THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF PHOSPHORUS

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During the past year considerable new knowledge has been added to that previously available concerning the function of phosphate compounds in metabolism. The well-known relationship between oxidation and phosphorylation has been further consolidated and extended, and some completely new reactions involving phosphate transfer have been discovered, such as the cleavage of thio-ether linkages by adenosine-triphosphate, and the ability of the latter to phosphorylate pyridoxal.

Inasmuch as other chapters in this volume are devoted to nucleic acids and phospholipoids, only brief mention will be given in this chapter to studies dealing with these compounds. It is hardly necessary to add that European literature during the year is covered only incompletely due to the difficulty of obtaining European journals. For the same reason it has been necessary to include European publications which are one or two years old. My thanks are due to Professor J. Runnström, Wennergren Institute of Experimental Biology, Stockholm, Sweden, for valuable information regarding research in Sweden during the past few years.

DETERMINATION OF PHOSPHATE

A modification of the usual method for determination of phosphate has been described by Borei (1). Norberg (2) describes an ultramicro determination of total phosphorus using a photoelectric microscope described by Caspersson (3). Lowry & Bessey (4) have devised another ultramicro phosphorus determination using the Beckman spectrophotometer and narrow cells (1 or 2 mm. wide and 10 mm. deep).

The method for phosphoglycerol determination has been found to lack specificity (5). Haas (6) has described a spectrophotometric micro method for the determination of glucose-6-phosphate using dichlorophenolindophenol as hydrogen acceptor and the specific dehydrogenase as catalyst. The method is also useful for the study of dehydrogenase.

Phosphatases

Determination.—The King-Armstrong (7) method for the determination of phosphatase has been modified by Binkley, Shank & Hoagland (8). Phosphotyrosine is used as substrate and the liberated tyrosine is determined colorimetrically. By this method phosphatase can be determined in 1 ml. of plasma. Determination of phosphatase in very small quantities of blood has been made possible by the ingenious method of Lowry & Bessey (4) in which a nitrophenyl phosphate reagent is used. Three c.mm. of plasma at pH 10 are incubated with 50 c.mm. of the reagent, and after the addition of sodium hydroxide the intensity of the color (400 mμ.) produced by the liberation of the free sodium salt of nitrophenol is measured directly in a photoelectric colorimeter. The method is simple and direct, and particularly useful when large numbers of determinations are required.

Menten *et al.* (9) have modified the histochemical phosphatase test. They used a mono-aryl phosphate which is hydrolyzed by phosphatase. The liberated aryl group reacts with a diazotized amine, forming a highly colored insoluble dye. Barium-β-naphthol phosphate was used as the phosphoric ester and the diazotized α-naphthol amine as the coupling substance.

Histochemical techniques have been used by a number of investigators to study phosphatases in normal and pathological tissues. Wilmer (10) using a histochemical technique found that the renal tubules in the cortex of aglomerular kidneys do not contain alkaline phosphatase, whereas the connective tissue contains considerable amounts of the enzyme. This finding is of interest in connection with the mechanism of sugar reabsorption proposed by Lundsgaard (11) which involves a phosphorylation-dephosphorylation cycle. If this theory is correct, it is only logical that kidneys in which no filtration takes place, and consequently no reabsorption, are devoid of phosphatase. The occurrence of an acid phosphatase in these kidneys still remains a possibility. Wilmer (10) also found a decrease in phosphatase in the tubular cells when the tubular function was destroyed, as for instance in hydronephrosis or spontaneous interstitial nephritis. Wachstein (12) studied renal phosphatases in hemorrhagic kidney due to choline deficiency. He found a marked decrease in alkaline phosphatase in the damaged atrophic tubules and some increase in phosphatase in the vessels and in the glomeruli. Alkaline phosphatase was also found in ovarian follicles and corpora lutea (13). Bodian & Mellors (14) found a marked

increase in acid phosphatase in the region of the cytoplasm of nerve cells in which chromatolysis had been produced by axon section. Adrenalectomy caused a decrease in kidney and liver phosphatase (15). The phosphatase content returned to normal following injection of adrenal cortex extract. Inclusion bodies of vaccinia, herpes simplex, fowl pox, etc. were also investigated by histochemical techniques and found to contain no phosphatase (16).

Changes in serum phosphatase under various conditions have been studied by a large number of investigators. Drill *et al.* (17) found that sodium cyanide in concentrations of 0.0001 to 0.1 molar has only slight inhibitory effect on serum phosphatase activity of normal dogs. However, when the serum phosphatase values were increased as a result of liver damage, the addition of sodium cyanide inhibited the extra phosphatase activity markedly. Gould (18) found that fat feeding causes an increase in serum phosphatase.

Herbert (19) found that prostate phosphatase can be distinguished from other acid serum phosphatases by its great lability. Thus one hour incubation of serum at 37° at pH 7.4 inactivates the prostate phosphatases, whereas the other acid phosphatases are not affected. Addition of 0.4 vol. of ethanol to prostate phosphatase in buffers from pH 4.8 to 7.4 renders it completely inactive after half hour incubation at room temperature, whereas the other acid phosphatases remain unaltered.

Delory & King (20) have studied the kinetics of phosphatase action at different pH's and with different substrates. They found that the rate of hydrolysis of phosphate esters having low ionization constants is higher than that of esters having high constants. Moreover, the pH optimum is more alkaline for the former than for the latter. These observations are consistent with the hypothesis of Martland & Robison (21) that the enzyme is a weak base whose undissociated molecule combines with a substrate to form a compound which breaks down into the products of hydrolysis. Alkaline phosphatase is apparently only active if amino groups as well as phenol groups are intact (18). Its behavior towards ketene treatment is similar to that of the lactogenic hormone.

Phosphatase from rat sarcoma has a pH optimum of 5.6 in the absence of magnesium or manganese. In the presence of those two metals the pH optimum is around 4.8 (22).

The more specific phosphatases will be dealt with in another chapter.

FORMATION OF PHOSPHATE ANHYDRIDES

Mann (23) has conducted some interesting studies on the phosphorus metabolism of the mold *Aspergillus niger*. A specific metaphosphatase was isolated from extracts of the mold. Inorganic pyrophosphate and polymetaphosphates were isolated as ammonium and barium salts. The metaphosphates are readily converted into inorganic pyrophosphate during the isolation procedure. The metaphosphate could be separated from the inorganic pyrophosphate by chemical procedures as well as by enzymatic techniques. Thus pyrophosphatase splits only inorganic pyrophosphate, whereas the metaphosphatase from mold hydrolyzes metaphosphate as well as pyrophosphate. The metaphosphate isolated acted as a precipitating agent for proteins. Inorganic pyrophosphate has been isolated from yeast (24), and recently Cori & Ochoa (25) have isolated considerable amounts of inorganic pyrophosphate from liver extracts which oxidized dicarboxylic acids. It is not definitely established yet whether the pyrophosphate, the polymetaphosphate or perhaps an organic complex represent the compound originally present in the cell.

PHOSPHATE COMPOUNDS AND ENZYMES INVOLVED IN CARBOHYDRATE METABOLISM

Formation of phosphohexoses.—Phosphorylase has been studied by Sumner, Somers & Sisler (26). They found that the nature of the products synthesized from Cori ester by plant phosphorylase depends upon the kind and amount of polysaccharide added to prime the reaction. Thus a small quantity of achroodextrin will lead to the production of a substance giving a blue color with iodine. A larger quantity of achroodextrin will cause a product to be formed giving a red color with iodine, while addition of a very large amount of the dextrin will cause the production of a substance which gives no color at all with iodine. In each case the quantity of inorganic phosphate liberated is practically the same. The authors interpret their findings as follows: the enzyme adds anhydro-*d*-glucose molecules to whatever foundation it finds present. If a few dextrin molecules are present it forms a chain sufficiently long to give a blue color with iodine. If many dextrin molecules are present, phosphorylase forms many polysaccharide chains of intermediate length and the product resembles erythrodextrin. In this connection the experiments of Hidy & Day

(27) are of interest. They find that the priming effect of polysaccharides is markedly increased by partial hydrolysis. The staining reaction with iodine disappears completely about the time the polysaccharide begins to decline in activating power. All these observations are in agreement with the formulation proposed by Cori *et al.* (28) as a result of their experiments with crystalline muscle phosphorylase incubated with varying amounts of glycogen. Cori *et al.* formulated the polysaccharide formation catalyzed by phosphorylase as follows: glucose-1-phosphate plus terminal glucose units $\rightleftharpoons$ maltosidic chain units plus inorganic phosphate. The terminal glucose units are the end groups of the highly branched glycogen molecule which serves as the primer of the polysaccharide formation.

Sumner & Somers (29) have described a simplified method for the preparation of glucose-1-phosphate employing purified potato phosphorylase.

Doudoroff, Hassid & Barker (30) described the synthesis of two new sugars which appear to be analogs of sucrose. A phosphorylase prepared from *Pseudomonas saccharophila* catalyzes the reversible splitting of sucrose into Cori ester. If *l*-sorbose or *d*-ketoxylose is incubated with the enzyme in the presence of Cori ester, a reaction similar to that observed in the presence of fructose and Cori ester takes place, i.e., inorganic phosphate is liberated and the amount of sugar decreases. The synthetic compound appears to be the disaccharide glucoside-sorboside.

Shapiro & Wertheimer (31) investigated phosphorylase activity in various animal tissues. They found a highly active phosphorylase in subcutaneous tissue. Phosphorylase was not found in the muscle of rats of less than ten days of age. In fourteen-day-old rats the enzyme is already active. No decrease in the glycogen phosphorylase of muscle could be demonstrated in adrenalectomized or in thyroidectomized rats. The inhibitory effect of glucose on muscle phosphorylase was diminished by adenylic acid. No glucose inhibition was found with potato phosphorylase.

Transphosphorylation of hexoses.—The transphosphorylation of glucose to hexose monophosphate and of the latter to hexose diphosphate by adenosinetriphosphate has been given much attention during recent years. Youngburg (32) studied aerobic phosphorylation of sugars in kidney cortex extract. He found that whereas hexoses were readily phosphorylated, no phosphorylation of pentoses took place.

Klein (33), studying the metabolism of brain tissue, observed that the oxidation of fructose is accompanied by a phosphorylation. Huszak (34) found that the white and gray matter of the brain show a different carbohydrate metabolism. The white matter used preferentially glycogen as metabolite, whereas free glucose was not utilized. The main metabolite in the gray matter was glucose, which was phosphorylated by adenosinetriphosphate and subsequently oxidized. Phosphopyruvic acid is also able to phosphorylate glucose, but only through the adenylic acid system. Lindberg (35), working in Runnström's Institute, studied the carbohydrate metabolism of sea-urchin eggs during fertilization. He found that the dehydrogenases from ground sea-urchin eggs are strongly stimulated by the addition of hexose phosphates, phosphoribose, and phosphogluconic acid. Lindberg furthermore described a phosphoric ester, occurring in sea-urchin eggs, which has an activating effect upon the carbohydrate metabolism. This phosphoric ester was also found in and isolated from beef brain. The phosphoric ester was crystallized both as a brucine and an acridine salt, and the molecular weight was reported to be about 150. The substance, which is acid stable, behaves in many ways like glycerophosphate. In experimenting with egg pulp, it was found that the ester had a strongly enhancing effect on respiration. Furthermore, it caused a temporary accumulation of pentoses and of an unidentified acid. The amount of acid formed was of the same order of magnitude as that of the carbohydrates broken down. The author compared the phenomenon with that which occurs after fertilization. In both processes formation of acids takes place presumably by oxidative decarboxylation of hexose. Greville & Lehmann (36) studied phosphate and carbohydrate metabolism in extract of human muscle. They observed the well-known transphosphorylations and dephosphorylations.

The enzymes catalyzing the phosphorylation of hexoses to hexose mono- or diphosphates are of considerable interest for an understanding of the regulation of carbohydrate metabolism. Until recently the phosphorylation of glucose to hexose monophosphate (catalyzed by the enzyme hexokinase) had never been clearly demonstrated in muscle extracts. The phosphorylation of hexose monophosphate to diphosphate by adenosinetriphosphate was demonstrated in muscle extracts several years ago by Ostern (37) and his co-workers. Now, Colowick & Price (38) have succeeded in demonstrating hexokinase in extracts from rat muscle. They furthermore report that the transphosphorylation of glucose and hexose monophosphate requires the presence of

reduced cozymase, oxidized cozymase being without activating effect. The coenzyme of the transphosphatase seems to be destroyed rapidly by an enzyme in the muscle extract which has not yet been identified. However, it is known that animal tissues contain a specific nucleosidase which splits off the pyridine base in pyridine nucleotides (39). The finding that the reduced cozymase is the activator of these transphosphorylations may be of importance for the understanding of the so-called Pasteur effect, i.e., the suppression of fermentation by oxygen. In this connection some recent experiments of Engelhardt & Sakov (40) are of interest. They found that the addition of phenol, phenol oxidase, and the cytochrome system completely inactivates the transphosphorylation of hexose monophosphate. Kubowitz (41) has shown that the phenol oxidase system can reoxidize reduced cozymase.

The nature of the coenzyme of the hexose monophosphate fermentation (42) has not been further clarified.

Hotchkiss has continued his investigation of the effect of gramicidin on bacterial metabolism (43). He found that gramicidin increases the oxygen uptake of intact bacteria, provided glucose is the substrate, and that the uptake of phosphate is completely inhibited. In kidney extract, Hotchkiss (44) was able to show that the aerobic phosphorylation of glucose is also completely inhibited in the presence of small amounts of gramicidin (30 to 40 µg. per ml.).

Meyerhof & Beck (45) have purified the phosphotriose isomerase by ammonium sulfate fractionation and adsorption on cupric oxide. The preparation obtained was free of phosphohexose isomerase and of aldolase. The activity of the preparation was high but the stability low.

Coupling between oxidation-reduction and uptake or liberation of phosphate.—The enzymatic formation of 1,3-diphosphoglyceric acid (phosphoglycerylphosphate) from phosphotriose discovered by Warburg & Christian (46) and Negelein & Brömel (47) has been reviewed in previous volumes. Bücher, continuing these studies, has purified and crystallized the enzyme catalyzing the equilibrium between diphosphoglyceric acid and the adenylic acid system (48). The enzyme was precipitated as a nucleoprotein from acidified alcoholic solution and subsequently crystallized in alkaline ammonium sulfate (0.6 saturated) containing inorganic pyrophosphate. This enzyme is the most active fermentation enzyme thus far isolated. An amount as small as 0.01 mg. per ml. can be detected readily in the optical test at 334 mµ. (the test for reduced cozymase). The equilibrium catalyzed by the enzyme can be expressed as follows: 1,3-diphosphoglyceric acid plus

adenosinediphosphate $\rightleftharpoons$ 3-phosphoglycerate plus adenosinetriphosphate.

Lipmann has continued his studies of the formation and the properties of acetyl phosphate and has been able to throw light on many interesting problems concerning bacterial metabolism. Acetyl phosphate was synthesized by Lipmann & Tuttle (49) according to a greatly simplified method in which monosilver dihydrogen phosphate reacts with acetyl chloride yielding monoacetyl phosphate. Acetyl phosphate is readily hydrolyzed both in the acid and in the alkaline range. At pH 5 the compound showed maximum stability. The addition of substances which combine with phosphate greatly increases the hydrolysis of acetyl phosphate. Thus molybdate increases the hydrolysis of acetyl phosphate in acid solution, whereas calcium ions which precipitate phosphate at alkaline reaction correspondingly increase the hydrolysis of the compound in the alkaline range.

The formation of acetyl phosphate by oxidation of pyruvate in the presence of dry bacteria (*Bacillus acidificans longissimus*) has also been described by Lipmann (50). Acetyl phosphate was isolated as a silver salt and identified as disilver monoacetyl phosphate. In analogy with the findings of Bücher (48), it was found that acetyl phosphate is also able to transfer its phosphate group to the adenylic acid system.

Hitherto the enzymatic formation of acetyl phosphate had been demonstrated only in the special system just mentioned. However, during the last year acetyl phosphate formation has been observed in preparations from other bacteria. Koepsell, Johnson & Meek (51) have succeeded in demonstrating the formation of acetyl phosphate in the oxidation of pyruvate by a dry preparation of *Clostridium butylicum*. They found that in the absence of glucose, inorganic phosphate is taken up and appears as labile phosphate. After fractionation with silver they found that the purified labile phosphate fraction contained both acetic and butyric acid, this fact indicating the formation of both acetyl and butyryl phosphates. If butyric acid was incubated with acetyl phosphate in the presence of the enzyme extract, considerable amounts of butyric acid were found in the silver precipitate. This was interpreted as indicating the presence of butyryl phosphate since absorption of free butyric acid by the silver precipitate had been excluded. They suggested the following reaction: acetyl phosphate plus butyrate → acetate plus butyryl phosphate.

Acetyl phosphate has also been shown to play a role in the phosphoroclastic splitting of pyruvate into acetate and formate catalyzed

by an enzyme from *Escherichia coli*. Utter & Werkman (52) found that the splitting of pyruvate proceeds according to the equation: pyruvate plus phosphate $\rightleftharpoons$ acetyl phosphate plus formate. Utter, Werkman & Lipmann (53) have been able to show that this phosphoroclastic splitting is reversible. An enzyme preparation from *E. coli* was incubated with formic acid containing an excess of C_{13}, and pyruvic acid containing ordinary carbon. After one hour the C_{13} concentration in the formate had decreased considerably and was accounted for in the carboxylic group of the pyruvate. The carbon dioxide did not contain any excess C_{13}, indicating the absence of Woods' equilibrium enzyme (54). Although the equilibrium of the phosphoroclastic reaction is far toward the formation of acetyl phosphate, it has nevertheless been possible to demonstrate chemically the formation of small amounts of pyruvate by incubating acetyl phosphate and formate with the enzyme preparation (55). The equilibrium constant is roughly 10^{-2} for the reversed phosphoroclastic reaction.

Acetyl phosphate may also play a role in animal tissue. Lipmann (56) has most recently described an enzyme occurring in skeletal muscle which rapidly and specifically dephosphorylates acetyl phosphate. The stability of the enzyme toward acid as well as its high specificity are features not ordinarily found among phosphatases. Its presence in tissue might very well interfere seriously with any demonstration of acetyl phosphate formation in animal tissue. The occurrence of such an enzyme in animal tissue, on the other hand, may also suggest that this compound actually is an intermediate in the carbohydrate metabolism of higher animals.

The role of acetyl phosphate in the formation of acetylcholine is not known. However, it has been found that adenosinetriphosphate under anaerobic conditions greatly stimulates the formation of acetylcholine in brain extracts (57, 58).

Ochoa (59) studied α-ketoglutarate dehydrogenase from cell free suspensions of washed heart muscle. The α-ketoglutarate was oxidized only one step, i.e., to succinate and carbon dioxide, provided that the succinic dehydrogenase was inhibited by malonate. In the presence of glucose, three mols of phosphate were transferred to the sugar (forming hexose diphosphate) for each mol ketoglutarate oxidized to succinate and carbon dioxide. Synthetic succinyl phosphate did not give rise to any phosphorylation of sugar but was rapidly dephosphorylated. Inorganic phosphate, magnesium ions, and muscle adenylic acid, or adenylpyrophosphate, were required for the activity of α-ketoglutarate

dehydrogenase. Adenosinetriphosphate was five times as efficient an activator as adenylic acid. This difference was attributed to destruction of adenylic acid by deaminase action.

Long (60) studied the oxidation of α-ketobutyrate on minced pigeon brain. He likewise found inorganic phosphate to be an essential component in the oxidation of pyruvate as well as of α-ketobutyrate. Adenine nucleotides markedly increase the oxidation of pyruvate, provided inorganic phosphate is present. The oxygen-pyruvate ratio is 1:2 for the part of the pyruvate oxidation catalyzed by adenine nucleotides.

Leloir & Muñoz (61) have studied the oxidation of butyric acid in liver extract. They found that the oxidation of this fatty acid is stimulated by the presence of a number of dicarboxylic acids. All the active dicarboxylic acids when added alone to liver extract are readily oxidized and give rise to the formation of phospho-enol pyruvic acid. No phosphopyruvate is formed in the absence of adenylic acid, cytochome-c, or inorganic phosphate. Malonate inhibits phosphopyruvate formation from succinate, fumarate, or citrate. Phosphopyruvate can replace dicarboxylic acids in increasing the rate of butyrate oxidation, provided that carbon dioxide is present, indicating that phosphopyruvate may be carboxylated.

Lehninger (62) has studied fatty acid oxidation in homogenized liver preparations. He found that the oxidation of saturated fatty acids having four to eight carbon atoms by homogenized rat liver requires the presence of adenosinetri- or adenosinediphosphate. Adenylic acid is inactive. This finding is in agreement with the observations of Lang (63) and of Shapiro & Wertheimer (64) who demonstrated that palmitic acid dehydrogenase from liver extract requires the presence of adenylpyrophosphate.

Lardy, Hansen & Phillips (65) found a phosphate uptake in sperm cells which is coupled to oxidations other than those of carbohydrate metabolism. The ultilization of phospholipids was suggested as a possibility.

Enolase.—The work of Warburg & Christian (66) on crystalline enolase was reviewed last year (67). It will be recalled that the amount of magnesium present in purified enolase was analyzed and found to be one gram atom magnesium per 52,000 grams enolase. Bücher (68), working in Warburg's laboratory, has recently determined the molecular weight of crystalline enolase and found it to be 62,000, which means that one molecule of enolase when fully activated

by magnesium contains one atom of the metal. Bücher likewise found that the crystalline mercury salt of enolase contains one atom of mercury per molecule of enolase. The molecular weight of enolase was determined by a specially constructed apparatus applying the Tyndall effect as a measure of molecular size. Edestin, the molecular weight of which was determined by diffusion and sedimentation as well as by the Tyndall method, served as a standard. Bücher found that the molecular weight of the enolase decreased after dialysis at pH 5 and the activity disappeared; if salt were added the molecular weight increased to the original value and activity was fully restored.

NUCLEOTIDES AND NUCLEIC ACIDS

Analysis of nucleotides and nucleic acid in tissues.—Analyses of tissues like muscle, liver, and kidneys have been made both by ordinary chemical methods, by enzymatic methods, and by optical methods. Caspersson & Thorell (69) using the photoelectric quartz microscope studied the ultraviolet absorption at various wavelengths of muscle fibers from *Drosophila funebris* which possesses large segments, making it possible to investigate the isotropic and anisotropic sections separately. He found that the ratio between the absorption at 260 mμ. to that at 280 mμ. was much higher in the isotropic than in the anisotropic part, indicating that the adenine nucleotides (which according to Parnas (70) constitute more than 90 per cent of the muscle purines) may be confined exclusively to the isotropic part of the resting muscle. There is reason to believe, as pointed out by Bernal (71), that myosin is present in both parts. Hoagland, Lavin & Shank (72) have reached the same conclusions as Caspersson, using a direct technique by which one part of the muscle fiber is photographed in polarized light and the rest of the fiber is simultaneously photographed in ultraviolet light (73). With this method, it was shown in human muscle fibers that the dark isotropic sections continue into the ultraviolet field as dark absorbing bands. It is hardly necessary to add that without the knowledge gathered by chemical analysis of muscle tissue, we would not be in a position to interpret the optical analyses.

A number of investigators have studied acid labile phosphorus in various tissues under various conditions. Wagtendonk (74) found a particularly marked lowering of the labile phosphate in liver and kidney of guinea pigs on a diet deficient in the so-called "antistiffness" factor. Whether the decrease in labile phosphate in this case is spe-

cific or nonspecific, it is difficult to decide. It is known that starvation of animals gives rise to the same phenomenon (75, 76). However, Wagtendonk's observations are of interest because the changes are much more marked than those observed in starved animals. There is reason to believe that the decrease in the labile phosphate is mainly due to a decrease of adenylpyrophosphate since the "adenylic acid fraction" was correspondingly increased. However, it would be of interest to know whether it is adenylic acid or inosinic acid which constitutes the main component of the so-called "adenylic acid fraction."

Rapoport *et al.* (77) found that red cells obtained during phenylhydrazine reticulocytosis showed a substantial increase in the concentration of adenylpyrophosphate as related to the hemoglobin.

Kabat (78) found an increase of acid labile phosphate in the brain of animals infected with poliomyelitis. Whether this labile phosphate can be identified with adenylpyrophosphate remains to be seen.

More precise information about the content of nucleotides in small tissue samples can be obtained by using enzymatic methods, provided the enzymes employed are sufficiently purified. Thus Schmidt & Engel (79) in 1933 initiated new methods for purine analyses in tissue samples by using purified deaminases and measuring the ammonia liberated from various purines. It has recently been possible to measure minute amounts of purine derivatives using Schmidt's enzymatic technique combined with ultraviolet spectroscopy. Thus a highly sensitive and specific method for the determination of muscle adenylic acid was developed by observing the change in the ultraviolet spectrum (the decrease in absorption at 265 mμ) which takes place when the nucleotide is deaminated by adenylic acid deaminase (80). If the deaminase has been freed from impurities of myokinase, no decrease of absorption takes place when adenosinetri- or diphosphate is added. However, if a few micrograms of a specific adenylpyrophosphate from potato are added to the system, adenylic acid is formed and subsequently deaminated, thus causing a fall in the absorption. By this method less than 0.5 mg. of muscle tissue can be analyzed for adenylic acid and adenylpyrophosphate (81). A similar sensitive method for hypoxanthine compounds has been developed (81) using the rise in absorption at 290 mμ which takes place when hypoxanthine is oxidized to uric acid by xanthine oxidase. Inosine requires the presence of nucleosidase, and inosinic acid requires both nucleosidase and phosphatase in addition to xanthine oxidase before any rise in absorption will take place. This combination of optical and enzymatic methods

might be a valuable tool for studying changes in the composition of nucleotides or nucleic acids, as well as their enzymes in animal tissue under pathological conditions.

Anfinson (82) studied the distribution of diphosphopyridine nucleotide (DPN) in retina using the Cartesian diver technique; triosephosphate dehydrogenase was used as catalyst. The higher concentrations of DPN (4 μg. per mg. fat free solid) were found in the two synaptic regions. The rods and the outer nuclear layer contained less and the nerve fibers were very low in DPN.

Davidson & Waymouth (83) studied the content of nucleotides and nucleic acids in various tissues by means of ordinary chemical methods. The concentration of nucleotides seems to be lower in tumor tissue than in the corresponding normal tissues.

Enzymatic reactions involving adenosinetriphosphate.—Adenosinetriphosphate can participate in reversible transphosphorylations and in irreversible transphosphorylations, and it can undergo simple hydrolysis. The first type of reaction includes the phosphorylation of amidines (creatine, arginine) and of carboxylic groups (phosphoglyceric acid, acetic acid), and the phosphorylation of adenosinediphosphate (phosphate dismutation). The irreversible transphosphorylations include the phosphorylation of hydroxy groups such as the 1- or 6-hydroxy groups of hexoses and that of pyridoxal. The phosphorylation of hexoses has already been discussed. The phosphorylation of pyridoxal is a very recent observation (84) and of great interest because the phosphorylated product is active as a coenzyme of the enzyme which brings about decarboxylation of tyrosine. A completely new type of transphosphorylation was discovered by Binkley (85), who found that the terminal group of adenosinetriphosphates splits the thio-ether linkage of cystathionine with the formation of cysteine and phosphohomoserine. The reaction is a strictly stoichiometric one, with one mol of phosphate being transferred per mol of cysteine liberated.

Adenylpyrophosphatases.—A highly active adenylpyrophosphatase was isolated from potatoes (86). The enzyme splits both labile phosphate groups from adenosinetriphosphate. It is activated by calcium ions. Inosinetriphosphate is hydrolyzed to inosinic acid. The rate is somewhat slower than for adenylpyrophosphate. The enzyme is readily adsorbed on myosin, a phenomenon of interest in the discussion of the possible identity of myosin with muscle adenosinetriphosphatase (ATP-tase) (87). Myosin might be identical with ATP-tase

but the possibility that the muscle ATP-tase is adsorbed on myosin has certainly not been excluded. The muscle deaminase has also been found in myosin even after three reprecipitations (88).

A large number of investigators have continued the study of the myosin adenylpyrophosphatases. Ziff & Moore (89) studied myosine ATP-tase by means of electrophoresis and ultracentrifugation. Myosin was found to consist electrophoretically of one component to which 90 per cent of the triphosphatase activity is associated.

The effect of oxidation and reduction on myosin ATP-tase has been studied extensively. Singer & Barron (90) found that mercaptide-forming compounds as well as mild oxidizing agents inhibit ATP-tase. This inhibition was attributed to an oxidation of sulfhydryl groups since the inhibited enzyme could be reactivated by adding reduced glutathione. They found a close parallelism between the number of sulfhydryl groups attacked by mercuric *p*-chlorobenzoate and the degree of inhibition of enzyme activity, and interpret these findings as evidence in favor of Engelhardt's hypothesis that muscle ATP-tase is identical with myosin. Ziff (91) found that stored myosin loses its ATP-tase activity but can be partly reactivated by cystine or glutathione, which also reactivates oxidized myosin. Mehl (92) observed a greater decrease in ATP-tase activity in stored rat muscle myosin when the activity was estimated at a pH of 9 than when determined at pH 6 or 7. He likewise found that oxidation and reduction have a much greater effect on the activity measured in the alkaline range than when measured in the acid range. Binkley, Ward & Hoagland (93) studied myosin from persons afflicted with hereditary muscle dystrophy and found that the preparation contains an active ATP-tase. It was found that traces of copper completely inhibited the enzyme activity. The effect of copper was nullified by the addition of cyanide. Cyanide also increased the activity of fresh myosin preparations as well as preparations inactivated by oxidation with hydrogen peroxide. They devised a method of purification in which the myosin was precipitated with copper and redissolved in cyanide buffer. In this way they succeeded in obtaining ATP-tase preparations of more constant activity.

As might be expected, myokinase added to myosin ATP-tase results in the dephosphorylation of adenosinediphosphate (ADP) (86). This is merely due to the enzymatic conversion of ADP into adenylic acid and ATP, which is then subsequently hydrolyzed by ATP-tase.

Dainty *et al.* (94) studied the particle shape of myosin by means

of anomalous viscosity and flow birefringence. When a myosin solution was incubated with a small amount of ATP its birefringence was decreased about half, and the relative viscosity was slightly decreased. The full effect of ATP was obtained at a 0.004 molar concentration. Although other substances can cause a decrease in flow birefringence, to do so they must be present at a much higher concentration than this. The changes of physical and chemical properties of myosin brought about by ATP-tase are spontaneous and reversible, and seem to be connected with the enzymatic action of the protein as an ATP-tase. Effects similar to those of ATP have been obtained so far only with inosinetriphosphate, whereas inorganic triphosphate, although hydrolyzed by myosin phosphatase, has no effects on the physicochemical properties of myosin.

Important contributions to our knowledge in this field have also been made from the Institute of Medical Chemistry in Szged by Szent-Györgyi and his group (95). They observed a marked difference in the physico-chemical as well as the enzymatic properties of myosin, depending on the method of extraction. The myosin obtained by extracting skeletal muscle ten minutes with potassium chloride shows a low viscosity; this preparation is called myosin "A" by the Hungarian group. If the muscle, on the other hand, is extracted for several hours with alkaline potassium chloride, a myosin preparation is obtained which is highly viscous and which readily forms fibers when injected into distilled water. The second type of myosin is called myosin "B" (96). If a myosin B fiber is placed in a freshly prepared water extract of muscle it contracts and becomes opaque. A myosin A fiber shows no change under such circumstances. Three components are necessary for the effect on the myosin B fiber: potassium, magnesium, and adenosinetriphosphate (ATP). If a contracted myosin B fiber is subsequently suspended in 0.2 M potassium chloride (containing magnesium ions) and ATP is added, the fiber relaxes. However, if the relaxed fiber is suspended in 0.1 M potassium chloride (containing magnesium ions) addition of ATP now produces contraction. Thus ATP addition can give rise to either contraction or relaxation, depending upon the potassium chloride concentration. In order to bring a contracted fiber into a state of relaxation in the absence of ATP, potassium chloride concentrations as high as 0.6 M are required, and it is necessary to adjust to a quite alkaline pH range.

Straub (97) has isolated a protein called "actin" from muscle which is soluble in alkalies. An actin solution remains liquid in the absence

of salts. Upon addition of salt, the viscosity as well as the birefringence is greatly increased. Actin is able to combine with myosin, forming more or less viscous complexes, depending on the viscosity of the original "actin." For a given actin preparation the maximal viscosity is reached by mixing one part actin to three parts of myosin, a ratio which according to the authors is very nearly the same as that found in skeletal muscle. If ATP is added to a viscous solution of an actin-myosin complex it causes a marked decrease in viscosity, approaching the viscosity found for myosin A. Szent-Györgyi concludes from this observation that ATP separates myosin B into actin and myosin A. After ATP has been hydrolyzed by phosphatase action, the viscosity is found to increase again, indicating that after the disappearance of ATP, actin and myosin A are again able to form a complex (myosin B). One mol ATP is able to effect a decrease in viscosity of 100,000 grams of myosin, which indicates that if the molecular weight of myosin is around 100,000, one mol of ATP reacts with one mol myosin (98). Szent-Györgyi emphasizes that the ATP reacts with the myosin component and not with the actin component. This is further indicated by the fact that ATP is also able to decrease the viscosity of free myosin (myosin A) in salt solution.

Szent-Györgyi interprets the difference in myosin obtained by various methods of extraction as follows. Extraction of fresh muscle, containing a large amount of ATP, with saline yields myosin A, leaving the actin in the insoluble residue. If, on the other hand, the muscle is extracted with alkaline potassium chloride overnight, the ATP is hydrolyzed, and a myosin-actin complex, myosin B, is obtained. Suspensions of myosin A or B in potassium chloride solutions of a strength between 0.1 and 0.2 M are flocculated by the addition of ATP. Suspensions of myosin in potassium chloride solutions stronger than 0.2 M go into solution upon addition of ATP, an effect which is otherwise obtained only by raising the potassium chloride concentration to 0.6 M and making the reaction alkaline. These observations are similar to those just described for the myosin fiber.

The effect of ATP on isolated muscle fibers has also been studied (99). It was found that minute amounts of ATP applied directly to the isolated muscle fiber cause a rapid contraction. Intra-arterial injection of ATP likewise gives rise to muscle contractions accompanied by electrical activity (100). The curarized muscle is just as sensitive to ATP as the non-curarized. ADP has the same effect as ATP, whereas adenylic acid, though just as effective in the non-curarized

muscle, is much less effective in the curarized. Inorganic phosphate is inactive, whereas inorganic triphosphate and pyrophosphate release contractions. In smooth muscle, only ATP is active.

Pharmacological effect.—Green & Stoner (101) report that the toxic effect of ATP on rats is potentiated by magnesium salts. The studies of Bollmann & Flock (102) are of interest in connection with the problem whether ATP plays a major role in tourniquet shock and traumatic shock. They found that ATP is almost completely hydrolyzed in muscles deprived of their blood supply. If the occlusion lasts more than three hours, little if any rephosphorylation takes place after the blood supply has been re-established. The decomposition products are relatively nontoxic and, presumably, consist mainly of inosinic acid. Tourniquet shock is, therefore, not caused by release of adenylic acid derivatives into the blood stream. Moreover, it is unlikely that adenylic acid compounds play a major role in traumatic shock, even though it has been found by means of the spectroscopic deaminase method just described (80) that the adenosine derivative concentration of the blood coming from a traumatized extremity is increased (103). That this influx of adenosine compounds is insufficient to exert a depressor effect is shown by the failure of injections of adenosine deaminase plus phosphatase to effect the low blood pressure accompanying traumatic shock (103). And yet, this enzyme combination exerts a marked antagonistic effect on the fall in arterial blood pressure caused by infusion of ATP. Release of adenylic acid or its derivatives into the blood stream seems, therefore, to be at the most a secondary factor in the traumatic shock.

Prosthetic groups.—Phosphorylated compounds have been identified as prosthetic groups in two important enzymes. Ratner et al. (104) found that the flavine adenine dinucleotide is the prosthetic group of glycine oxidase. Gunsalus et al. (84) found that phosphorylated pyridoxal can activate the decarboxylation of tyrosine. They were able to obtain an active coenzyme, both by chemical phosphorylation as well as by enzymatic phosphorylation, using ATP as a phosphate donor.

The experiments of Westenbrink & Veldman (105) indicate that phosphothiamine synthesized in the yeast cell is not all bound to carboxylase, although it is present in a form in which it is attacked much more slowly by yeast phosphatase than is free phosphothiamine. Thus the phosphothiamine content of yeast may be increased 1700 per cent without increase in carboxylase activity. One must, however, bear in

mind that phosphothiamine seems to be the prosthetic group of a number of dehydrogenases as well as carboxylases.

Nucleic acids.—Davidson & Waymouth (106) studied a factor in pancreatin which increased nucleoprotein phosphorus of fibroblasts *in vitro*. The active material seems to be a mixture of polypeptide and nucleotide derivatives. Gulland *et al.* (107) reviewed critically previous claims concerning the structure of nucleic acids in the dividing cell. Claude (108) found nucleic acid associated with the formed elements of the cell. Woodward (109) observed ribonuclease in the plague bacillus. Bain & Rusch (110) described a manometric determination of ribonuclease.

Desoxyribonucleic acid.—Avery, MacLeod & McCarty (111) isolated in a highly purified form a factor from type III pneumococci which is able to transform the unencapsulated R variant of Pneumococcus type II into the fully encapsulated cells of type III. The active factor was shown to be a highly polymerized specific desoxyribonucleic acid which is destroyed by phosphatase and by minute amounts of purified desoxyribonuclease. The wide significance of this work will be discussed elsewhere.

Phospholipids and Their Constituents

Baer & McArthur (112) have synthesized phosphorylcholine by phosphorylation of choline halide with diphenylphosphoryl chloride in pyridine, with subsequent isolation of the diphenylphosphorylcholine as the chloroaurate. The latter compound is decomposed with metallic silver, yielding the free diphenylphosphorylcholine which in turn may be readily catalytically hydrogenated to the free phosphorylcholine.

Riley (113) studied the metabolism of phosphorylcholine. The betaine is readily dephosphorylated *in vivo* and the inorganic phosphate is excreted in the urine. Phosphorylcholine exerts an inhibition of the turnover of phospholipid in the liver. The inhibition appears to be limited to the noncholine phosphatide fraction. Phosphorylcholine, as a unit, is probably not utilized in the synthesis of phospholipids. Wagner-Jauregg & Lennartz (114) have synthesized dicholesterol pyrophosphate.

Elliott & Lebet (115) have studied oxidation of phosphatides in brain extract. Ascorbic acid and iron salts or iron protein complexes greatly stimulate the oxygen intake.

Studies of the Metabolism of Phosphate Compounds in Vivo

Studies of carbohydrate metabolism in vivo by means of radioactive phosphate.—A number of publications dealing with the study of carbohydrate metabolism and with the rejuvenation *in vivo* of phosphate compounds such as adenosine polyphosphates and phosphocreatine have appeared this year. It will be recalled that Sacks in 1940 (116) expressed the belief that, contrary to what had been found *in vitro*, the formation of lactic acid in the working muscle *in vivo* is independent of phosphorylations. The main evidence brought forward against the occurrence of a phosphate cycle in the working muscle was the finding that phosphocreatine and pyrophosphate from the muscle of animals injected with radioactive phosphate contained the same concentration of P_{32} whether the muscle was working or resting, that is, whether much or little lactic acid was being produced. The isotope concentrations of the two fractions were much lower than that found in the inorganic phosphate. Bollmann & Flock (117) obtained essentially the same results and drew the same conclusions. It was, however, soon realized that the conditions under which these experiments were conducted did not permit valid conclusions about the rate of phosphate turnover in the phosphate compounds of the working muscle. The main difficulty encountered in these studies has always been, and still is, to obtain reliable figures for the isotopic concentration of the inorganic phosphate in the muscle fiber. The reason for this difficulty must first of all be attributed to the very slow penetration of phosphate into the muscle fiber (Hevesy, 118). As a consequence of this, the isotopic concentration of the extracellular inorganic phosphate is manifoldly higher than that of the inorganic phosphate inside the muscle fiber. The very low isotope concentration of pyrophosphate and phosphocreatine as compared to inorganic phosphate, observed by Sacks, and Bollmann & Flock must, therefore, be ascribed to a contamination of the cellular inorganic phosphate with the highly radioactive extracellular phosphate. In a later paper Sacks & Altschuler (119) have taken the extracellular phosphate into account but, nevertheless, still maintain that there is an essential difference in metabolism between the resting and the working muscle with respect to the phosphate cycle.

It seems justifiable to raise the question whether there actually is any conflict between *in vitro* and *in vivo* studies in this special case.

So far, we do not know of any experimental data from *in vivo* studies of phosphate metabolism which cannot be accounted for by the so-called Embden-Meyerhof scheme.

One way of studying the rate of rejuvenation of phosphate compounds in muscle is to remove the extracellular phosphate by perfusing the muscle with saline. If, under these circumstances, the P_{32} concentrations of phosphocreatine and adenylpyrophosphate are compared with that of the true intracellular inorganic phosphate, one finds a very rapid rejuvenation of the phosphate compounds of the muscle (120). Thus only twenty minutes after an intravenous injection of P_{32} the phosphocreatine phosphorus has an isotope concentration about 60 per cent that of the inorganic phosphate; the same results are obtained for adenylpyrophosphate. In other words, the phosphorus turnover of this labile phosphate compound is already so high in resting muscle that with present techniques it would be quite difficult to discover any substantial increase in the turnover during or after muscular work. This technical failure does not, however, justify us in claiming that a further increase of phosphorus turnover does not actually take place during muscular contraction. The rate of rejuvenation of adenylpyrophosphate phosphorus and of phosphocreatine phosphorus in resting rabbit muscle amounts to 20 to 30 µg. phosphorus per minute per g. of muscle, and there is every reason to expect that the rate will be manifoldly higher in working muscle. In the liver the rate of rejuvenation of adenylpyrophosphate phosphorus is about the same order of magnitude as that of resting muscle.

The rate of rejuvenation of the two labile phosphates in adenosine-triphosphate has been studied using hexokinase as an instrument to differentiate between the terminal and the second phosphate group (121). In the resting rabbit muscle the P_{32} concentration was always found to be the same in both of the labile phosphate groups even when investigated shortly after the injection of radioactive phosphate. However, Flock & Bollmann (122), using myosin ATP-tase as a tool to differentiate between the two labile groups of ATP, have found a distinctly higher P_{32} concentration in the terminal phosphate group as compared with that of the second group.

In the studies of Kalckar *et al.* (120) the relative isotope concentration of hexosemonophosphate phosphorus in resting muscle varied greatly but was usually found to be considerably lower than that of the pyrophosphate. However, in certain cases the isotope concentration of this ester (which was purified as the calcium salt and "washed" with inert inorganic phosphate) was found to be as much as three times that of the pyrophosphate and more than twice that of the inorganic phosphate (120). The same observation has been made by Sacks (119), who has been able to throw further light on this finding (123, 124). The interpretation of Sacks' figures are, however, somewhat difficult because no values for the P_{32} concentrations of the inorganic phosphate were presented. Undoubtedly, the most important observation is that the high P_{32} concentrations of the "glucose monophosphate" phosphorus are never found in fed animals (in the post-absorptive phase) but only in fasted animals. The results might be interpreted as indicating that, before entering the cell, some form of hexose combines with extracellular phosphate, which is, of course, very rich in isotopic phosphate. The possibility suggested by Hotchkiss (43) that phosphate from the environment reacts with a polysaccharide complex in the cell wall of microorganisms forming a hexosephosphate might very well be considered in the case of muscle. Sacks discusses a similar hypothesis. The observations made by Myrbäck & Vasseur (125) that certain enzymes in the yeast cell seem to be located inside and certain other enzymes outside a barrier may also be of interest in this connection.

Kaplan & Greenberg (126, 127) have published a number of papers dealing with the determination of phosphoric esters in liver by barium and mercury fractionation, and by hydrolysis curves. The justification for identifying the "seven minutes acid-hydrolyzable phosphorus" in the fraction of water-insoluble barium salt with the labile phosphorus of pyrophosphate is open to criticism. The identification of these two fractions is justified when applied to acid filtrates from skeletal muscle, but the picture in the liver is much more complex. Inorganic pyrophosphate, for instance, has been found in liver (25), and other yet unknown acid labile phosphoric esters having insoluble barium salts may belong to this fraction. The authors report that insulin (in the presence of glucose) increases the amount, as well as the P_{32} content, of the labile groups of ATP. The changes observed are small and may be secondary or nonspecific. It is known that reduction of food intake causes a decrease of the acid-labile phosphorus in the liver (75), an observation actually confirmed by Kaplan & Greenberg (76). It is, therefore, conceivable that the effect of insulin may be the result of its known effect in increasing the food intake.

Rejuvenation of phosphorus in nucleic acids.—Brues *et al.* (128) investigated the turnover of phosphorus in the desoxyribonucleic acid

(nuclear nucleic acid) from liver and found that the rate of rejuvenation in non-growing liver is very slow (10 to 11 per cent rejuvenation after three days). As shown by Marshak (129) the rate of rejuvenation of ribonucleic acid (cytoplasmic nucleic acid) phosphorus is very rapid. Euler & Hevesy (130, 131, 132) studied the rate of rejuvenation of nucleic acid phosphorus in tumors. The radioactivity per mg. nucleic acid phosphorus was compared with that of the free inorganic phosphate. In two hours 2 to 3 per cent of the total nucleic acid phosphorus of a Jensen sarcoma had been rejuvenated. The phosphorus of desoxyribonucleic acid of growing sarcoma was also turned over at a considerable rate (1.5 per cent per hour). Irradiation with 1,000 International Roentgen units decreased the rate of rejuvenation of nucleic acid phosphorus to half or one-third of that of the untreated sarcoma. This effect of x-rays appears before the fall in the number of mitoses occurs.

Phosphate turnover of phospholipids.—Two independent groups have confirmed and extended previous reports (133) that choline deficiency causes a decrease in the synthesis of phospholipids in the liver and kidney of young rats in which damage of the two organs has been produced by the dietary regime. Patterson *et al.* (134) found the rate of rejuvenation of phospholipid phosphorus decreases in choline deficient rats. Boxer & Stetten (135) found correspondingly that the daily replacement of choline in phospholipids, which in normal rats amounts to 3.9 mg., is decreased to one-third in choline deficient rats. The effect consists in a retardation of the incorporation of new choline into the phosphatide without altering the quantity of choline present in the phosphatides.

The reader is referred to the chapter on lipid metabolism for further information about this interesting topic.

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