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A LONG LIFE IN TIMES OF GREAT UPHEAVAL

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CHILDHOOD, SCHOOL YEARS, AND STUDY OF MEDICINE

I was born in the middle of the last year of the last century, in the city of Königsberg, the capital of East Prussia. It is now Russian and its name is Kaliningrad. I lived through the two world wars and was lucky to leave Germany in 1931 to live in Denmark, where I worked for seven years. In

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1939, shortly before the outbreak of the second world war, I got the opportunity to come and settle in the United States when I was already 40 years old. My work was not unknown, but also not well known.

Königsberg, when I was born, was the largest city near the Russian border, and a minor harbor in the row of Hanseatic cities; it is connected by a sound to the Baltic Sea. It was only a short train ride to the seashore, where we spent all our summer vacations. As Königsberg was a large border town, the German government saw to it that its university and cultural life were of high quality. The university had a particularly good medical faculty. The head of medicine, Professor Mathes, was quite outstanding; he had written a fine book on differential diagnosis, which was translated into English. Later, while studying in Berlin and Munich for some semesters, I listened to great surgeons and psychiatrists, and (in Munich) to Friedrich von Müller, who pioneered in connecting medicine to biochemistry. This happened, however, after the end of the first world war, and I would like to fill in here on my early life and school years to the beginning of my study of medicine.

I can still remember the peaceful years in the beginning of the 20th century, when carriages were horsedrawn, including the taxis, called *droschkes*, a word borrowed from the Russian. When I was four or five, during visits of the Kaiser, the nurse took me to town, where I was fascinated seeing him driven through the streets in a four horse–drawn carriage by a coachman with a plumed helmet. However, during my teens the *droschke* and the Kaiser's coach turned into automobiles. By then I had started to go to a classical *gymnasium* where for ten years I learned Latin; however, it was only toward the end that I started to appreciate it for its precision and beauty. For six years we were also coached in Greek, which, I am somewhat ashamed to say, I liked much less than the Latin. I was never very good in school, nor later at the university—just average. After finishing the *gymnasium* I chose, for various reasons, medicine. The youngest brother of my mother was a very lovable man, and a well-liked pediatrician. He died young from a burst appendix long before the age of antibiotics. He had been one of my heros. Many of my father's friends were prominent physicians. My father, whom I was very fond of, was a lawyer. He once confessed to me that he was not enough of a crook to be an outstanding lawyer. Owing to his charming personality, nevertheless, he did quite well. I am grateful for his support through my student years and the early period after finishing medical school when, in Germany, one could not expect to be paid.

Before leaving my early history, I have to introduce two who had great influence on me during my formative years. One was my brother, Heinz, who was two years older than I. We two were the only children. We were rather different; he was blond, popular, and outgoing. I was dark and

somewhat clammed up. He was early interested in the theater, and he composed poems. I rather played with toy trains and building blocks.

After graduation from the *gymnasium* he went to Munich to study literature; later I joined him there in 1919 to spend a semester studying medicine. In Germany, one was permitted to change universities. Munich was at that time artistically an important center, close in rank to Berlin. During my semester in Munich, I lived in its version of Greenwich Village, called the Schwabing. I was admitted to my brother's circle of artist friends. The memory of this time has remained with me.

My other friend, whom I met when still in school, was Siegfried (Friedel) Sebba, a gifted painter who later became much interested in the theater. He and I remained in close contact all our lives; he died only a few years ago. I shall tell more about these two later.

Soon after I had started studying medicine in 1917, in the last year of World War I, about May 1918, I was called up to serve in the army. As a medical student, I was lucky enough to join the medical service; it became my first adventure far away from home. After a brief indoctrination with about 20 medics, I went on a long train trip, not knowing whereto. We ended up in Sedan, a nice, small French town on the Marne, far from the front. We were distributed among the hospitals there and had a rather easy life. Only at the very end did I get a brief taste of war. I had to serve in an improvised field lazarett in a church not far from the fighting. I could hear cannon fire. They were short of help there, and I had the duty to supervise about 40 seriously wounded men. I had to learn to exert authority. It was not an easy job, but a grim experience, with freshly wounded men badly taken care of.

Returning to Königsberg after the war's end, I met there the murderous influenza epidemic that, I understand, killed a similar number of people as had been lost in the war. I was not yet dismissed then from the army, because there were hotels requisitioned in my hometown for taking care of soldiers with this disease. I was assigned to one of them. I witnessed many people dying of the dreaded pneumonia, not only the soldier patients, but also the personnel—the head nurse and one of our doctors. In retrospect, it is a miracle that I did not get it, being for months constantly in contact with the patients.

Finally, in March 1919, I got out of the army and could go back to medicine. Medical study in Germany was then divided into two distinct phases: two years preclinical work with a lot of anatomy, introductory physics, chemistry, zoology, and botany, followed by examinations in these disciplines, and two years of clinical work. Since I had finished *gymnasium* early (mid-1917) I already had one year of preclinical before induction and, by returning to medicine in March, I could get my first part finished in the

summer of 1919, with some shortcuts allowed for service. It was then that I had my glorious experience outside and in medicine for a semester in Munich, and the next half-year in Berlin. I had, eventually, to return for finals to Königsberg and finished medicine in 1920. The required practical year, half of which I spent in the municipal hospital of Königsberg, was most interesting; when not serving on the wards I reviewed the stream of cases of alcoholics, accidents, and derelicts flowing through the emergency and out-patient departments. While studying medicine, I was quite absorbed with learning what is going on inside the human body. But I had a glimpse into chemistry through our teacher, Professor Klinger, who presented us, in his preclinical lectures, with a dramatized view of the field.

Turn From Medicine to Biochemistry

Toward the end of my studies in medicine, doubts appeared in my mind if I really wanted to become a practicing physician. I was uneasy with the prospect of charging people money for trying to make them healthy. Yet, I spent three months in the pathology department of a hospital in Berlin-Friedrickshain with a rather well known pathologist, Ludwig Pick. Then, during the last part of the practical year, I took a very popular three-month course in modern biochemistry in Berlin, taught by Peter Rona, a collaborator of the famous Leonor Michaelis. After the course, I continued working with Rona on my first published paper (1), which was on an aspect of the then very popular colloid chemistry. This became my required M.D. thesis.

A fortunate opportunity then came along to work on a stipend for a half-year in pharmacology at the University of Amsterdam. I was happy to escape from the inflation that was then rampant in Germany. In Amsterdam, I became acquainted with biochemical problems and with being in a biological laboratory. This decided me to turn to biochemistry; I felt, however, that I needed more chemistry. Meanwhile, Hans Meerwein, an excellent organic chemist, had been appointed the new head of the chemistry department in the university at Königsberg. This made me decide to return home to live cheaply with my parents and study chemistry with Meerwein. This I did for three years. Most instructive were Meerwein's lectures, which every member of the department and all students had to attend. I spent a good deal of time in the practice of inorganic chemistry. It was very instructive to realize the individualities of the important atoms by learning to analyze the mixtures of their salts. I also learned the basics of organic chemistry, analysis as well as some synthesis. Meerwein's lectures were exciting and the repetition and progress during the three-year period gave me the feeling for chemistry, which I thought I needed for going into biochemistry.

Apprenticeship in Meyerhof's Laboratory

After the three years, I passed the *Staatsexamen*, and now had to find for my chemical thesis a good biochemical laboratory where work went on that seemed to deal with the moving edge of biochemical understanding in intermediary metabolism. I chose Otto Meyerhof's laboratory at one of the Kaiser Wilhelm Institutes (now Max-Planck Institutes) in Berlin-Dahlem. When I entered it, it was only about 25 years since the cell-free fermentation of sugar to alcohol had been discovered in yeast by the Buchner brothers, as described by Kohler (2), and Harden (3) had started work on its mechanism by finding a fixation of phosphate in intermediaries on the way to yield two moles of ethanol and two of CO_2 from one mole of glucose. Meyerhof and Karl Lohmann studied the glycolysis of glucose to form two moles of lactic acid in frog muscle extracts. Except for the last steps, it was quite analogous to alcoholic fermentation. Here, pyruvic acid is not decarboxylated as in yeast to CO_2 and acetaldehyde, which then is reduced to ethanol by NADH, Harden's coenzyme. Instead, in muscle extract, the pyruvic acid is directly reduced by NADH to lactic acid, which is here the end product. Work by Hill and Meyerhof on heat production in an anaerobically working muscle indicated a rather exact equivalence between lactic acid formation and contraction. This connection of intermediary metabolism with energy production for the first time began to be understood in mechanistic terms. It was this kind of approach that made me choose Meyerhof's laboratory to get my introduction into intermediary metabolism.

My early work there, however, was not too successful. When I asked him, in 1927, if I could work in his laboratory, he agreed to take me; probably he liked that I had spent some time in chemistry. Fortunately, I could use the work I did there for my chemical thesis. The first two tasks he gave me were interesting shots in the dark that did not yield a much wanted understanding of the role of a new compound, creatine phosphate (CrP), which had been found in muscle. It was chemically identified as CrP by Fiske & Subbarow (4), and Eggleton & Eggleton (5) had simultaneously observed the presence of an acid-unstable phosphate, which they had not characterized, and called it phosphagen. The work indicated some relation of CrP to muscle contraction. However, such a connection was then difficult to define. Meyerhof's fishing for some use of CrP was not without justification because normal contraction definitely showed a rather irregular breakdown of CrP.

Then I started a somewhat more successful piece of work on metabolic effects of fluoride, a known inhibitor of muscle contraction. I did not resolve the mechanism of its inhibition of contraction, but observed that it inhibited

phosphatases in muscle and yeast extracts. I confirmed inhibition of glycolysis and found, to a lesser degree, also inhibition of respiration. I noticed that fluoride had been reported to react with methemoglobin to form a specific fluoromethemoglobin and thought it worthwhile to look into this rather interesting interaction. Eventually, the combined papers on metabolic fluoride effects were used as my chemical doctorate thesis. Of those connected with the Dahlem Institutes, only Karl Neuberg, also a professor at the Charlottenburg Institute of Technology, could accept students for promotion. Thus, he was kind enough to arrange for me to be accepted as his doctorand, based on the work done in Meyerhof's laboratory, as he was working on parallel problems in yeast fermentation. Therefore, in 1929 I obtained the additional degree of Ph.D. in chemistry.

Before expanding on a more detailed description of the dramatic

Figure 1 Portrait of Fritz and Heinz Lipmann, 1926, by Siegfried Sebba.

developments in muscle contraction at that time, I pause here briefly to describe the unusually active artistic and scientific life in Berlin in the 1920s, before Hitler's breaking it up. It was indeed the cultural center of Europe in the arts and in the sciences.

My brother by then had moved to Berlin to become the dramaturge with the intendant of the Staatstheater, Leopold Jessner. There, a group of great actors presented exciting performances. As in Munich, I again had even closer contact with the people of the theater; they are a clan, closed up and agitated by their problems and intrigues. Among them I could mix with unusual characters and beautiful women, often astonishingly intelligent. They are a caste not unsimilar to the scientists.

Also, my friend Friedel Sebba had then come to Berlin. He had meanwhile concentrated on oil painting. I saw Friedel often and learned from him about the difficulty with achieving the expression in his pictures as he wanted them. He had a good understanding of the kind of work I did. He knew that I worked with frogs then and once asked me, "Did the frog speak today?" That was a good question, because that is what we want to do, make the frog "speak." Later, only did the living cell start to tell me something; it took me quite a while to get there.

I show here an example of Friedel's paintings, in a general way representative of the paintings at the time in Germany. It is a black-and-white reproduction of his 1926 portrait of my brother and me; he smuggled himself in as the mask my brother is holding. He put us into costumes for a masked ball, which was a prominent part of the night entertainment in those Berlin years. At one of them I met my wife-to-be, Freda Hall. That was early in the winter of 1929, when I had just finished the quite strenuous Ph.D. examinations.

Use of Phosphate Bond Energy in Muscle

Hill and Meyerhof had earlier successfully determined a constant ratio in living muscle between glycolysis, measured by lactic acid formation, and heat production in the anaerobically contracting muscle. It seemed, thus, to be quite a surprise when Einar Lundsgaard, from the University of Copenhagen, reported a very curious effect of the injection of mono-iodoacetate into rats in amounts he found completely to inhibit glycolysis. These rats ran around quite normally for 5–10 minites, but then collapsed with their muscles going into a rigor similar to the rigor mortis. However, these muscles in contrast to normal ones had been exhausted without lactic acid formation. Inhibition of glycolysis by the iodoacetate caused, rather, a complete decomposition of available creatine phosphate. Now, in Meyerhof's laboratory, it had been shown that CrP hydrolysis developed an unusually high amount of heat. Thus, Lundsgaard's experiments could

only indicate that in absence of glycolysis, the breakdown energy of CrP
could be used by the muscle for contraction. Lundsgaard cautiously
concluded that the use of this phosphate bond energy was nearer to
contraction than the production of lactic acid. The final explanation was
that CrP was in equilibrium with ATP; the terminal phosphate bond of
ATP had, by its heat production, already been found in Meyerhof's
laboratory to be on an equal energy level as the one in CrP. On the other
hand, the energy yield of glycolysis, we now know, appears as the phosphate
bond energy of ATP, which in turn is formed from the energy-rich
phosphate of P $\sim$ enol pyruvate and phosphoglyceryl $\sim$ phosphate. In
Lundsgaard's experiment with blocked glycolysis by iodacetate, CrP acted
in the short normal period of the injected rat as energy donor to ADP,
acting thus as an energy buffer, substituting for glycolysis. In this respect
Lohmann's finding is important that in a muscle extract the adenylic acid
system acted as a coenzyme accepting the $\sim$ P of CrP. I use here the
squiggle $\sim$ to indicate an energy-rich bond. This was introduced in my
review on phosphate bond energy (21). It is discussed later as indicating a
free energy of $\Delta G^{o'}$ of 10 ± 3 to 5 Kcal of hydrolysis at physiological
conditions.

All this revealed that the end product of glycolysis, lactic acid, did not
play a role in the contraction mechanism, and it removed the previous
temptation to connect the mechanism of contraction with acidification
caused by the formation of lactic acid. The evolving evidence for ATP as the
driving force of the muscle is discussed later.

Since fluoride, like iodoacetate, inhibits muscle contraction, I injected
frogs with fluoride and after stimulation observed Cr $\sim$ P and some ATP
breakdown replacing the inhibited glycolysis, just as in Lundsgaard's
iodoacetate injection, ending in a rigor of the muscle after exhaustion of the
Cr $\sim$ P.

Toward mid-1930 the Meyerhof laboratory moved into what was called
the Physiology Section of a new, beautiful Kaiser Wilhelm Institute in
Heidelberg. After settling there, Meyerhof suggested that I do experiments
with frog muscle on CrP breakdown at acidic pH, which was known to
inhibit glycolysis. One of the drawbacks of previous experiments on CrP
breakdown during contraction was that the determinations of metabolic
reactions had been done by workup of the muscle after the experiments, e.g.
by pulverizing the deep-frozen muscle and deproteinizing with ice-cold
trichloracetic acid. The supernatant was used for lactic acid and CrP
determination. It was known, however, that particularly CrP breakdown
occurred during the workup. In the experiments reported with the new
procedure, both lactic acid and CrP determinations were done manometri-
cally with frog sartorius muscle anaerobically suspended in Ringer

bicarbonate buffer between pH 6 and 8. About 100 mg frog sartorious muscle was used; the diameter was less than 1 mm. This dimension allowed an easy equilibration with the suspension fluid. Comparing the electrotitration curves of free and split CrP, due to the liberation of the strong base creatine on split of CrP, a sizable alkalinization takes place. It amounts in base equivalents per mole of CrP split depending on pH to 0.8 at pH 6 and nearly 0 at pH 8; the alkalinization declined almost linearily between these two pH values. The measurements at 15°C showed curves that, on appropriate stimulation by way of platinum threads melted into a Warburg-manometer vessel, indicated in the first hour a nearly linear CO_2 absorption bending into CO_2 liberation for the next hour. The levels of the turnover points depend on the pH varied anaerobically in mixtures of N_2 with CO_2 of 1.1%–11.5% with constant 0.02 m bicarbonate as mentioned. The CO_2 absorption and evolution corresponded to an initial CrP breakdown and subsequently produced lactic acid. The chemical determination of CrP and lactate confirmed the manometric results. In an experiment with iodoacetate-poisoned muscle at pH 6.45, only CO_2 absorption and of similar magnitude as in a pH-equivalent of normal muscle was·observed. However, it was not followed by the CO_2 evolution by lactic acid due to poisoned glycolysis. This furnished confirmation of the validity of our manometry. It indicated at the low pH range an initial CrP-linked contraction period as long as CrP was available, followed by a turn to· lactic acid formation, the degree of alkalinization of CrP utilization depending on pH. These results confirm the early observation with muscle, by D..Nachmansohn, that by a single two-second tetanus contraction, the extent of CrP breakdown was four times that of the formation of lactate, while by continued stimulation the use of CrP was gradually replaced by lactate formation. Our experiments now removed any doubts about a use of CrP as source of energy for the contraction in a normal muscle and also removed acidification as such, as a possible reaction connected with contraction (6).

Tissue Culturing with Albert Fischer in Berlin

Since the time I was still with Meyerhof in Berlin it became clear that after a half-year in Heidelberg I had to get another job. I had been in Meyerhof's laboratory during the last two years on a fellowship that could not be renewed. I was very eager to find a place in Berlin after this interval in Heidelberg, during which Freda Hall and I saw each other only a few times in places between Heidelberg and Berlin. Therefore, I was very happy to get a job in Berlin with Albert Fischer, and to join her again. Fischer's laboratory was located within the Kaiser Wilhelm Institute for Biology, where Meyerhof had worked; the return to Berlin was, therefore, not a

great change for me laboratory-wise. Tissue culture was a rather interesting new field, in which Fischer had become a leader. He wanted me to use metabolism as a method to measure cell growth. It turned out to be a feasible approach by using the fairly sizable aerobic glycolysis found in the tissue cultures of fibroblasts. The measurement of actual growth in tissue cultures is not easy. A true measure could, at that time, only be obtained by tediously counting under the microscope the cells in the state of mitosis. To use metabolism as a measure looked promising, and I worked out an easier manometric method showing essentially linear increase of aerobic glycolysis or respiration in Warburg vessels for tissue cultures. In view of the long periods of several days, differential manometers had to be used to avoid interference by the daily change in air pressure. The culture of fibroblasts was kept in plasma and appropriate fluid laid over it ; the thickness of tissue and the plasma layer were kept at dimensions appropriate for a saturation with oxygen. The compensatory vessel contained the same plasma level without tissue overlaid with the same fluid layer.

First experiments were made using respiratory O_2 uptake by heart fibroblasts in phosphate buffer in presence of KOH to absorb the respiratory CO_2 in a side vessel, separated from the tissue compartment. The O_2 absorption was low, but constant 0.91 cmm per hour.

A better increase of rather high aerobic glycolysis was found with osteoblast cultures using bicarbonate-CO_2 buffer aerobically and measuring CO_2 evolution manometrically. As background, omitting the essential growth-promoting embryonic extract, in 37 hours a barely measurable increase of 0.12 ccm of hourly CO_2 production compared to a quite constant hourly increase of 2.3 ccm over 47 hours with embryonal extracts. Yet, as a routine determination this method under aseptic conditions was too complex.

During the first year I spent in Fischer's laboratory, the new field interested me a good deal. Particularly the results I had obtained prompted me to do experiments on the large aerobic glycolysis in normal embryonic fibroblasts. Such a glycolytic activity was in the foreground of interest because of Otto Warburg's discovery of high aerobic glycolysis in malignant tissues. This high glycolysis in normal fast growing tissues may be indicative that in both cases a large supply of anaerobic energy was needed as a safeguard against the possibility of limited oxygen supply.

After I had been with Fischer for less than a year, he was offered a new institute in Copenhagen and wanted to close the laboratory in Berlin. He wanted to spend the following year supervising the construction of his new institute funded jointly by the Rockefeller and the Carlsberg Foundations. The latter was a great force in Danish science in that the income of the prosperous Carlsberg brewery went through a Carlsberg Foundation into a

generous support of science as well as the arts. Fischer wanted to take me with him to Copenhagen, which, with the threat of Hitler's influence growing rapidly in Germany, was a great stroke of luck. He arranged for me to get a Rockefeller fellowship for a year, which I could spend wherever I wanted and in any way I chose. I decided to join Dr. P. A. Levene at the Rockefeller Institute for Medical Research in New York. He had published a paper on the phosphate content of egg yolk protein, which was amazingly high. The protein isolated from egg yolk, which he called vitellinic acid, contained 10% bound phosphate.

In Meyerhof's laboratory I had become familiar with hydroxyester phosphates of glucose and fructose and gained an intimate experience with the energized N-P link in creatine phosphate. Since proteins contain a lot of nitrogen, I first guessed it to be such a N-P link. Levene had not made attempts to identify the linkage of the phosphate in this vitellinic acid. I therefore wrote to him and he accepted me to work on this identification in his laboratory. Freda Hall and I had become quite closely attached to each other and before leaving for New York we decided to marry and go together to America.

First Time in America, 1931–1932

We took a boat, as there were no airplanes yet, and arrived in September 1931. We were told that we should look for an apartment in Long Island. We regretted that later, after renting a rather dingy place in Jackson Heights. To get to the city, I had every morning a half-hour subway ride and then a 10–15 minute walk to the institute. There was then in the United States a depression, which, I must confess, we did not notice much. Levene was a cultured and interesting person. The atmosphere at the institute, however, was quite restrained. A few Europeans were rather friendly, and of the Americans, Alfred Mirsky and his wife, Riba, and M. L. Anson took a little care of us. Anson took me to the Princeton section of the institute, now long abolished.

Work at Rockefeller Institute

There were two prominent phosphorylated proteins known at that time. One in egg yolk, and the other one in the casein in the milk of mammals. The latter contained only 3% phosphorus; it was a mixture of various proteins. Therefore, in the first attempt to identify the phosphate linkage, a protein containing such high amounts of phosphorus was most promising with the 10% of P in vitellinic acid. It could, moreover, be isolated easily in pure form. These compounds interested me altogether because both served as food for the tissues of growing animals with which I had started to work.

Becoming a little better acquainted with the literature, I soon found out

that Plimmer & Bayliss (7) had worked on many phosphorylated proteins and found as a general property a considerable lability of the phosphate toward alkaline reaction. This more or less excluded an N-P link because the N-P link is stable to alkali, but unstable to acid. They had also already found that the phosphate in egg yolk and casein was, in contrast, quite stable to hydrolysis by acid. It was therefore decided to use limited acid hydrolysis in order to isolate the phosphorylated amino acid. I soon learned to obtain the vitellinic acid; it was relatively easy to purify. My preparation contained approximately 10% phosphate, and I started with its hydrolysis in a boiling water bath for 10 hr with 2.5 molar hydrochloric acid. I used the method of Lohmann and enclosed equal samples in a number of soft glass tubes and sealed the tubes over the flame to keep the concentration of HCL constant. After 10 hr a sample was analyzed and a good deal of inorganic phosphate, short of 50%, was found. By further hydrolysis for 10 hr, I seemed to have reached the stage where most of amino acid phosphate seemed to be in free form. From this hydrolysate I tried now to isolate the amino acid phosphate. This I precipitated with barium and after some reprecipitation, analysis showed its composition to be quite consistent with the barium salt of serine phosphate. In the meantime, I had found that S. and T. Posternak had published an isolation of what they thought was a tetrapeptide of serine phosphate as it contained equal amounts of serine and phosphate. However, C. Remington had worked with casein, which on hydrolysis behaved, according to Plimmer & Bayliss, very similarly to the phosphate in vitellinic acid, but he claimed it to be bound there to different amino acids. It was, however, most likely that serine was also a carrier of phosphate in casein.

Woods Hole in Summer of 1932

In June the institute emptied out and everybody went to Woods Hole on Cape Cod, in Massachusetts. Leonor Michaelis, then a member of the institute, offered me space in the rooms he regularly occupied at the well-known marine laboratory of Woods Hole. We went there by boat; it took almost half a day to New Bedford, Massachusetts, and from there, I don't remember how, to Woods Hole. I met many interesting people; almost everybody of consequence showed up. I met Guzman Barron, an interesting man with whom I often met later on. John Runnstrom, a Swede, who worked with Michaelis at Rockefeller, was there. Later, during our Danish period, we became good friends with him and his wife. It was particularly exciting to meet with Linderstrøm-Lang, who had worked with Morgan in Pasadena, and had driven across the country to Woods Hole. He was one of the Danes we later got to know well—one of the most exciting persons in

Copenhagen, as a scientist and as a human being. Recently, I was pleased to be invited to write about him (8). There, I expanded on my memory of this extraordinarily gifted man. He died, sadly, when he was still in his prime.

At the end of the summer we returned to New York by train, and I finished the work on vitellinic acid (9). We then returned to Europe.

Return to Europe: Copenhagen

STOP IN LONDON   On a visit to London, while traveling to Copenhagen, I went to the Lister Institute and saw Robert Robison. He was quite interested in this "new hexosediphosphate" Lohmann and I (10) had investigated as being formed from fructose diphosphate on incubation in muscle extract. I was then quite unsure about the nature of this product, feeling that we had too little information on it, apart from the considerable increase in acid stability of the ester phosphate. My uneasiness proved to be well founded. Shortly afterward, G. Embden became interested in this "transformation" of ours and connected it with Ragnar Nilson's phosphoglycerate formation from hexose-diphosphate + acetaldehyde with yeast, because the phosphate of phosphoglyceric acid was very difficult to hydrolyze. He confirmed the formation if the expected phosphoglyceric acid and found in addition, phosphoglycerol. Embden guessed rightly that these two three-carbon compounds, both very difficult to hydrolyze, were the products of our reaction, rather than a new hexosediphosphate. He postulated they were formed by a dismutation of the trioses phosphoglyceraldehyde and phosphodihydroxyacetone, to which fructosediphosphate was split enzymatically. This ingenious interpretation, soon fully confirmed, earned him rightly the companionship in the name of the Embden-Meyerhof pathway for the glycolytic cycle. I described this development much later in a contribution to a volume in honor of Gerhard Schmidt, a pupil of Embden with whom we became quite friendly during the time we were in Boston (11).

From England we took a boat to Jutland and the train. In Copenhagen, we found the new institute ready and, as it was there the custom for the first assistant, they had prepared for us an apartment on top of the institute to be supplied with some furniture. There was ample space to live in, and a guest room where we could house visitors, among them Friedel Sebba, who had moved to Sweden, and Dean Burk, a colleague of mine in Meyerhof's laboratory. He had become interested in my work on the Pasteur effect, to be described later. I had ample laboratory space, and eventually such co-workers as Jorgen Lehman from Sweden, Brecke, a young student from Norway, Gertrud Perlmann from Austria, and others.

I was very eager first to continue with the analysis of casein for the

phosphorylated amino acid. Using the same hydrolysis procedure with 2.5 n HCl, I easily isolated again a quite pure preparation of barium and silver phosphoserine.

PASTEUR EFFECT   Through the above described finding of high aerobic glycolysis in chicken fibroblasts, I became interested in the aerobic repression of the substrate-wasteful energy supply by glycolysis through the highly economical respiratory energy production. Warburg suggested that the high aerobic glycolysis in tumors was due to abolition of the generally observed inhibitory effect of respiration on glycolysis in normal tissue. He called it the Pasteur effect, because Pasteur had first described it in facultative anaerobic yeast. For testing in an in vitro system for an oxidative inhibition of glycolysis and fermentation, I added positive oxidation reduction indicators of the indophenol blue type to particle-free fermentatively active yeast extracts with negligible oxygen uptake, which in air remained colored, but by exclusion of air were easily decolorized by reduction. It appeared that they lost the activity to ferment glucose in air, but not in anaerobiosis. A glycolyzing muscle extract behaved similarly. These extracts did not respire. I thought this to be an "artificial" Pasteur effect due to oxidation of an enzyme. At that time I had played around a great deal with measuring the redox potential in fermenting yeast and glycolyzing muscle extracts. I used first quinone and iodine as oxidants, and measured, upon gradual addition, the disappearance of the nitroprusside reaction for SH-, presumably that of glutathione. The disappearance of glycolysis in these extracts coincided with the disappearance of SH- groups. Even a slight remainder of SH- groups permitted almost normal glycolysis. Today, one would argue that this oxidation of the SH- group in glutathione opens the access of the oxidant to the essential -SH in phosphoglyceraldehyde dehydrogenase. Thereto, the phosphoglyceric acid has to attach intermediately in the oxidation process to accept the phosphate for eventual phosphoryl transfer to ADP. With a strong oxidant, this reaction seems irreversible.

I then became generally interested in the energy metabolism of embryonic cells. This was prompted by the finding of high glycolysis in normal chick embryo fibroblasts similar to the one in malignant tissue. I proposed that in both cases the high glycolysis may be used to overcome the possibility of oxygen deficiency (12).

Recent work suggests that the respiratory inhibition of glycolysis and fermentation is due to a feedback inhibition by high concentration of ATP produced by respiration. The phosphorylation of fructose-6-phosphate by phosphofructokinase is inhibited (13). My early experiments were largely designed to emphasize the unlikelihood that the Pasteur effect is due to a

cycle of lactate, formed glycolytically, being resynthesized by respiration to glucose. Such resynthesis of glucose from lactate takes place in liver, but not in muscle.

Pyruvic Acid Oxidation: Acetyl Phosphate

Since in a respiring cell, the fate of pyruvate is changed from a reduction to lactate to an oxidation to acetate and CO_2, I wanted to study the mechanism of pyruvate oxidation. At that time, in 1937, the only case of cell-free oxidation of pyruvate had been reported with an acetone powder of a *Lactobacillus delbrueckii* acidificans longissimus, which I obtained from Germany. Our laboratory was equipped for growing tissue, but not bacteria. However, I managed to get a good growth of this organism, which likes a temperature of about 40°C by growing it near the water heater in the cellar, and centrifuged it off in a milk centrifuge. A desiccator-dried powdered preparation was found best for extraction. When purified by ammonium sulfate fractionation, the enzyme lost two cofactors, thiamin pyrophosphate and flavin adenine dinucleotide, the restoration of which was needed. The most important, rather accidental, observation was made when the medium was on one occasion switched from phosphate, generally used as buffer, to bicarbonate-CO_2. Then I did not get the oxidation of pyruvate. It returned on addition of inorganic phosphate (P_i). Suspecting an energy-rich intermediary, I added radioactive P_i and ademylic acid in a crude extract and found pyruvate oxidation yielded ATP. After finding an old method for synthesizing acetyl phosphate, a crude sample of it was synthesized. With a dry preparation of *L. delbrueckii* shaken with adenylate and acetylphosphate (AcP), an approximately similar amount of AcP disappeared and ATP appeared. AcP, like CrP assays in the P_i determination as inorganic P. The description of all these experiments, done while still in Copenhagen, was presented at the 7th Cold Spring Harbor Symposium during 1939, in the USA (14).

Back to America

Toward the end of 1938, Hitler's fascism was slowly expanding into Denmark. Our Danish friends, foreseeing the danger of a war and the occupation of Denmark by Hitler's Germany, urged us to try to leave. I got in touch with Dean Burk, my American colleague in Meyerhof's laboratory, who had visited us in Denmark. I thought he might be helpful in view of our common interest in the mechanism of the Pasteur effect and I knew that he was moving in 1939 to a laboratory in DuVigneaud's department at Cornell Medical School, at the New York Hospital, and he could choose two assistants. I wrote to him that I needed a job in the United States, because Denmark had become unsafe due to the developments in

Hitler's Germany. Linderstrøm-Lang, who knew DuVigneaud well, was most helpful in recommending me to him. Luckily, my invitation to become Burk's assistant in DuVigneaud's laboratory was arranged, which was necessary for me to enter the United States. It turned out to be helpful that my wife, who had been born in Ohio, already had an American passport.

When we came to New York, a small apartment was rented for us in Tudor City. But very soon we went to Cold Spring Harbor on Long Island, where I was to give, as mentioned, a talk on all I had found with pyruvic oxidation (14). It was a great meeting with the Cori's, the Needham's and many American and other European scientists. In the middle of this meeting, the second world war started and the Europeans were eager to hurry home.

We stayed on in Cold Spring Harbor and rested a little after all the excitement. Returning to New York, we moved into one of the two large laboratories reserved for Dean Burk, Richard Winzler, and myself. Our work was then concerned with cancer. At that time, the surprising claim by F. Kögl, a well known Dutch biochemist, that cancer tissue contained D-amino acids, was causing much excitement. While the evidence was not very convincing, it was difficult to disprove. I proposed to assay hydrolysates of cancer tissue with the D-amino acid oxidase to obtain an undisputable result. I prepared a quite active enzyme and the result was definitely negative (15, 16). This was seemingly essential, but not very interesting work.

I met here again Rollin Hotchkiss, whom I had first encountered in Denmark. He was now working nearby at the Rockefeller Institute with René Dubos. He had isolated the two earliest bacterial polypeptide antibiotics, tyrocidin and gramicidin, from a Bacillus brevis as a quite pure mixture. Having the D-amino acid oxidase handy, we thought the hydrolysate of these antibiotics was worth analyzing for a possible presence of D-amino acids. I was quite surprised and pleased to obtain a vigorous O_2-uptake, which clearly indicated their presence in sizable amounts (17).

After finishing these refreshingly successful experiments during the spring of 1940, we went for a vacation to a nice place on Lake Iroquois in Vermont, not far from Burlington. I had time there to meditate on the metabolic appearance of a compound like acetyl phosphate. It had become clear from Lundsgaard's discovery (18) that the energy for the muscle contraction apparatus was by way of ATP (19, 20). On the other hand, Schoenheimer and colleagues had found deuterated acetate to be transferred by, presumably, biosynthetic pathways into amino acids, fatty acids, and steroids. In the bacterial system, I had observed that acetate, by action of an acetokinase, was in equilibrium with ATP. Therefore, acetate could be activated by pyruvate oxidation or by transfer of phosphoryl from

metabolically formed ATP and used for anabolic purposes. Here it appeared then that acetyl phosphate might in animal tissues be a prospective acetyl donor in the biosynthesis of essential metabolites. I proposed the generalized use of ATP as energy carrier in an essay I started in 1940 in Vermont. It was called "Metabolic Generation and Utilization of Phosphate Bond Energy" (21). In this paper, I introduced a special sign, the so-called squiggle $\sim$ for energy-rich phosphate, $\sim$ P. It is now much used for other energy-rich linkages, e.g. thioester. It has been helpful to define group activation of the C $\sim$ P link either to donate P to ADP or of the carboxyl group for acetyl donation. I argued that generally ATP was active in group activation, as of amino acids in protein synthesis; I used as presumptive examples the activation of acetate in fat, steroid, and amino acid synthesis, as indicated by the mentioned experiments by Schoenheimer and colleagues. Thus, a general use of ATP in biosynthesis of macromolecules was foreseen. The transfer of the phosphoryl potential from ATP, or indirectly by $\sim$ P donation from creatine phosphate, to energize muscle to contract was one of the prime examples of the role of ATP in energy transmission.

During the last part of my stay with Burk, I had returned to work on the acetylation problem. In the middle of 1941, my two years with Burk at DuVigneaud's laboratory ended and Burk then moved to Washington. After many unsuccessful trials, I eventually landed with a Ciba fellowship at the Department of Surgery of the Massachusetts General Hospital (M.G.H.) in Boston, one of the hospitals connected with Harvard Medical School. The grant was given to Oliver Cope, who was interested in endocrinology. Therewith, I entered the unusual Department of Surgery led by Edward Churchill. It had a quite potent research floor and became the forerunner of a development of great expansion into research at the M.G.H. Oliver Cope, the second in command, gave me a spacious room.

The appearance of the much discussed essay on phosphate bond energy increased my reputation in the biological sciences. Quite a few biologists and chemists recognized the essay as an illumination of the relationship between metabolic energy production and its utilization. I was very pleased to find the main idea in this essay paraphrased in Bernal's delightful little book, *The Physical Basis of Life* (22), in the following manner: Bernal wished to avoid troubles with defining life by limiting it partially to a common material, the proteins, and one common physicochemical process, a stepwise catalysis of organic compounds carried out practically isothermally by quantum jumps between 3 and 16 kilocalories/M, comparing it with the jumps of ca 300 in laboratory chemistry. In Figure 2, I like to express differently the essentially same energetic cycle. My paper on phosphate bond energy also induced Dr. H. A. Barker, the well known
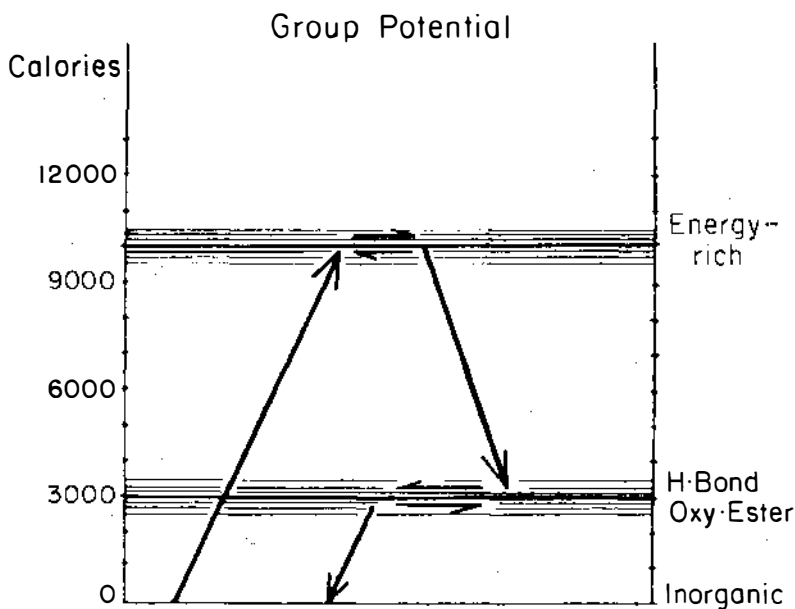
Figure 2   Scheme for group activation in group potentials between 3 and 12 kcal.

Berkeley bacteriologist, to ask in 1942, right after my arrival at the M.G.H., to work for a year with me. Bacteriologists started then to find acetyl phosphate formed in other organisms, i.e. Utter & Werkman (23), and Koepsell & Johnson (24) in Clostridia. Werkman invited me for a few months to the Iowa Agricultural Experiment station at Ames, where I increased my knowledge of bacteriology.

Most important was the increasing support of my work by the Commonwealth Fund, which allowed me to get technical assistance. I was lucky to find Constance Tuttle, who helped me a great deal for many good years.

Fortunately, I had brought from Copenhagen a good supply of *Lactobacillus* pyruvic oxidase and had in the second year in New York found time to work out a method to determine differentially acetyl phosphate in presence of inorganic phosphate (P_i). With this now, I definitely identified the acetyl phosphate as the product of pyruvic acid oxidation in the lactobacillus.

Massachusetts General Hospital: Coenzyme A

I learned more easily to prepare acetyl phosphate (Ac ∼ P) from silver monophosphate and phosphorus oxychloride, and then most urgently needed an assay system for acetyl transfer in animal tissue. For this purpose

I isolated a very potent enzyme from pigeon liver extract suitable for easy colorimetric determination of the known acetylation of sulfonamides and aromatic amides in general (25). With this assay, I first tried ATP + acetate, which I expected to form acetyl phosphate as in microorganisms, and found it active. But then I tried acetyl phosphate itself, which unexpectedly in animal tissues was almost explosively hydrolyzed by a heat-stable enzyme. Yet, if Ac ~ P would have been active in transacetylation, since it could be used under conditions where some reaction should have been obtained, it was rather convincingly nonreactive. It appeared, however, that on autolysis the liver extract easily lost activity with ATP + acetate. This indicated the activity of an acetyl carrier, since boiled liver extract caused full reactivation. Our group, with increasing help from Commonwealth funds, had been fortified by the addition of two excellent co-workers, Drs. Nate Kaplan and Dave Novelli. With further help from the Upjohn Company, we obtained a pure enough preparation of the coenzyme to carry out a preliminary analysis. We observed the presence of adenylic acid and of a sulphydryl-containing compound. All of the known coenzymes were inactive. We were most eager for an analysis to check on the presence of a vitamin in this new coenzyme preparation. From two pharmaceutical companies we obtained negative answers. However, encouraged that Roger Williams, the discoverer of pantothenic acid, had considered it likely to be involved in intermediary metabolism, I sent him a sample of the coenzyme. He gave it to Dr. Beverley Guirard, who was familiar with the difficulties of finding pantothenic acid in crude tissue extracts, and instead hydrolyzed it for assay of the β-alanine part of pantothenic acid. She found quantities of it in our coenzyme equivalent to its content of adenylic acid. We found, then, an enzyme in liver extract together with alkaline phosphatease to liberate all pantothenic acid from CoA (26). Concurrently, a cofactor for acetylation of choline was found by Nachmansohn & John (27) and Feldberg & Mann (28) in their work on acetylation of choline with brain extract plus acetate and ATP. We tested our purified coenzyme on this acetylation and found it to be active in choline acetylation with dialyzed brain extracts (29). Therefore, we called it CoA, with "A" for activation of acetate. Of great importance, however, would have been the identification of the SH-derivative in CoA, which we observed, but neglected. Eventually, it was identified in Snell's laboratory as part of the Lactobacillus bulgaricus factor (LBF) needed by this organism instead of pantothenic acid. They found it to be a peptide of pantothenic acid with thioethylamine and called it pantetheine. In view of our observation of an SH- derivative in highly purified CoA, they considered panthetheine to be a precursor of CoA. Meanwhile, Lynen had attempted to isolate not free, but rather acetyl CoA. Our observations had convincingly shown that acetyl CoA carried an

activated, or let us call it energy-rich, acetyl, but we had not identified the energy-rich link between CoA and acetyl. Lynen & Reichert (30) used our sulfonamide acetylation system for assay on purification of acetyl-CoA and eventually found that it was linked to CoA by a thioester link. This important observation introduced the thioester bond as a new energy-rich bond.

With the identification of pantetheine (pa) as part of CoA, the link from it to adenylic acid, presumably by a pyrophosphate bridge, was still to be defined. For this purpose, Baddily & Thain had synthesized the likely 4′-phosphate of pantetheine on the terminal of its four hydroxyl groups. A pure preparation of D-L-pantetheine 4′-phosphate was synthesized in Baddily's laboratory using a pigeon liver enzyme fraction isolated by Levintow & Novelli, and called pantetheine-kinase. This was condensed with ATP by Baddily et al (31) yielding a 45% conversion into CoA. Then the same experiment was carried out with pure D + 4′-phosphopantetheine yielding an 82% conversion. This confirmed the complete structure of CoA with the third then unsure phosphate on the ribose of adenylic acid identified in Kaplan's laboratory (32) as being in 3′-position by use of 3′-specific Rye grass phosphatase.

CHEMICAL STRUCTURE OF CoA   This now well-known structure was presented in a review that also surveyed the CoA linked acyl-transfer reactions elaborated by Novelli, Kaplan, Soodak, Stadtman, Klein, and others in this laboratory (33). The CoA-linked reactions included fatty acid and steroid synthesis and, most importantly, citrate synthesis from oxaloacetate. The latter was the definition of the role of "active acetate" in the initiation of the citric acid cycle of H. A. Krebs.

We recognized further connections with CoA-linked reactions after Stadtman joined us. The Stadtman-Barker bacterial trans-acetylase that had been identified by Ac $\sim$ P $\rightleftharpoons$ ^{32}P exchange and by arsenate-induced hydrolysis of acetyl phosphate was found to be CoA dependent (34). Thus, this transacetylase was a bacterial type of acetyl CoA synthesis. By efforts largely of Jones, Black, Hoagland, Novelli, and Chou, the complex CoA-ATP-acetate reaction had been found to result in what we called a pyrophosphate split of ATP, which Paul Berg later showed to yield enzyme-bound acetyl-adenylate (35).

In very informative experiments, Chou (36) separated by combined acetone and protamine precipitation the donor ATP-CoA-acetate reaction and the acceptor enzymes for arylamine acetylation, citrate and ac-etoacetate synthesis. There also could be shown that the bacterial transacetylase with CoA and acetyl phosphate could with pigeon acceptor enzymes replace the ATP-CoA-acetate reaction. Thus, it was shown that

the activation of acetate, as well as its utilization, was due to independent enzyme systems and that a mixture of bacterial activation and animal utilization enzymes could easily interact. When Doudoroff came to the laboratory, he and Stadtman (37) made use of such a mixed system to show that with [^{14}C]acetyl phosphate as donor, by way of transacetylase, the synthesis of acetoacetate is effected by a liver enzyme, and two [^{14}C]acetate residues appeared in acetoacetate. This result made us conclude that in this condensation both acetyl residues had to be activated.

In the meantime, many laboratories had become interested in CoA activity and we were ready to leave the field with the observation, with Srere (38), of a citrate split by ATP and CoA, yielding acetyl CoA and oxaloacetate in pigeon liver extract we made as our last contribution. The complex problem of mechanism of this reaction was resolved by Walsh & Spector (39). The overall split of ATP here is to ADP and P_i. Srere has since also elaborated extensively on its mechanism.

In the fall of 1953, something extraordinary happened: I received notice that I was awarded the Nobel Prize for Medicine and Physiology for my work on Coenzyme A. I was paired with Hans Krebs presumably because with acetyl CoA, the "active acetate" that starts the citric acid cycle had been defined.

I have enlarged on that somewhat in a book, *Wanderings of a Biochemist* (40), which includes a photograph of my wife and Stephen, our son who was born in 1945, and myself, all smiling on our departure to Stockholm. That is now over 30 years ago, and although the event has lost some of its glamour, I still look back fondly on it.

ROLE OF PHOSPHORYLATION IN OTHER GROUP ACTIVITIES

The metabolic appearance of acetyl phosphate had led me to a consideration of the general use of group activation by way of phosphorylation as a common intermediary reaction in biosynthesis. To document this, we have been searching for such cases of group activation.

Carbamyl Phosphate

A phosphorylation seemed to be involved in the first step of the Kreb's urea cycle, the conversion of ornithine and ammonium carbonate ⇌ carbamate and then to citrulline. I attended a Gordon conference, where there were a number of groups reporting on the phosphorolysis of citrulline ($+P_i$ $+ADP$) yielding ATP. This indicated a presumably unstable carbamyl phosphate as intermediary. We obtained, therefore, a culture of *Streptococcus faecalis* R, active in the phosphorolysis of citrulline. Mary

Ellen Jones incubated its extract with ammonium carbonate and P-enolpyruvate + a pure pyruvate kinase, and also the same mixture with addition of ornithine. In the mixture without ornithine we found an unstable P that was not too quickly hydrolyzed in the Fiske-Subbarow P determination, but was destroyed by one minute at 100°C in 0.01 N HCl. This differential was used for determining the unstable intermediary, and after 30 min incubation 0.75 μM of such phosphate was formed, which was presumed to be carbamyl phosphate. On addition to the same mixture and ornithine, citrulline in amounts nearly equivalent to the P_i liberated from phosphoenol pyruvate was found.

Thereafter, realizing the difficulty of obtaining enough unstable compound to analyze it, we approached Leonard Spector, a clever chemist with the Huntington Laboratory, one floor below us. He had the brilliant idea to try cyanate as a phosphate acceptor in the hope that isocyanate by incubation for 30 min at 30°C would condense with phosphate. This proved to be very successful (41, 42) and gave the opportunity to synthesize quite pure lithium carbamyl $\sim$ P by repeated alcohol precipitation. We also succeeded with the carbamylation of aspartic acid in rat liver extract with carbamyl phosphate as the donor. This is the first intermediary in the synthesis of pyrimidine and its nucleotides. Cohen & Grisolia (43) working with animal tissue, had obtained an unidentified ATP-linked precursor that had been found active in citrulline and carbamylaspartate synthesis.

Sulfate Activation

It is not surprising that in the 1950s ATP was applied in many cases of group transfer, including sulfate activation. In my review in *Science* (44) I summed up the early work by DeMeio, Bernstein, and McGilvery, and others who obtained sulfate transfer by addition of ATP in a cell-free system. It was also recognized that the activation and transfer using phenol as acceptor was due to a two-step reaction. Bandurski had gone further and isolated from yeast two fractions that had to be combined for activation. Hilz, in our laboratory, using ^{35}S, marked sulfate and found that the primary reaction yielded adenylsulfate (APS) and pyrophosphate (PP_i) analogously to the ATP-CoA-acetate reaction. After Hilz left, Phil Robbins took over and succeeded in complete analysis of what we now call the active sulfate. He found the second step to be a second phosphorylation of the APS to form PAPS, which using Kaplan's 3'-specific Rye grass phosphatease, was identified as in the 3'-position of the ribose of A. Robbins describes the methodology he worked out for preparation of sulfate-activating enzymes from liver and Baker's yeast. Therefrom he also isolated PAP[^{35}S]. Most of the work done on this problem, by us and others, is included with references in a review by Gregory & Robbins (45).

Chick embryonic cartilage was introduced for the assay of enzymatic transfer of sulfate into chondroitin sulfate (CHS) with d'Abramo (46). A survey of our work on sulfate activation and transfer was described in detail in the mentioned review in *Science* (44). This includes work by Irving Goldberg on the ceribroside sulfate in brain. The latter is of clinical interest as, in man, a debilitating deficiency disease is known where the lysosomal hydrolase is missing and in all organs large amounts of this ceribroside sulfate accumulates. We found another aberration in a chondrosarcoma that was observed. There are normally three CHS—A, B, and C—differing in the manner of sulfate esterfication. We obtained a chondrosarcoma from Sloan-Kettering Cancer Institute that had sizably increased activity to produce CHS-C only (47). Recently, a frequent binding of sulfate to tyrosine in protein has been found that earlier was observed only in rare cases (48). We are now starting to explore possible differences in this process between normal and malignant cell types.

Move to Rockefeller University

In the mid-1950s I became restless at the Massachusetts General Hospital. There was, as I have experienced, a certain friction between the teaching and research departments in the same university; a certain envy by the former of the latter. Luckily, just at that time Dr. Detlev Bronk had become the president of the Rockefeller Institute, which he converted into a graduate university, with relatively low demand for teaching duties. He invited me to join the university as a member, or as it was then already called, a professor. The transition was very easy because John Gregory, who had been at the Rockefeller Institute and had joined me at Massachusetts General Hospital, moved back with me and excellently organized the laboratory facilities there by moving a half-year ahead of us early in 1957. When we joined him a few months later, we were able, without interruption, to start work there. Quite a few of my co-workers did move with me and the laboratory was generously supported by N.I.H., and our support was transferred with us with one exception that I regretted. Dr. Warren Weaver, director of the Rockefeller Foundation, had a few years earlier spontaneously offered me a personal grant of $5,000 a year to make use of however I wanted, without need for any report. This had been the nicest money ever given to me; unfortunately, it could not be transferred between these two Rockefeller institutions.

Protein Synthesis

After moving to Rockefeller we took up almost exclusively the analysis of protein biosynthesis. Already in my review of 1941, in the discussion of group activation for polymerization, I had proposed that amino acids were

likely to be activated by phosphorylation of the carboxyl group. We had learned then that in animal tissue acetate activation was more complex, and was due to a pyrophosphate split in ATP, rather than a phosphorylation by ATP to Ac $\sim$ P as in bacteria. Its reversibility was shown by pyrophosphate exchange in what we called the ATP-CoA-acetate reaction. Hoagland then had been working in our laboratory on acetate activation. After returning to Zamecnik's laboratory, he assayed the supernatant of liver homogenates, which supplanted polypeptide synthesis for pyrophosphate exchange with ATP in presence of amino acids. As in the ATP-CoA-acetate reaction he found a pyrophosphate (PP_i) exchange with ATP and amino acid. This indicated an activation of amino acids by an $AMP-PP_i$ split. Later, for both acetate and amino acid activation, Paul Berg showed that it involved an enzyme-bound acyl-adenylate intermediary.

The Hoagland-Zamecnik group then made rapid progress in the field of amino acid activation by confirming Crick's prediction that short RNAs containing presumably amino acid–specific triplets of nucleotide codons would join the amino acids. With this clever formulation, Crick foresaw that positioning of amino acids (aa) in a prescribed order in proteins would be like the construction of the double helix by polynucleotide synthesis, a function of a pyrimidine-purine hydrogen bonding between the specific sequence in or attached to the units. The need for such a hydrogen bonding between triplets of pyrimidine and purine nucleotides was deduced from the number 20 for the amino acids, for which doublets were insufficient. These ideas were in the air as it became clear that the ribosome could not be responsible for sequencing the aa's. There was found in the soluble supplement to ribosomes a soluble RNA first called sRNA, soon to be changed to tRNA (t for transfer). They contain about 70 nucleotides and are specific for particular amino acids (49). It appeared to have a common O-terminal of two cytidylic acids (C's) terminating with adenylic acid (A), and that the amino acids were all linked to the O-terminal adenylic acid. Zachau et al (50) isolated adenosine-linked amino acid from the ribonuclease digest of liver amino acyl transfer RNAs. We use the now commonly preferred tRNA.

In summary, the ATP-linked activation of amino acids, with the amino acyl adenylates as intermediary, and the discovery of the amino acid binding eventually to a tRNA in an energy-rich linkage by way of the glycolic 3'-OH or either 2'- or 3'-OH on the ribose (51, 52). Furthermore, we had confirmed the expected amino acid specificity of activating enzymes and proposed the base pairing by hydrogen bonding. Crick's prediction was further confirmed by isolation of a messenger RNA (mRNA), discovered in three laboratories simultaneously; this includes Sam Weiss,

who, when he left us for the University of Chicago, told me he would look
for it.

The further development to 1962 is reviewed by Nathans et al (53), by
examining the protein synthesis from amino acyl tRNA. They discuss the
function of what is then still called a supernatant factor. Its bacterial and
eukaryotic specificity is described and contrasted with an apparent
nonspecificity of amino acid transfer RNAs. There was emphasized the
specific participation of GTP discovered by Keller & Zamecnik as an
important observation although the role of it was then still unknown.
Furthermore, the increasing evidence for a messenger RNA serving as a
template for protein synthesis is mentioned, referring to the discovery of
Nierenberg & Matthei of synthetic polynucleotides to serve as templates for
polypeptide synthesis on *Escherichia coli* ribosomes. They first discovered
that polyphenylalanine is synthesized on poly U.

Polypeptide Chain Elongation

The understanding of the mechanism of polypeptide synthesis had
proceeded then to distinguish three phases of the process, eventually found
in all polymerization reactions producing chains with specific subunit
sequence: (a) initiation, (b) polymerization, and (c) termination. In protein
synthesis the 20 amino acids found in most proteins are linked on a
ribosome in an initially straight chain. This eventually folds up into the final
three-dimensional structure. We worked mostly on the middle phase of
chain elongation in *E. coli* homogenates. Choosing a simple system, we
worked predominantly on polymerization of phenylalanine (phe) on a
template of polyuridylic acid. There the mechanism of elongation to
polyphenylalanine chains on ribosomes could be studied rather in-
dependently of the complex initiation and termination periods.

Separation of the Three Elongation Factors, Tu, Ts, and G

The supernatant of the *E. coli* homogenate contained, as shown by Nathans
in our laboratory, the elongation complements that could be separated by
Allende et al into subunits by DEAE chromatography. They obtained two
fractions: a heat-stable factor T and a heat-unstable G. These were more
definitely identified by Nishizuka by using DEAE Sephadex and a
potassium gradient. This yielded three peaks, (a) a now unstable factor T, (b)
a mixture of factors G and T, and (c) a peak that contained the now stable
factor G, so named for its inducing a ribosome-linked GTP split. In this
chromatography, in contrast to the earlier one where heat instability was
found in the late appearing factor G, it had now shifted to the early T. We
interpreted this change as indicating that our now isolated factor T

contained, in addition to the earlier stable T we now called Ts, an unstable Tu, which in the earlier chromatogram had joined with the now stable G. This proposition was confirmed by experiments with Lucas-Lenard (54) who separated T into Tu and Ts. Using mostly our simplified poly-U–linked poly-phe synthesis, the addition of a new amino acid is catalyzed in four phases on the ribosome. A phe-tRNA may initiate the process instead of the complex initiation involving special I-factors and in bacteria as a peptidyl substitute, the formyl methionyl-tRNA having been elaborated in other laboratories. Lucas-Lenard, in this laboratory, found, however, with the simplified phe-system that N-acetyl-phe-tRNA was a better initiator than plain phe-RNA (55), and this was used in most experiments. In Phase 1, the start of the polymerization cycle, the acetyl-phe-tRNA is bonded to the left, the "donor" or D-section of the upper 50s part of the ribosome and with an A-triplet on tRNA to a U-triplet on poly-U on the 30s part of the ribosome. The initiating acetyl-phe-tRNA serves in Phase 2 with its activated carboxyl group linked to tRNA from D as donor to the amino group of added phe-tRNA to A-site. Thereby, the carboxyl link of acetyl-phe to tRNA gives way to a new peptide bond as polymerization progresses by the addition of the new phe-tRNA. It is followed by Phase 3, the translocation from A to the D site of the newly elongated peptidyl-tRNA, which was still sitting on the "wrong," or the A-site. This translocation is catalyzed by the G-factor with GTP splitting, promoting the translocation after a new addition from acceptor to donor site. The translocation and removal of the free tRNA released as discussed from acetyl-phe-tRNA by transpeptidation simultaneously with translocation is completed in transition to Phase 4. Phase 4 is identical with Phase 1, except that the peptide chain is elongated by one phe.

I have described here in an abbreviated form the work here and elsewhere between 1962–1969 on protein biosynthesis, which is discussed in detail in my 1969 paper in *Science* (56).

The most elaborate description from our laboratory of protein biosynthesis in 1971 was presented by Lucas-Lenard and myself (57). We attempted there not only to describe, but also to rationalize all the knowledge available up to 1971. Much has been added since and much additional information is found in the book *Molecular Mechanism of Protein Biosynthesis* (58).

Most recently, an illuminating paper by Kaziro (59) on this topic has appeared, entitled "Molecular Mechanism of Protein Synthesis and an Approach to the Mechanism of Energy Transduction." Kaziro describes and discusses new results that led him to conclude that the GTP-linked prokaryote elongation steps involving both the Tu-Ts pair binding the amino acyl-tRNA to the ribosome, and the G-promoted translocation of

the newly elongated polypeptidyl-tRNA are due to protein transformations caused by binding of the terminal $\sim$ P in GTP. The latter, involving apparently a mechanical move of a ligand on the ribosome, is proposed to be comparable to the role of ATP in muscle contraction. This proposition, founded on Kaziro's new results, goes far to explain the role of GTP in protein synthesis. Also, in the book mentioned before, isolation of elongation factors analogous to Tu-Ts and G is described in the eukaryote supernatant fractions. They are, however, not interchangeable as mentioned before (58).

Increase of Phosphoryl Potential of Serine-O-Phosphate and Tyrosine-O-Phosphate by Binding into Protein

SERINE PHOSPHATE   In earlier work, I had become rather familiar with the serine phosphate in phosvitin and casein. Thus, we expected this phosphorylation to be originally catalyzed by a phosphate transfer from ATP. We tried with Murray Rabinowitz to reproduce this reaction with the egg yolk protein (60). In this protein, nearly every second amino acid is serine, and serine and phosphate are present in nearly equal amounts. The compound used had a phosphate content of 10%, similar to the one I had used in earlier experiments.

In order to obtain a phosphorylation of phosvitin with ^{32}P, one had to remove part of the phosphate. For this purpose, we used a spleen phosphatase and for rephosphorylation we obtained good results with purified protein phosphokinases prepared either from brewer's yeast or calf brain. Dephosphorylation with alkali destroyed the serine in protein and, therefore, the enzymatic method had to be used.

The results of the reverse transfer to ADP from 20%–40% enzymatically dephosphorylated phosvitin may be summarized as follows: A strong affinity was already indicated by near equality of rephosphorylation from ADP to ATP and ADP + hexokinase + glucose to glucosephosphate, while, generally, the latter with ADP as catalyst is more effective. Furthermore, the specific activity of rephosphorylated phosvitin and the phosphate transferred back to ADP differed widely in favor of that of the terminal P in ATP thus formed. This made it clear that the protein phosphokinase discriminated for a transfer from special sites in phosvitin. The results actually indicated that the newly transferred phosphates may be the ones preferred for return.

This is also confirmed by the finding that dephosphorylation of the P in phosvitin of over 70% abolished the reverse transfer of phosphate by protein phosphokinase. We were then reminded of the finding in Sanger's laboratory (61), both in phosvitin and casein, of serine phosphate sequences of up to six and that the phosphoryl potential of phosphate in these blocks

might be higher. This suggestion was recently confirmed by Fisher et al (62), who extended our experiments by using in addition to phosvitin a tryptic digest of casein and isolated the ser-leu-ser-ser-ser peptide. This they showed, in contrast to free serine phosphate, to transfer easily in presence of phosphokinase its phosphate to ADP. Therefore, it appears that in the serine in the blocks in phosvitin and casein the phosphate becomes energy rich.

TYROSINE PHOSPHATE   Dr. J. M. Sturtevant of Yale University and Dr. R. Epand of McMaster University, with samples of tyrosine (tyr-P) phosphate, prepared by Fukami (63), found the $\Delta H°$ of tyr-P-hydrolysis to be -2.8 Kcal p/mole, which is considerably below the value of $\Delta H°$ for γP in ATP that Sturtevant earlier determined.

Tyrosine phosphate had become of great interest because Erikson & Brugge (64) had discovered that the translation product of the transforming gene of Rous sarcoma virus (RSV) RNA was a protein phosphokinase specific for transfer of phosphate (P) to tyrosine in proteins (65). The transphosphorylation had been found first in transfer of P to an immunoglobulin (IgG) to sarcoma-bearing rabbits (TBR). In this paper the TBR-IgG was to be used as acceptor of P by use of the transferase isolated by Fukami from tumor tissue.

Fukami & Lipmann (66) could show first that the phosphorylation of specific IgG was easily revertible to ADP, using this system for determining the equilibrium constant, K_{eq}, of the reaction between $ATP + IgG \rightleftharpoons ADP + IgGP$ as described in the paper. The value of K_{eq} at optimal pH was used to determine the change of free energy, $\Delta G°'$ of hydrolysis of $\Delta G°'$ enzyme-bound tyr-P. It was found to be -9.48 kcal/mole. This is very near to the $\Delta G°'$ of the hydrolysis for γP of ATP at analogous conditions, which is approximately 10 kcal/mole.

The result indicates that the tyr-P is bound in this protein by an energy-rich bond in contrast to free tyr-P, to judge from its low heat of hydrolysis of tyr-P.

BACTERIAL PRODUCTION OF POLYPEPTIDE ANTIBIOTICS BY A THIOL-LINKED ACTIVATION AND POLYMERIZATION

In 1963, I was invited to participate in a conference on the Origins of Prebiological Systems. I presented there a paper entitled "Projecting Backward from the Present State of Evolution of Biosynthesis" (67). There was much discussion then about the priority of proteins or polynucleotides in the earliest prebiotic evolution. In any case, the production of proteins

appears to be the final goal in the polynucleotide translation of the four-letter codes of DNA to messenger RNA, which is to be expressed into the 20-letter language of the proteins. Only by this expression does the translation product become usable as living matter. Yet, it remains likely that the polynucleotide system and the polyamino acid system should have to develop in parallel. Nevertheless, I found it worthwhile to search for an alternate, less complex mechanism for the production of polypeptides without the need of nucleic acids. When attempting to find such a mechanism, I noticed the recent report on an apparently nonribosomal polypeptide synthesis. Extracts exhaustively treated with RNase had been found in several laboratories (68) to synthesize bacterial antibiotic polypeptides. Thus, with Wieland Gevers and Horst Kleinkauf (69) we started the synthesis with the smallest of a group of related antibiotics, the gramicidin S (GS) in extracts of a *B. brevis*. GS is composed of two identical pentapeptides that cyclize head to tail.

The amino acids (aa) are activated by the reaction of ATP + aa = AMP $\sim$ aa + PP_i. The synthesis of the half molecule, the pentapeptide sequence of D-Phe-Pro-Val-Orn-Leu is carried out on two proteins. The L-Phe is ATP-activated and linked to a thiol on a protein of M_r 100,00 that we call the light enzyme. The four following amino acids are thiol-linked to a polyenzyme that contains one mole pantetheine (Pan) bound to it by a protein of $M_r \sim$ 17,000–20,000 first isolated from tyrocidin-synthesizing polenzymes (70). The M_r 100,000 light enzyme contains a racemase and when charged with L-Phe initiates the synthesis of GS with the D-Phe. This initiation process had been further analyzed (71) and it begins by the Pan of the polyenzyme displacing by higher affinity the Pro from its -SH and carrying S $\sim$ Pro to the initiating Phe that then reacts with its thio-activated carboxyl group with the amino group of proline to form the first peptide link Phe-Pro carried on the Pro + thio-linked to pantetheine. The Pan-Phe-Pro then reacts with Pro's neighbor Val's NH_2 to form the tripeptide temporarily hanging by the following protein and transthiolites to Pan:

$$\begin{array}{c} \text{O} \\ \parallel \\ \text{S} \sim \text{C}\!-\!\text{Val}\!-\!\text{Pro}\!-\!\text{Phe} \end{array}$$
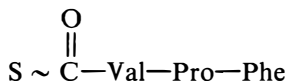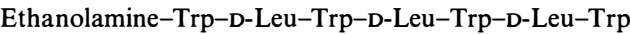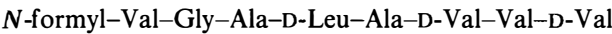
This alternation of transthiolation and transpeptidation yields the penta-peptide half of GS and cyclizes head to tail by the S-linked carboxyl of leucine reacting with the free-NH_2 of Phe. The alternation with trans-peptidation is presented in Figure 3. The process is the same in GS, the later-analyzed tyrocidin (Ty), and linear gramicidin (LG), which was partially synthesized on polyenzymes. Actually, the details were most

strongly recognized with Ty analysis. We obtained a more complex, but analogous biosynthesis of Ty with its dekapeptide cyclyzing much more slowly. Tabulating the enzyme structure of both the GS and Ty we get:

Antibiotic	No.	M_r of enzymes	Amino acids activated and fixed in sequence	Subunit M_r per amino acid	Pan
GS	1	100	D-Phe	100	None
	2	280	Pro-Val-Orn-Leu	70	1
Ty	1	100	D-Phe	100	None
	2	230	Pro-Phe-D-Phe	76	1
Dekacycle (slow)	3	440	Arn-Gln-Phe-Val-Orn-Leu	74	1

Pantetheine is a rather long molecule of $\sim 20\text{Å}$; according to the clever proposal by Lynen it transacts a specific bond formation as a swinging arm as shown in Figure 3. This process is comparable to the translocation of the peptidyl from a donor to acceptor site on the ribosome.

Linear gramicidin is not yet quite resolved. Kurahashi's laboratory (72) discovered the manner of its initiation by finding that the initiating valine is formylated. The GL structure is:

N-formyl–Val–Gly–Ala–D-Leu–Ala–D-Val–Val–D-Val

Ethanolamine–Trp–D-Leu–Trp–D-Leu–Trp–D-Leu–Trp

Bauer et al (73) carried out its synthesis in a biosynthesis with thioethanolamine synthesized onto it by using a high concentration of it reacting chemically with the following structure and releasing the peptide from the enzyme:

$$\text{Enz—S—}\overset{\displaystyle\overset{\textstyle O}{\|}}{\text{C}}\text{—tryptophan—terminal}$$

I would like to add here that, with the GS system, it has been possible to react the light enzyme with preactivated phenylalanine by its chemical thioesterfication without adding ATP. To recognize the synthesis of GS we combined the thioester of cold phenylanine transferred to light enzyme with the heavy enzyme that had been charged with radioactive amino acids. It appeared, thus, that the Phe-thioester was able to transthiolate to the enzyme-SH and to initiate synthesis. Trials with other amino acyl thioesters of Val or Leu to charge the heavy enzyme were unsuccessful.

We began with Wieland Gevers, and soon afterwards Horst Kleinkauf joined us. Roskowski came somewhat later, and Lee has done a great deal
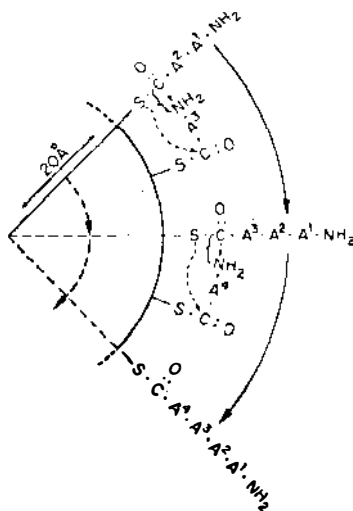
Figure 3 A schematic representation of transpeptidation and transthiolation, mediated by enzyme-bound pantetheine in antibiotic peptide biosynthesis.

of very fine work during the last few years. Bauer worked quite successfully on the chemical-enzymatic synthesis of linear gramicidin, as did Akers et al (74) on the analysis of charging its two first polyenzymes with amino acids.

We initiated these experiments with the aim of finding a less complex method for peptide synthesis. In prebiological evolution this might have preceded the present complex protein synthesis. I am no longer too happy with this proposition, but recently I was encouraged not to forget about it. I received a note from Dr. Onsager (cited in 75) indicating that since his retirement from Yale he had moved to Miami and become productively interested in the origin of life. In a talk at a Nobel meeting in Lindau in 1973, he apologized in his note for having discussed a thioesterification of carboxylic acids for activation as an early event in prebiology. He had gotten this idea without knowing about our work on antibiotic synthesis. At a later meeting, I had the opportunity to talk with him. I was pleased with this contact, since he had spent a longer period at Rockefeller, where I had observed and liked him, but had never spoken with him.

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