity. This was calculated by dividing the specific activity of a fraction from the nonstimulated into that obtained from the stimulated gland in the same animal. The average S/N ratios of the specific activities (Table I) were 1.7 for the total acid-soluble phosphorus fractions (TAS), 1.4 for 7-minute hydrolyzable phosphorus (terminal P of ATP), 3.9 for phospholipid fraction, and 2.5 for the phosphoinositide complex. The specific activity of the latter fraction was 6-8 times that of the phospholipid fraction.

Table I shows the effect of electrical stimulation, during atropine blockade, on the same fractions. Both secretion and augmented phosphate turnover were inhibited. The small increase in phosphate turnover is explainable on the basis of increased blood flow, since, according to Hilton and Lewis(13), atropine does not block vasodilation during *chorda lingual* stimulation. Average stimulated-to-control ratios of specific activities were 1.3 for TAS, 1.3 for 7-minute hydrolyzable, 1.4 for phospholipid fraction, and 1.4 for the phosphoinositide complex fraction. Relative to the TAS and 7-minute hydrolyzable phosphorus fractions, pretreatment with atropine consistently resulted in a diminished specific activity of the phospholipid and phosphoinositide complex fractions, both in stimulated and in control glands.

Aliquots of phospholipid and phosphoinositide complex extracts representing equal weights of phosphorus from stimulated and nonstimulated glands were applied to TLC plates, and the individual components were separated by ascending chromatography. The data presented in Table 2 represent the incorporation of radio phosphorus into the different components of the phosphoinositide complex and phospholipid fraction. Monophosphoinositide showed about a 6-fold increase, the other "phosphoinositides" about a 3-fold increase, and the remaining phospholipids only a 1.5-2.0-fold increase.

The data obtained above with respect to the monophosphoinositide would suggest that it is the phosphate atom, present as the phos-

TABLE II. Incorporation of Radiophosphorus into the Different Components of the Phosphoinositide Complex and Phospholipid Fraction of the Submaxillary Gland of the Cat After Unilateral Electrical Stimulation of the *Chorda Lingual* Nerve.

	Total radioactivity					
	Phosphoinositide complex			Phospholipid		
	S	N	S/N	S	N	S/N
Triphosphoinositide	1180	434	2.7	356	119	3.0
Diphosphoinositide	181	70	2.7	200	100	2.0
Phosphatidyl serine	0	0	0	30	18	1.6
Monophosphoinositide	372	66	4.7	522	86	6.1
Phosphotidyl choline	0	0		81	39	2.1
Phosphotidyl ethanolamine	0	0		66	41	1.6
Phosphatidic acid	0	0		0	0	

Twenty μg of phosphorus from the phosphoinositide complex and 100 μg from the phospholipid fraction were applied to the TLC plates, which were developed by the ascending technique in the solvent system, $CHCl_3$: CH_3OH : 7*N* NH_4OH (60 : 35 : 5, v/v).
S = Stimulated organ. N = Non-stimulated organ.

phodiester, which was specifically affected rather than the monophosphate esters present in di- and triphosphoinositide. To obtain more information in this regard, the phosphoinositide complex from stimulated and non-stimulated glands was subjected to alkaline hydrolysis as described by Andrade and Huggins(2). The water-soluble phosphates were then separated by descending paper chromatography, and the active areas were located by radioactive scanning of the developed chromatogram. Table III shows the radioactivity data found in the different phosphorus fractions obtained by alkaline hydrolysis of the phosphoinositide complex. These data, as well as thin layer chromatography, suggest that the diesterified phosphate atom linking glycerol to inositol turns over more rapidly than do the other phosphate esters in the same molecule during secretion (Tables II and III).

Discussion. The secretion of hexosamine (mucin), sodium, and potassium has been studied in saliva from the submaxillary gland of the cat(5). Burford and Huggins(4) found that the level of hexosamine secreted was related to the degree of stimulation of incorporation of radiophosphorus into the phospholipid fraction and into the phosphoinositide complex. Even though there was no stoichiometry between augmented phosphate turnover and mucin secretion in this present study, both were elevated in the stimulated or secreting gland. Although more hexosamine-containing compounds and cations were secreted under *chorda lingual* stimulation, their concentrations were higher in sympathetically elaborated saliva. Potassium concentration in the saliva exceeded that in the plasma and the concentration of this ion in sympathetically elaborated saliva was about twice that under *chorda lingual* provocation. There was no measurable salivary flow from the control gland under these conditions.

Specific activity is defined as the amount of radioactive phosphorus in counts-per-minute per microgram of phosphorus in the compound under study and has been used to indicate changes in metabolic activity of the TAS, 7-minute hydrolyzable, phospholipid, and phosphoinositide complex fractions. The TAS fraction contains the phosphorylated intermediates of metabolism, and the 7-minute

TABLE III. Incorporation of Radiophosphorus into the Phosphate Esters Obtained by Alkaline Hydrolysis of the Phosphoinositide Complex from Submaxillary Glands of the Cat After Unilateral Electrical Stimulation* of the *Chorda Lingual* Nerve.

		Total radioactivity (counts/min)		
	R_{fgp}	S	N	Ratio S/N
Origin	.0	706	173	4.1
Inositol triphosphate	.2	192	56	3.4
" diphosphate	.6	760	216	3.5
" monophosphate	.85	1080	216	5.0
Glycerophosphate	1.0	673	106	6.4

The phosphoinositide complex was subjected to alkaline hydrolysis (0.5 *N* NaOH 100°) for 45 min and the resulting phosphate esters were separated by descending chromatography (30 hr at 30°C).
S = Stimulated. N = Non-stimulated.
* The *chorda lingual* nerve was stimulated supramaximally for 60 min.

hydrolyzable phosphorus fraction represents the terminal high-energy phosphate group of nucleotide triphosphates. These fractions reflect, therefore, the metabolic machinery of the cell and the precursor phosphate involved in the synthesis of phospholipid. The specific activity values for the phosphoinositide complex were 10-15% of the TAS and, therefore, were considerably higher than such values for the phospholipid fraction.

Folch(10) has reported that a phosphatido-peptide fraction could be obtained by the extraction of a trypsin-resistant protein residue of beef brain with acidified chloroform-methanol. Recently, Dawson(7) has reported that "phosphatido-peptide"-like complexes could be formed by mixing calcium triphosphoinositides with protein. It has also been shown by Huggins(18,19) that a similar fraction can be obtained from kidney and secretory organs such as pancreas and submaxillary glands. More recently, Andrade and Huggins(1,2) have presented data which show that the phosphatido-peptide fraction contains inositol mono-, di- and triphosphate, indicating that this fraction should more properly be called a phosphoinositide complex.

The data presented above reveal consistently greater values for the specific activity of the phosphoinositide complex than for the phospholipid fraction under conditions of salivary secretion from electrically stimulated submaxillary glands. The phosphoinositide complex, which is composed of mono-, di-, and triphosphoinositides, contains only about one tenth as much phosphorus, and has a specific activity of 6-8 times that of the phospholipid fraction. Therefore, under these conditions, the inositides have a much greater turnover rate than do the serine, choline, and ethanolamine phosphatides, and, in this system, phosphatidic acid. The latter exhibited a remarkably low turnover. The largest stimulated rate of turnover occurred in monophosphoinositide.

The use of the muscarinic inhibitor (atropine) blocked salivary secretion as well as the increase in specific activity of the phospholipid fraction and the phosphoinositide complex during *chorda lingual* stimulation. Electrical stimulation of the sympathetic trunk produced similar but less dramatic effects both in salivary volume and phosphorus turnover in the various fractions. These effects were also blocked by the parenteral injection of dihydroergotamine (50 μg/kg). These data would rule out the possibility of vasodilation as being the reason for the increased secretion and metabolism of phosphoinositides in the supersensitive submaxillary gland of the cat(13).

In conclusion, the phosphoinositides exhibited a high rate of turnover in submaxillary glands which was augmented during electrical stimulation of the preganglionic sympathetic and parasympathetic innervations, *in vivo*. This stimulatory effect on phosphorus metabolism (occurring at the phosphodiester bridge) was obtained in addition to the already high metabolic activity of the monophosphate esters of the polyphosphoinositides. The possible relationships of these compounds, in submaxillary glands, to protein transport, as proposed by Hokin(14-17) for the superior cervical ganglion, will require further study.

Summary. After subcutaneous injection of radiophosphorus, secretion was induced by electrical stimulation of the preganglionic sympathetic and parasympathetic innervation to the submaxillary gland of the cat. Saliva was collected from the stimulated submaxillary gland and analyzed for Na^+, K^+ and hexosamine (mucin). Analysis for phosphorus and radioactivity on the different phosphorus-containing fractions have shown an augmentation in the specific activity of the phosphoinositide complex and phospholipid fractions from the electrically stimulated gland. No increase was apparent in the radioactivity of the total acid-soluble or 7-minute hydrolyzable fractions. By means of TLC chromatography, monophosphoinositide was found to be the fraction responsible for the augmented turnover. The data obtained to date indicate that there are two types of phosphorus atoms in the phosphoinositide complex: one type being a monophosphate ester which is responsible for the relatively high metabolic activity and the other being the phosphodiester between glycerol and inositol which appears to be involved in secretory phenomena. It is believed that the phosphoinositide in conjunction with a membrane protein is actively involved in transport

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Megakaryocytopoiesis in the Rat with Transfusion-Induced Thrombocytosis.* (31323)

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Tritiated thymidine (^{3}THdR) has been used to study maturation of megakaryocytes in rat bone marrow(1,2). These observations showed that the compartment of recognizable megakaryocytes could be morphologically divided into 3 stages of maturity. It was also clear from evaluation of grain counts that megakaryocytes were not self-maintaining, but were maintained by continuous influx from an unrecognized precursor compartment.

The level of circulating blood platelets is determined by a balance between rate of production from megakaryocytes and rate of destruction. The possibility that this balance is governed by a negative feed-back mechanism, affecting influx from a precursor compartment or the maturation of megakaryocytes was evaluated by observing labeling of megakaryocytes with ^{3}HTdR and differential counts of megakaryocytes in rats with transfusion-induced thrombocytosis.

Materials and methods. Female Sprague-Dawley rats weighing 148-182 g were used as test animals. Larger rats of the same strain served as blood donors for platelet transfusions. Platelet counts were done on cardiac blood, anticoagulated with K_2EDTA, by the method of Brecher and Cronkite(3).

Platelet transfusions were prepared by a modification of the method of Dillard *et al* (4). Cardiac blood was drawn into 0.05 volume of EDTA (1.5% Na_2EDTA and 0.7%

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