Lipmann Symposium

Lipmann Symposium

Energy, Regulation and Biosynthesis in Molecular Biology

Editor Dietmar Richter

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Fritz Lipmann

Dedicated to the 75th Birthday of Dr. Fritz Lipmann

Preface

Dr. Fritz Lipmann celebrated his 75th birthday on June 12th, 1974. To mark this event a symposium was arranged and took place from July 7th to 9th at the Max-Planck-Institut für Molekulare Genetik in Berlin-Dahlem, the city where, fifty years ago, the career of this great scientist began. About 80 Lipmann alumni, guests and friends of the Lipmann family foregathered to meet their teacher and mentor, to discuss the past, present and future of biochemistry, and to exchange memories of their days in the Lipmann Laboratory. They came from all over Europe and the United States, and from countries as far away as Chile, Japan, and Australia.

Because of the special nature of this versatile biochemist, the lectures of this Symposium covered many fields of biochemistry. The book begins with vivid and amusing accounts by the two "old-timers" of biochemistry, Sir Hans Krebs and Fritz Lipmann of life and conditions in the "Golden Twenties" in the laboratories of Warburg and Meyerhof at the former Kaiser-Wilhelm-Institut. The volume closes with the lecture on "Structures of Biotin Enzymes" by Dr. F. Lynen. This lecture marks the beginning of a new event in the scientific calendar, the "Fritz Lipmann Lecture", which will now be given annually, and in this connection I am especially grateful to the Boehringer Mannheim Corporation for their financial support.

As editor of this volume I am indebted to Dr. Fritz Lipmann for the time that I spent in his laboratory at the Rockefeller University in New York. As Sir Hans Krebs so aptly put it, "The desire of the followers to express a sense of loyalty, gratitude and affection" was by no means the least of the reasons why the Symposium was such a success.

It is one thing to conceive the idea of arranging such a Symposium, but its management and organization is quite another matter; it has required the assistance and support of a number of people and institutions whom I would like to thank. In particular, I would like to mention Dr. F. Lynen and Dr. H.U. Bergmeyer, both of whom made the initiation

of the annual Fritz Lipmann Lecture possible; also Dr. E. Helmreich the chairman, Dr. E. Auhagen the treasurer, and Dr. H. Gibian the secretary of the Gesellschaft für Biologische Chemie. The meeting would have been much more difficult to organize without the advice and cooperation of Dr. H.G. Wittmann. The financial support given by the pharmaceutical companies Schering AG and Boehringer Mannheim Corporation, as well as the Volkswagen Stiftung, Max-Planck-Gesellschaft, Gesellschaft für Biologische Chemie and Walter de Gruyter Press is gratefully acknowledged.

The planning and organization would have been inconceivable without the help, support and continuous encouragement of my wife Heidi. For this I am very thankful.

I am grateful to the Walter de Gruyter Press for making it possible for us to publish the contributions in their entirety in a special birthday volume which is dedicated to Dr. Fritz Lipmann with best wishes.

I do not want to end this Preface without remarking on how Dr. Lipmann appeared to his former colleagues, many of whom may not have seen him for some years; everyone was pleased to see that his own personal store of high-energy phosphate bonds appears to be sufficient to keep him going for a good while yet. We wish him sincerely many more creative and energetic years.

July 1974 Dietmar Richter

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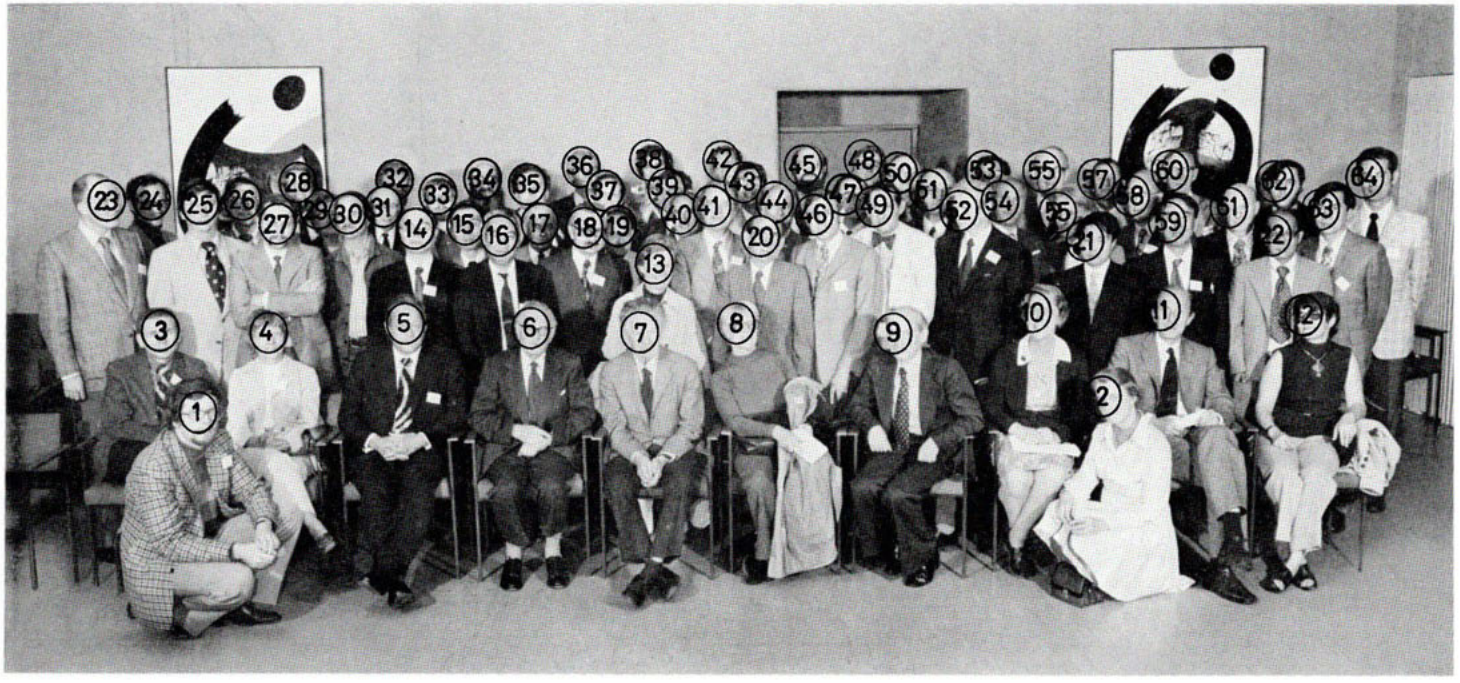

Lipmann Lab Photo in Berlin 1974:
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Welcome to Professor Lipmann

Ernst J. M. Helmreich
Vorstand der Gesellschaft für Biologische Chemie

It is indeed a memorable event to honor Professor Fritz Lipmann at the occasion of his 75th birthday here in Berlin. In 1970 Professor Lipmann wrote a personal account of his life as a Biochemist which was published by Wiley Interscience 1971 with the title: "Wanderings of a Biochemist". This delightful little book, because it is so honest and unpretentious, has impressed me as I am certain it has impressed every Biochemist who has worked at least in part during this period. Moreover, it makes clear why to honor Professor Lipmann here in Berlin, fits the occasion because it was in Berlin at the Kaiser Wilhelm Institute for Biology at Dahlem, when he joined Otto Meyerhof's Laboratory in 1927 where he was exposed to perhaps the most important intellectual influences in his life which have molded his scientific philosophy and interests. As Professor Lipmann writes: "In the Freudien sense all that I did later was subconsciously mapped out here and it started to mature between 1930 and 1940 and was more elaborately realized from then on". It is not my task to enumerate the many discoveries which Professor Lipmann and his associates have made over the years in so many different areas of Biochemistry. They are biochemical history and are taught all over the world to students. When I have selected a few examples of Professor Lipmann's contribution it is obvious that my choice was arbitrary and dictated by my own research interests. But perhaps in a time where many younger colleagues and students lack sense for historical relationships, it might be worthwhile to show on a few examples how influential Professor Lipmann's work was. This might help to arrive at a more balanced view of the events which set the pace and helped shape the Biology of our age.

Among the two examples which I have chosen one dates back to 1932/33. It was the characterization of the phosphoryl seryl ester bond in certain proteins. Professor Lipmann writes in his memoirs: "When 40 years ago I chose to probe into the binding of the phosphate in these phosphoproteins,

unwittingly, it now turns out, I struck a gold mine". Phosphorylation not only of enzymes but of a variety of proteins, so different as the histone and non-histone proteins of chromatin, or membrane and contractile proteins are among the most interesting control devices which have emerged in the evolution of living systems. However, these and many other important discoveries are overshadowed by perhaps Professor Lipmann's most important contribution. That is the concept of the energy rich bond and the idea of a phosphoryl group transfer potential. There are some of us here who had the honor to be invited to contribute to the Fritz Lipmann dedicatory volume "Current Aspects of Biochemical Energetics" which was edited by Nathan O.Kaplan and Eugene P. Kennedy at the 25th Anniversary of the Publication of Fritz Lipmann's classic paper on the "Metabolic Generation and Utilization of Phosphate Bond Energy". On the cover of that book it was rightly stated that this contribution had opened the way to an understanding of the energetic basis of biosynthetic reactions and greatly clarified the relation between exergonic and endergonic processes in the living cell.

But aside from his scientific achievements there is another aspect which should not be forgotten at this occasion. We only have to look at the program of this Symposium to realize what Professor Lipmann has done for Biochemistry, especially also for European Biochemistry. The contributors to the Scientific Program of this Symposium are his students and biochemical research in Europe has greatly benefitted from them. Many of them are Heads of Departments or lead independent research groups. I am pleased to announce today that the Gesellschaft für Biologische Chemie as a token of our gratitude has installed an Annual Fritz Lipmann Lecture made possible by a generous financial contribution of Boehringer Mannheim Company. I am especially indebted to Professor H. U. Bergmeyer without its help this would not have been possible. Professor Lynen will give the first Fritz Lipmann Lecture at the conclusion of this symposium.

As a biochemist, coming from Würzburg I was thinking about something related with that University and with Biochemistry and which might be of interest to Professor Lipmann. Then I recalled that my colleague Guido Hartmann sometime ago when he was still at Würzburg University had presented me with reprints of two papers published by Robert E. Kohler

from the Department of History of Science at Harvard University. The papers appeared in the Journal of the History of Biology 1971, 1972 and are entitled: "The Background to Eduard Buchner's Discovery of Cell Free Fermentation and the Reception of Eduard Buchner's Discovery of Cell Free Fermentation. As Professor Lipmann writes in his recollections: "What I delight in here is Buchner's preoccupation with the accidental". Kohler's historical study quite convincingly shows that Hans Buchner, who was an Immunologist and Professor of Medical Microbiology and Public Health in Munich was the intellectual driving force and that the discovery of zymase actually emerged from Hans Buchner's immunological studies. Eduard Buchner's experiments were actually undertaken to elucidate the role of Protoplasmic proteins for immunity. It was in the course of these studies that Eduard Buchner who later became professor of biochemistry and organic chemistry in Würzburg together with Martin Hahn developed a method for getting press juice which facilitated the study of intercellular enzymes and proved cell free fermentation in 1897. This discovery has long been recognized as the resolution of one of the most famous scientific controversies of the 19th century, namely the controversy between Louis Pasteur and Justus von Liebig over the nature of alcoholic fermentation. Therefore Guido Hartmann and I, two Biochemists from Würzburg take the pleasure to present to Professor Lipmann today together with these two reprints a reprint of a paper "Über Zellfreie Gärung" in which Eduard Buchner himself gave an account of his discovery in a lecture given 1909 and published in the Zeitschrift des "Österreichischen Ingenieur und Architekten Vereins". In this lecture Eduard Buchner said: "Der misslichste Punkt ist aber jedenfalls der, daß wir durchaus nicht in der Lage sind, die Enzyme zu isolieren. Bei Reinigungsbestrebungen ist der erste Erfolg meist der, daß auch die Wirksamkeit des betreffenden Präparates abnimmt". This sounds familiar to every enzymologist but it also shows that thanks to Professor Lipmann and the biochemists of his time we have finally solved this and many other problems because we have followed Goethe's advice with which Eduard Buchner concluded its lecture. He said: "Wenn aber einmal unsere Bemühungen tatsächlich jahrelang ohne Erfolg bleiben sollten, dann lassen Sie uns recht der Worte Goethes eingedenk sein: "Der Mensch muß bei dem Gedanken verharren, daß das Unbegreifliche begreiflich sei; er würde sonst nicht forschen!"

Before Professor Wittmann our host here in Berlin will officially open the Fritz Lipmann Symposium which was organized by Dr. Dietmar Richter, I wish to acknowledge the very generous contributions of the VW Foundation, the Schering A.G. and the Walter de Gruyter Verlag, Berlin. I am sure that all the participants are looking forward to be with friends and for having the opportunity to discuss science and reminisce about the time they spent together and with Professor Lipmann in the laboratory. I wish Professor Lipmann, his students and his friends a very good and enjoyable time while here in Berlin.

Welcome to the Max-Planck-Institut

H.G. Wittmann
Max-Planck-Institut für Molekulare Genetik
Berlin-Dahlem, Germany

Dr. Lipmann, Ladies and Gentlemen:

It gives me great pleasure to welcome you all to the Max-Planck-Institut für Molekulare Genetik in Berlin-Dahlem. It was here in Dahlem where Fritz Lipmann, our guest of honour today, began his scientific career as a biochemist almost fifty years ago. At that time, Dahlem was the scientific center in Germany. This was mainly due to the work done in the various Kaiser-Wilhelm-Institutes located here. You will hear more about this period of time in the next two lectures.

At the end of the war, many of these institutes moved from Berlin to West-Germany. During the last 25 years, the number of the institutes (renamed the Max-Planck-Institutes) increased in number to about 50 staffed by approximately 11.000 scientists, engineers and technicians. If you are interested in obtaining more details about the Max-Planck-Gesellschaft and the institutes, there is a booklet available containing information about the organization and the budget of the MPG, as well as the scientific work done in each of the institutes.

The work in our institute is mainly concerned with the biochemical and genetic aspects of nucleic acid and protein biosynthesis in bacteria and phages. There are about 200 people (scientists, technicians and graduate students) working on the following subjects: DNA replication; prophage-induction; DNA-membrane interation; mechanism of cell division and conjugation; recombination, transfection and transformation; structure and function of ribosomes, which especially includes studies on the primary structure of ribosomal proteins, on the topography of the subunits and on the functional role of the ribosomal components; protein-RNA interaction; mechanism and re-

gulation of translation; DNA dependent protein biosynthesis in vitro. Besides these studies on prokaryotes there are two groups working on eukaryotic systems, namely on phototropism in Phycomyces and conjugation in Ciliata.

Anybody who is interested in details of the work done in our institute, he is welcome to either approach directly the group in which he is interested or, if he is unsure where to go, please come and see me so that I can make the appropriate introductions. Please regard our institute as an open house and we will try our best to make your visit in Berlin and in our institute as enjoyable as possible Let me end by wishing all of our guests a pleasant time in Berlin and Dr. Lipmann a very happy seventy-fifth birthday.

Dahlem in the Late Nineteen Twenties

Hans A. Krebs and Fritz Lipmann

Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, England and The Rockefeller University, New York, USA

Hans A. Krebs

It was Dr. Richter who suggested that I might reminisce on the life at Dahlem in the days when Fritz Lipmann and I were working here in the laboratories of Otto Meyerhof and Otto Warburg. I was in Dahlem from 1926 to 1930, and Fritz Lipmann from 1927-1930. So I will make an attempt to convey something of the atmosphere, of the working and living conditions, of our attitudes towards our work and of our outlook in general.

The Kaiser Wilhelm Gesellschaft

Dahlem was at that time the main campus of the Kaiser Wilhelm Gesellschaft (in 1948 renamed Max-Planck-Gesellschaft). This Society was initiated in 1910 with the intention of providing outstanding scientists with first-rate research facilities. The attitude of the founders was clearly expressed by Emil Fischer when he tried to persuade - successfully - Richard Willstätter to abandon his professorship at Zürich and to join the Society. Fischer, according to Willstätter (1), described the attitude in these words: "You will be completely independent. No-one will ever trouble you. No-one will ever interfere. You may walk in the woods for a few years, if you like; you may ponder over something beautiful". On the whole this policy (based on utmost care and competence in selecting the right people) has paid magnificent dividends: Otto Warburg, Otto Meyerhof, Albert Einstein, Max von Laue, Fritz Haber, Otto Hahn, Lise Meitner, Carl Erich Correns,

Richard Goldschmidt, Michael Polanyi, Carl Neuberg and many others made the fullest use of the opportunities.

By the late 1920's, within 15 years of its foundation, and despite the upset caused by World War I, Dahlem had become one of the world centres of scientific research. Not only did it attract many of the best scientists in Germany but also young people from all over the world.

Collaborators of Warburg and Meyerhof, 1926-1930

During my stay at Dahlem the people working in the laboratory of Warburg included Erwin Negelein, Hans Gaffron, Robert Emerson, Fritz Kubowitz, Werner Cremer, Erwin Haas, Walter Christian, Walter Kempner, Akiji Fujita and several other Japanese. Meyerhof's laboratory, accommodated in the same building, a few steps away, included Karl Lohmann, Karl Meyer, Fritz Lipmann, Hermann Blaschko, Severo Ochoa, Frank Schmitt, Ralph Gerard, Dean Burk, David Nachmansohn, Louis Génévois, Ken Iwasaki. Other young biologists working in the same building were Victor Hamburger, Curt Stern and Joachim Hämmerling. Many of these left their mark on later scientific developments.

Achievements of the Laboratories of Warburg and Meyerhof in the Nineteen Twenties

The discoveries made in the middle and later part of the 1920's in Warburg's laboratory included the discovery of the aerobic glycolysis of tumours, the general occurrence of the Pasteur effect, the accurate quantitative measurements of cell respiration and cell glycolysis, the carbon monoxide inhibition of cell respiration and the light sensitivity of this inhibition which made it possible to measure the action spectrum of the oxygen transferring enzyme in respiration (now referred to as cytochrome a_3) and to identify the catalyst as an iron porphyrin, the development of spectrophotometric methods of analysis (twenty years later commercially incorporated by Beckman into his black box), the discovery of copper in blood serum and the fall of its concentration in anaemias.

Meyerhof's laboratory made decisive contributions to what is now called the Embden-Meyerhof pathway of gycolysis. It laid the groundwork leading to the discovery of hexokinase,

aldolase and other enzymes. Monumental discoveries by Lohmann were those of ATP, first identified as a cofactor of glycolysis and of the "Lohmann reaction" - the interaction between ATP and creatine.

One of the secrets of these outstanding achievements in both laboratories was the creation of new methods, such as the tissue slice technique, manometry and spectrophotometry by Warburg, and Lohmann's method of distinguishing between the many different phosphate esters by measuring their rate of hydrolysis at 100° in 2 N HCl.

Now, some 45 years later, we can assess the achievements of Warburg and Meyerhof in proper perspective. Many scientific papers may seem to be very important at the time of their appearance but as the field develops it is appreciated that they were less significant than was at first thought; as time goes on the really significant contributions stand out as lasting landmarks.

An amazing feature of the teams of Warburg and Meyerhof was their smallness. Altogether there were hardly more than two or three dozen people who participated in these great developments I have listed. Meyerhof had four or five small rooms and the total number of his collaborators at any one time was not more than five. There was only one trained technician, Walter Schulz, and a part-time typist. The technician was Meyerhof's personal assistant and together with a "Diener" he looked after the general laboratory affairs such as maintenance of apparatus and ordering of materials. When I joined Warburg there was one large room for six people in all, plus several instrument rooms, plus one Diener . Warburg had no secretarial help - we all typed ourselves. There was no technician in the ordinary sense of helpers. It is true Warburg's long-standing collaborators - Negelein, Kubowitz, Christian, Haas - were originally technicians but not in the ordinary sense. They were research assistants who had been primarily trained as instrument mechanics in work shops in the factories of the Siemens Company in Berlin. They knew how to handle instruments and how to make accurate measurements, and Warburg taught them all the chemistry they needed. In 1928 Warburg obtained one more room for four extra people and

when he moved into his new Institute in 1931 there were a few more places but the total number remained deliberately small. Meyerhof of course also had more space and more staff when he moved to Heidelberg in 1930.

To those who participated what we did seemed to us quite normal and natural, a matter of course. Warburg, however, was always fully conscious of the monumental nature of his contributions. He has stated this in writing more than once, for intellectual modesty was not his strongest point. He considered himself to be in direct line with the giants of biology and in particular the chemically orientated biologists - a direct successor of Pasteur, with no-one of comparable calibre between him and Pasteur (1822-1895). Meyerhof appeared much more humble, and not concerned with his own assessment of his position in the history of science. He was content to leave this to his peers and to posterity.

And not only did the genius of leadership by Warburg and Meyerhof make outstanding contribution to the subject and inspire, the small band of deeply motivated and committed young collaborators. In the process Warburg and Meyerhof also educated (I do not say "train" because "educate" means more than train; educating includes the transmission of an outlook, not merely of technicalities) a future generation of leading scientists. This happened without actually aiming at doing it, without any policy of postgraduate training programmes. It happened naturally, and I believe something of this sort will always happen naturally. Born leaders attract born followers who develop into leaders - as long as bureaucracy and the erroneous concepts of equality do not interfere.

Day-to-Day Life in the Laboratories

We all worked very hard and intensively, though the atmosphere was relaxed. In Warburg's laboratory the working hours were from 8 a.m. to 6 p.m. for six days a week. Most of the reading and most of the writing had to be done at home in the evenings, at weekends and during the summer vacation. Warburg and Meyerhof were in attendance more or less all the time and always accessible to their collaborators. There were no committee meetings and hardly any academic tourism. There was a brief luncheon interval where the younger people from different departments (especially from

the laboratories of Warburg and Meyerhof) met in a common room for a simple snack consisting usually of eggs, sandwiches and milk. Coffee and tea breaks were unknown. The main vacation was rather long. Warburg closed his laboratory for 8 weeks during August and September but during this time he wrote most of his papers while on his estate in the Island of Rügen. Warburg liked to point out that the working hours were much less than they had been in his younger days. When he worked in Heidelberg in Krehl's Department of Medicine, Krehl often made a round of the laboratories on Sunday evenings and expected most of the workers to be in attendance. In Meyerhof's laboratory working hours were less rigid but hardly shorter.

Warburg's control of the laboratory was very autocratic but we never questioned the justification of his authoritarian rule because we thought he was entitled to this on account of his outstanding intellect, his achievements and his integrity, qualities which we admired enormously. On the whole his rule was benevolent but it could also be fierce. On one occasion he dismissed a research worker instantaneously after an incident in which Warburg thought he had not shown proper respect and courtesy. For Warburg autocratic control was essential in the interest of high standards of the work as well as of personal conduct. His was autocratic rule at its best. He never exploited the junior, as does autocratic rule at its worst. Democratic rule may at best make full use of the pooled resources but at worst it may create a situation where ignorance and obstruction prevails over competence and efficiency. Warburg was most generous in giving credit to his collaborators. Many pieces of research to which he had made the main contribution and which he had written were published without his name, except perhaps in an acknowledgement by the author. A review of his work (2) which established the oxygen transferring catalyst of cell respiration as an iron porphyrin ended with the passage "In concluding I wish to emphasize that the results which I have presented are largely due to the work of my collaborators, Drs. Negelein and Krebs". Yet the whole work was conceived by Warburg himself and the greater part of the critical experiments were carried out by him with his own hands.

I know of at least one specific incident where Meyerhof was

also very fair. In 1929 Lipmann had discovered (after Einar Lundsgaard had reported muscular contractions without lactate production in the presence of iodoacetate) that on anaerobic contraction muscle becomes initially alkaline even in the absence of iodoacetate, the rise of pH being due to the hydrolysis of creatine phosphate. Lipmann measured the pH change manometrically by the uptake of CO_2 which reacts with the OH^- ions formed.

This was an important finding because it helped to establish the now generally accepted concept that creatine-P, through the Lohmann reaction, can energise contraction. As it was very important to him to find a job he was anxious that he should get proper recognition and he said in a somewhat resigned spirit to one of his colleagues, Hermann Blaschko. "This will be just another paper by Meyerhof and Lipmann". Blaschko then encouraged Lipmann to ask Meyerhof whether he may not be the first author. Meyerhof's immediate reply was "But of course" (3). And Fritz was the sole author of a second paper printed directly after the joint one, in which he showed that fluoride can act similarily to iodoacetate (4).

Financial Position of Young Research Workers

Our financial position was very restricted and quite a few people in the laboratory received no salary or grant at all. Hermann Blaschko tells me that when he asked Meyerhof to be accepted in his laboratory Meyerhof eventually agreed to have him but he told him "I cannot give you any payment" whereupon Blaschko replied "I did not expect one". Fritz Lipmann was not paid during the first 2 years of his stay with Meyerhof, nor was Severo Ochoa paid. I was lucky and received a starting salary of 300 marks which rose to 400 after one year. It is difficult to equate this with present prices but it meant that we had to live frugally and count every penny. If we were careful we could afford one modest holiday a year; we could afford concerts and theatres in the cheapest seats. There were no travel grants for attending meetings. This did not mean that we were isolated because there were plenty of opportunities for learning something about new scientific developments within Berlin itself, through the colloquia at Dahlem and through the Berlin Chemical and Medical Societies. Who then, financed our maintenance? As far as I know our parents, even though they could ill afford

it, for inflation had devalued the pre-war Mark by a factor of 10^{12} (a million million) by the end of 1923. Parents were willing to make sacrifices for a good training of their sons. Of course, we felt very uncomfortable to be a burden to our parents when we were between 25 and 30 years of age. Franz Knoop impressed me in 1920 by saying during his lectures to medical students that he had earned nothing until he was 37 years of age, although he had made an outstanding discovery - that of β-oxidation - when he was 30. It was understood that an academic career meant willingness to put up with very modest material standards of living. We were motivated by a keen dedication to our work and we were maintained by the hope that when we had received a thorough training - which we expected would last until we were about 30 - we would eventually get a worthwhile job - satisfying professionally as well as financially. The sacrifices needed for a long period of training meant also that only the keenest did not give up. Most of us were medical graduates and could, if we wanted to, at any time branch off into a relatively lucrative medical career - but we preferred research.

The financial and some other aspects of the scene at Dahlem were of course not unique. Erwin Chargaff - my contemporary working at that time in Vienna - recently wrote (5) "No-one who entered science within the past 30 years or so can imagine how small the scientific establishment then was. The selection process operated mainly through a form of an initial vow of poverty. Apart from industrial employment in a few scientific disciplines, such as chemistry, there were few university posts, and they were mostly ill-paid".

Nowadays there are many undergraduate students who insist on their "rights" to be fully supported by the state. We expected no rights even at the post-graduate and post-doctoral level. We were satisfied if we could work hard under reasonable conditions and learn. We did not feel entitled to expect much, let alone make demands, before we had learned a lot.

In spite of our restricted economic circumstances we were on the whole very happy because we felt that we were receiving a first rate training and were doing something worthwhile. We had no undue worries about our long-term future

although this looked very uncertain in view of the economic and political difficulties in Germany. I am not aware that any of us anticipated a particularly successful career. Being close to giants of science we felt very small and Warburg himself did not do much to encourage our self-confidence. In fact when I had to leave his laboratory he told me that he considered my chances in biochemistry as slight and advised me to return to clinical medicine (which I did).

Relations between Dahlem and German Universities

Of a peculiar sort were the relations of Warburg and Meyerhof to the official representatives of German physiology and biochemistry, i. e. the German university departments. Warburg regarded himself as an outsider, and Meyerhof too, but perhaps less so. It is remarkable that German universities had not appreciated officially Meyerhof's qualities. By the time he got the Nobel Prize in 1923, at the age of 39, he held the post of an assistant in the Physiological Institute of Kiel University; he had been passed over for a junior professorial appointment (Professor Extraordinary) in favour of a man called Pütter, whose claim to distinction remained slight. After the award of the Nobel Prize to Meyerhof, Warburg succeeded in persuading the Kaiser Wilhelm Gesellschaft to offer Meyerhof a post.

This sense of being outsiders had to do with the Cinderella treatment of biochemistry by the German universities. The number of chairs and departments of biochemistry or physiological chemistry was very small in Germany. Independent departments existed in four universities only, in Frankfurt with Embden at its head, at Freiburg, Tübingen and Leipzig. In other universities biochemistry was a sub-section of physiology and the heads of these sub-sections did not have the rank of full professor. In some universities the professor of physiology was essentially a biochemist. This applied, for instance, to Heidelberg where Kossel was the Professor of Physiology and where first-rate work was done on protein chemistry.

Thus it came about that Leonor Michaelis, one of the brightest biochemists of his time could not be absorbed into the German university system and therefore left Germany in 1921 for Japan, to move later to Johns Hopkins University and the

Rockefeller Institute. While in Germany he had to earn his living as a clinical biochemist in one of the municipal hospitals in Berlin.

Hopkins, in a general address at the International Congress held in Stockholm in 1926 commented on the neglect of biochemistry in German universities and spoke about the importance of "specialised institutes of general biochemistry". He referred to the appeal by Hoppe-Seyler in Volume 1 of his Zeitschrift für Physiologische Chemie in 1877 that institutes of biochemistry should be generally set up in the universities. In 1877 the only institute of this kind was that in Strasbourg with Hoppe-Seyler at its head. Hoppe-Seyler's appeal was at once opposed by the physiologist E. Pflüger in his Journal, and 49 years later Hopkins remarked that in fact Hoppe-Seyler's appeal for the recognition of biochemistry as an independent subject had still not yet found proper response in his own country and he added "It is difficult to see how Germany can continue the lead along the path which for a long time she has almost trod alone". He emphasised that academic centres in general in Europe are in this respect behind those in America. These remarks, incidentally, were made at the suggestion of F. Knoop for the benefit of the German readership and they were reprinted in translation in the Münchener Medizinische Wochenschrift (6).

Now and Then

Today the scene of scientific research seems very different. In the 1920's pure research was still widely looked upon as a luxury or extravagance that did not deserve major support from the State. The Kaiser Wilhelm Gesellschaft, after all, was founded in 1910 as a private organisation (while the German Universities were all state-controlled).

Now, 50 years later, scientific research is regarded a necessity for the survival of a nation, and large sums are provided by Governments for training people and for pursuing research.

But although the scene has changed some fundamental principles governing successful research are still the same, and will always remain the same - the recognition of leadership in research, the value of long training, the need for hard

work and for dedication, an attitude of humility.

What has gone, among other things, are the biting polemics in science in which Warburg liked to indulge, hurting and ridiculing the opponents - there are many examples of these in Warburg's books and in the Biochemische Zeitschrift (7). Gone, also, has the autocratic rule which Warburg, his teachers and his contemporaries practiced. This kind of benevolent (or mostly benevolent) dictatorship at its best as I already stated, helped to maintain high standards - but it could easily degenerate into arbitrary injustices, exploitation and mismanagement.

Today a different basis of the relations between seniors and juniors has evolved. The master may still rule, and rule firmly, but the basis of his authority is now a natural respect, a natural mixture of admiration and affection which he has earned by his work and conduct; in a good laboratory authority is no longer based on the power invested in a head of a laboratory. I think this very occasion here is an illustration of the kind of human relations between the master and his followers which nowadays exist in a really ideal team. It was the desire of the followers to express a sense of loyality, gratitude and affection which has brought this symposium into being. This assembly of so many people from so far away, motivated by mutual goodwill, I find deeply gratifying and moving, especially here today in Dahlem where, within a stone's throw in the Free University, I am told, senseless and unpleasant confrontations between teachers and taught represent a menace to academic life, a menace liable to destroy the old academic ideals, the search, in a spirit of tolerance, for knowledge and truth.

The motives which brought us together remind me of a remark which Warburg made to me during a casual chat in the laboratory. For two years I shared an island bench with him, working opposite to each other in close proximity. Although both of us were not exactly talkative while preoccupied with our experiments there were occasional conversations touching on many aspects of life. One day he remarked "The worst defeat a scientist can suffer is to die early because the fruits of his labour mature very slowly". Although these words - like many other of his casual sayings are still deeply ingrained in my memory - I did not properly appreciate

their full meaning at the time. But today their meaning is clear to me. The fruits of a scientist's labour are of several kinds. Promotion to a good post and a Nobel award are some; a very important one is the long term response from students and collaborators. Much of a research scientist's time is spent in helping to shape the outlook, career and life of his juniors, and the seeds which he plants take a long time to grow. Today we see the rich harvest that has come to Fritz from helping and guiding his younger associates - a harvest not only in the form of gratitude and affection but also in the form of the intensely pleasing knowledge that there is a new generation willing to carry on the work and to uphold the standards and ideals which motivated the master.

Such kinds of thoughts, I suspect, must have been in Warburg's mind when he suddenly spoke about the importance of living to old age. Perhaps some experiences of his father (8) (a founder of a large and devoted school of physicists), had impressed him*.

* Further material on Dahlem in the 1920's and the personalities of Warburg and Meyerhof will be found in references (6), (9), (10), (11), (12) and (13).

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Fritz Lipmann

I am going now to describe my relationship to Dahlem when I was in Meyerhof's laboratory, and here I would also like to include Berlin. From my early youth Berlin, the great city, had been for me a magnet. I was born in a small town, Koenigsberg, then in East Prussia, and my first contact with Berlin was after absolvation of my abiturium, as we called the final examination ending gymnasium time and giving the right to enter into University. Then, my parents gave me as a gift, a week all on my own in Berlin to experience the theater and to experience the great city. I spent more time in Berlin later on studying medicine, and again I was impressed by the experience of what happens in that city.

And then I came back to Berlin for quite a long period after I had finished my medical studies. Still under the influence of medical friends, I spent the latter half of my practical year there, and the first three months with Ludwig Pick, an excellent pathologist, because it was thought that to become a physician one had to do some pathology. Then I heard about

a biochemistry course which was given at the Charité, the Medical School of Berlin University, where a large number of physicians who became very good biochemists, including Hans Krebs and David Nachmansohn, were trained. That was Rona's laboratory. When I went to Ludwig Pick to tell him that I would spend the last three months of my practical year taking the biochemistry course of Peter Rona, he threw his hands up in amazement. Biochemistry then was still an unknown entity in Germany.

Rona's name is probably known to very few of you; for quite a while he was a collaborator of Leonor Michaelis. One of the reasons I wanted to mention him was that I found an amusing picture of the members of the course when I took it and I would like to show you this (Figure 1).

Figure 1

In the center is Peter Rona; in the middle is H.H.Weber as a young man - he died a few weeks ago; and in the top right corner am I, with a bow tie - some people are disappointed that I don't wear it any more as I used to do in early years.

I think it was an extraordinary course. I learned there the latest advances in the biochemistry of that time: manometry, pH measurement, electrophoresis, and so on. Actually, I stayed on with Rona and did a medical doctorate thesis which

was obligatory in Germany. It did not need to be very important. Mine was on the electrophoretic behavior of iron oxide colloids, mainly concerned with the reversal of the positive charge to negative in the presence of citrate. Colloid chemistry was very modern in those days - many people used it to describe the protoplasm. It seemed enough then to call it a colloid to imply one understood something about it. We have learned better.

That was in 1921-1922. Then I decided to go back to my home town to learn chemistry since I was lucky enough to be able to do this with Hans Meerwein who was the professor of chemistry at the University. I should guess that his name is known to those of you conversant with organic chemists. He was a superior chemist, and later moved from Koenigsberg to Marburg. In three years I learned a great deal, particularly from lectures; all during those years all students had to attend his lectures and it was a tremendous pleasure. He gave them all himself; there was no substitution by assistants, and that gave us students a contact with his personality.

Then I got my Verbands-examen, which enabled me to start on a thesis. After that first step was finished I became somewhat restless. I felt it was now time for me to find a place where I could do biochemistry, for which I had been preparing myself all this time. It was not without other reasons that I chose Berlin; but I was mostly motivated by the existence in Berlin-Dahlem of the two institutions which at that time seemed to me to do work in the field I had begun to become interested in, intermediary metabolism. These were the laboratories of Carl Neuberg and Otto Meyerhof, and I debated for a time whether I should join Meyerhof or Neuberg. I eventually decided for Meyerhof because of his much more physiological leaning, and just as Hans Krebs has told you, I likewise didn't expect any salary and for the first two years I didn't get anything. I just asked him if I could work there and as I had studied chemistry and had some experience I was lucky enough to be accepted; he asked me, interestingly enough, if I had any problem to work on and I was ashamed to say that I hadn't. I had to get a problem from him.

The problems I worked on in the early days there were not very important. I did some work on fluoride inhibition

of glycolysis and fermentation, which was published in Biochemische Zeitschrift. These papers I could put together and use as my chemical doctor's thesis. It reflects interestingly on the status of the heads of laboratories at the Kaiser Wilhelm Institutes that Meyerhof was unable to be my doctor "father" because he couldn't accept graduate students although he was a titular professor. However, Neuberg, whose institute was next door, could; he was a professor at the Technische Hochschule and I actually had to farm out my thesis, so to say, to Neuberg, who became my doctoral "stepfather" in a way. He always treated me very kindly.

I will now say a little about the Meyerhof laboratory. While I was sitting here just now and thinking about my choice of laboratory, I am almost surprised to discover that although most impressed by Warburg, I never even dared to think of going to work with him. One of the important aspects of Meyerhof was that he was not as stern and was much looser than Warburg. But he was Warburg's pupil and there is a paper by Warburg and Meyerhof which came from the Naples Laboratory. The Marine Laboratory in Naples was one of the meeting grounds for biochemists in the same sense that Woods Hole is or was in the U.S.A. In his earlier years Meyerhof tended to be very interested in philosophy, had joined a school of philosophers, and wrote several papers of a philosophical character. It was the influence of Warburg, I understand, that decided him to become a biochemist; and he took all the traits of Warburg, the feeling that to do good work one needs the most exact methodology and has to have full confidence in one's results.

The work in his laboratory, as you have heard already, centered about the muscle and I worked during that time largely with muscle or muscle extracts, mostly related to glycolysis. It is only in the later period after the laboratory had moved to Heidelberg that I did a fairly nice piece of work on a determination of creatine phosphate breakdown in living muscle which I measured manometrically. These manometers that I used were somewhat difficult to construct because I wanted to stimulate the muscle in the manometer and one had to seal in platinum electrodes, which was very hard to do without a leak. This work started when Meyerhof suggested that I should try to see what happens in muscle contraction at relatively high acidity, that is, in a bicarbonate solution with CO_2

in the gas phase. It was then that I found, during the early phase of a series of contractions, an alkalinization, i.e., manometrically, CO_2 absorption instead of the expected CO_2 liberation from lactic acid formation. Chemical analysis of the muscle showed that the alkali that formed early corresponded very nicely to a creatine phosphate breakdown; this had been found to yield alkali because of the strong alkalinity of the guanidinium base liberated. This was an early proof that without inhibitor under these acid conditions creatine phosphate breakdown could cause contraction.

Figure 2

It is now time to show you Meyerhof. This picture (Figure 2) is rather typical because it shows that it was not easy to approach Meyerhof. We actually talked very little and what I learned from him was largely by diffusion. But this was to influence me all through my life. This picture of us together was taken in 1941 at a conference in Madison, and you might say we look a little uneasy, which was because I had given a lecture there on the Pasteur effect and had shown disagreement with his interpretation, and he wasn't too happy about it.

Our working hours were much more relaxed than those at Warburg. I remember that we took pretty long lunch intermissions and went rather often to a little restaurant, which

was next to a further-up subway station, and sat there and happily talked in the garden. When I say we, in the next picture (Figure 3) you can see some of the people who were "we".

Figure 3

You can see, from left to right, Ken Iwasaki, Karl Lohmann, Walter Schultz, and Schroeder, who was something in between a scientific assistant and a diener;then David Nachmansohn and Paul Rothschild; and again me, this time with a long tie. Ken Iwasaki, Nachmansohn, Paul Rothschild, and I were the ones who often went to that little restaurant, and not only that, we even went together to masquerade balls which were very fashionable and much attented at that time; but they were very good entertainment and as free in spirit as in present-day terms. At one of them, the socialist ball, which had nothing to do with socialists, I met Freda Hall who was to become my wife. So that was an important event during my Berlin days. Actually, at this particular ball, David Nachmansohn danced more with Freda than I did, but he was already married.

Now to return to the laboratory. I had some contact with Ralph Gerard - I think we shared a laboratory when I entered the Meyerhof Laboratory; we met again in later life and I was rather fond of him. Then I moved into another labo-

ratory and worked very close to Ken Iwasaki who spent a good deal of time in Berlin and I am sure had a very good time there; he was not married then. I am told that Mr. Takeda, Masao's father, became a very good friend of Ken Iwasaki; they spent much time together in Berlin and are still very good friends. Ken Iwasaki is now retired from his biochemistry professorship and has a laboratory in the Takeda Company, which is one of the largest pharmaceutical companies in Japan.

The topics on which the work was done in Meyerhof's laboratory were not too varied; it was mainly concentrated on the muscle, but it also included nitrogen fixation on which Dean Burk and Ken Iwasaki worked. The status of our understanding at that time may be shown by what Karl Meyer did. He was trying, in parallel to what had been called zymase by Harden, to isolate the "glycolytic enzyme"; it wasn't quite realized then that the glycolytic enzyme was of course composed, as we now know, of numerous enzymes and that these enzymes could eventually be separated. That came not much later, but in 1928 it was really surprising that one could even aim at thinking of isolating as a unit something like a glycolytic enzyme.

Then, shortly before we moved away from Berlin, I worked for a little while with Lohmann, and I learned much from him. As you heard already, he really was an artist in determining by acid hydrolysis the different phosphorylated compounds that were in a mixture. For example, in this way he discovered the equilibration of glucose 6-phosphate with fructose 6-phosphate. The latter has a much faster hydrolysis time than glucose 6-phosphate, which is one of the most difficult to hydrolyze phosphate esters. On the other hand, fructose diphosphate is completely hydrolyzed within three hours. Lohmann used the acid hydrolysis of phosphate esters very effectively and, as I said, he discovered many new compounds.

I am very eager to show once in a while an experiment from which the wrong conclusions were drawn. Table I shows such an experiment that I did with Lohmann and which eventually became of great consequence. In this experiment fructose diphosphate was incubated in muscle extract, and I came in to try and see if this conversion of fructose diphosphate would also go without fluoride that was added in Lohmann's

Table 1: Conversion of FDP into acid - stable phosphate ester in muscle extract of winter frogs after incubation at 20°

Incubation (min)	Phosphate Bound (mg P_i)	Phosphate Acid-Hydrolyzed in 3 hr (mg P_i)	Converted Phosphate (mg P_i)	(%)
0	0.48	0.48		
20	0.50	0.39	0.12	25
60	0.50	0.25	0.27	53
120	0.49	0.19	0.33	68

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earlier experiments. The table shows the change of fructose diphosphate without fluoride to what was called a difficult to hydrolyze hexose diphosphate. One can see that by the incubation in muscle extract phosphate is not released. However, the hydrolysis time of the added fructose diphosphate goes up if one uses the three hours mentioned above as a standard. One can see that with time it becomes converted, and eventually 70% of it is present as what was thought to be a different hexose diphosphate which was much more difficult to acid-hydrolyze.

To turn a little more to the history of this part of biochemistry as I did in the meeting at the Ciba conference last week where I also mentioned these experiments, Nilsson had shown a little earlier in Euler's laboratory that in the presence of fluoride, the same fructose diphosphate, with a yeast preparation, when paired with acetaldehyde, gave phosphoglyceric acid as the oxidation product parallel with reduction of acetaldehyde to ethanol. This was the first appearance of phosphoglyceric acid in the picture of fermentation and glycolysis and nobody at that time could appreciate why this compound was formed as an oxidation product of fructose diphosphate. Nilsson came to the Meyerhof laboratory and we discussed this strange compound, phosphoglyceric acid, without seeing the light. The right idea was Embden's who essentially repeated our experiment. We had just made barium precipitates

of what we considered a hexose diphosphate. He found that it was surely difficult to hydrolyze but that it was not hexose diphosphate but rather a mixture of phosphoglycerol and phosphoglyceric acid which he assumed to be formed by a dismutation reaction. Thus, we had misinterpreted the conversion of fructose diphosphate. However, in the hands of Embden it became the reason why we now talk about the Embden-Meyerhof cycle; from this reaction he then concluded the disruption of fructose diphosphate into an equilibrium mixture of two triose phosphates and mapped out the foundation of our present scheme, recognizing phosphoglyceric acid to be the oxidation product of phosphoglyceraldehyde, the biochemically dominant of the two triose phosphates formed.

That's just a sidelight on Lohmann's artistry with hydrolysis. As I said, I learned much from him to handle what I did soon afterwards. When I went to New York to work with Levene, I chose the phosphoproteins as an object of investigation. You heard in Dr. Helmreich's talk that I there isolated serine phosphate from the egg yolk phosphoprotein. Lohmann was the one who suggested that I work with Levene. He had in mind, I think, that I should work on nucleotides, but I chose the mentioned topic because Levene had isolated a very phosphate-rich protein from egg yolk which attracted my interest. The methods I used were actually borrowed from what I had learned from Lohmann. It appeared that the phosphate in the yolk protein is alkali-labile but very acid-stable, and choosing acid hydrolysis as a means of degradation of the protein, I thus isolated the serine phosphate.

Much later, I turned to what was the prominent interest in Meyerhof's laboratory, namely, bioenergetics. This came to be an underground well, so to say, that eventually opened up after I discovered acetyl phosphate and led to my writing the paper on generation and utilization of phosphate bond energy.

I would like to close by saying a little more about Berlin in those days. Before going to New York, Freda Hall and I were married, and we were amazed to see that there was such an enormous difference in the way of life between Berlin and New York, particularly among young women and young men. We read in the "Saturday Evening Post" three articles by Hergesheimer, who was then a rather fashion-

able novelist, in which he described Berlin as the center of Europe: people didn't go to Paris, they went, rather, to Berlin. There was the theater, there was the music, there was the dance; you could have everything. There was also a great freedom in Berlin in the late twenties, a similar freedom to that which has developed in America in recent years. It was due to the breakdown of the family ties by which young people were held because they had to depend on their families, and it had the effect that the young men and women interacted much more freely with each other. After I had moved away from Berlin and from Germany, it took a long time to forget the way of life we had experienced there.

I have been very pleased and moved by this meeting's coming about, and don't want to end without thanking the many people who have made possible this happy get-together. I first want to mention Dietmar Richter who really had the lion's share in thinking of it, writing all the letters, and arranging the whole proceedings. I wish to thank Dr. Wittmann for providing this Institute as a place for us to meet, and Dr. Helmreich for his nice words and also for the encouragement and support from Gesellschaft für Biologische Chemie. And of course I am most grateful for the support of the pharmaceutical companies of Boehringer and Schering, and of the Volkswagen Foundation, without which we could not have had it.

Thank you all very much.

Cascade Control of E. coli Glutamine Synthetase

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One important mechanism for the regulation of glutamine synthetase activity in E. coli is the covalent attachment and removal of AMP from a specific tyrosyl residue in each of the enzymes 12 identical subunits (1-4). Adenylylation of a subunit converts it to a less active form dependent upon Mn^{++} (1). The enzyme's activity is thus controlled by the average number of adenylylated subunits per molecule which can vary from zero to 12. Both adenylylation and deadenylylation of glutamine synthetase are catalyzed by single enzyme, adenylyltransferase (ATase) (5). Adenylylation involves the transfer of AMP from ATP into an AMP-O-tyrosyl linkage (4), whereas deadenylylation involves a phosphorolysis of this bond to yield ADP (6). Although ATase catalyzes both reactions, its ability to adenylylate or deadenylylate glutamine synthetase (GS) is modulated by the regulatory protein P_{II} and metabolic effectors including α-ketoglutarate (α-KG), ATP, glutamine and Pi (4,7,8).

$$\begin{array}{ccc} \text{ATP + GS} & \xrightarrow[P_{IIA}]{\text{ATase}} & \text{AMP-GS + PPi} \\ & \downarrow\uparrow & \\ \text{AMP-GS + Pi} & \xrightarrow[\text{ATase}]{P_{IID}} & \text{GS + ADP} \end{array}$$

SCHEME I

The regulatory protein P_{II} exists in two interconvertible forms (7). One form, P_{IIA}, stimulates the ATase catalyzed adenylylation of glutamine synthetase, whereas the other form, P_{IID} is required for the ATase catalyzed deadenylylation (Scheme I). When P_{IIA} is incubated in the presence of UTP, ATP, α-KG, Mn^{++} or Mg^{++}, and another enzyme, uridylyltransferase (UTase), it is converted to P_{IID}; this involves the covalent attachment of UMP

to the protein (Reaction 1) (9).

$$UTP + P_{II} \xrightarrow[\alpha\text{-KG, ATP, } Mg^{++}]{\text{UTase}} UMP\text{-}P_{II} + PPi$$

REACTION 1

P_{IIA} can be regenerated from P_{IID} by a uridylyl removing enzyme activity (UR enzyme) (Reaction 2).

$$P_{II}\text{-UMP} \xrightarrow[Mn^{++}]{\text{UR enzyme}} P_{II} + UMP$$

REACTION 2

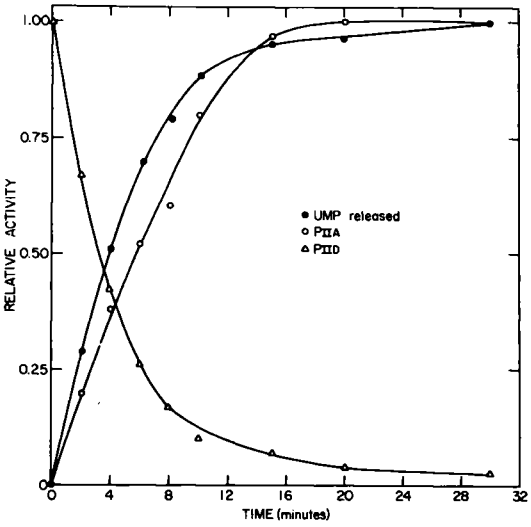

Figure 1. Reciprocal effects of UR-enzyme catalyzed deuridylylation of P_{IID}. H^3-UMP-P was incubated with partially purified preparation of UR-UTase. The P_{IIA} and P_{IID} activities as well as the release of [^{3}H]-UMP was followed. (Exp. details, see ref. 10).

Figure 1 illustrates the relationship between uridylylation and the capacity of P_{II} to stimulate adenylylation or deadenylylation of glutamine synthetase. When P_{IID} labelled with ^{3}H-UMP was

incubated with Mn^{++} and a partially purified extract containing UR enzyme activity, there was a rapid release of covalently bound ^{3}H-UMP from P_{II}. This release was accompanied by a parallel increase in the ability of P_{II} to stimulate the ATase catalyzed adenylylation of glutamine synthetase, and a concomitant loss in its ability to stimulate the ATase catalyzed deadenylylation of glutamine synthetase (10). The reciprocal changes in activity associated with deuridylylation of P_{IID} clearly demonstrate the role of P_{II} uridylylation in determining the adenylylation and deadenylylation capacity of ATase.

TABLE 1

Properties of the Regulatory Protein P_{II}

Property	Value
Molecular weight, native protein	44,000
Subunit molecular weight	11,000
Number of identical subunits	4
Number of tyrosines per subunit	2
Number of iodinatable tyrosines per subunit of P_{IIA}	2
Number of iodinatable tyrosines per subunit of P_{IID}	1
Moles of UMP per mole of P_{IID} subunit	1

The P_{II} protein has been purified to homogeneity from E. coli (11) and from Pseudomonas putida (12). Properties are shown in Table 1. The native protein has a mol. wt. of 44,000. Based on its homogeneous behavior during disc gel electrophoresis in the presence of SDS or 8 M urea (11) and during sedimentation in 6 M guanidine·HCl (13), and also the minimum mol. wt. calculated from amino acid composition, the native protein is a tetramer of identical subunits (11). This is supported also by the facts: (1) there are two tyrosyl residues per 11,000 mol. wt.; (2) following iodination of the tyrosyl residues of P_{IIA} with ^{125}I, tryptic digestion yields only two radioactive peptides in

equal molar amounts. Only one of these two peptides is obtained after tryptic digestion of iodinated, fully uridylylated P_{IID}. Since substitution of a tyrosyl hydroxyl group prevents iodination of the aromatic ring, this result indicates that in P_{IID} the UMP is covalently bound in phosphodiester linkage to the hydroxyl group of one of the two tryosyl residues in each subunit. This is supported also by the fact that treatment of P_{IID} with phosphodiesterase results in release of the covalently bound UMP and the stoichiometric appearance of phenolate ion (pH > 11.0) as measured by ultraviolet absorption spectroscopy (14). Thus, activity of P_{II} is modulated by covalent attachment of UMP to a specific tyrosyl residue in each subunit.

Table 2 shows the effect of cations and other effectors on the enzyme activities that catalyze the uridylylation of P_{IIA} (UTase) and the deuridylylation of P_{IID} (UR - enzyme).

TABLE 2

Effect of Divalent Cations and Other Effectors on UR and UTase Activities

Effector added	Relative Activity	
	UR activity	UTase activity
Mg, ATP, α-KG[1]	55%	100%
Mn, ATP, α-KG	85	100
Mn	100	0
Mg	0	0
Mg + Mn	100	0
Mg, ATP, α-KG, GLN	-	7

[1] The concentration of effectors used was: 1 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1 mM ATP, 5 mM α-ketoglutarate (α-KG), 18 mM glutamine, and 2 mM K_2Mg EDTA. These reaction mixtures also contain 2 mM K_2Mg EDTA to chelate traces of Mn^{++} that were present in the enzyme preparation. (For exp. details, see ref. 10).

Whereas Mn^{++} alone supports maximal UR activity, Mg^{++} cannot support UR activity except in the presence of ATP and

α-ketoglutarate; however, activity with Mg^{++} is less than with Mn^{++} alone. In contrast, Mn^{++} and Mg^{++} support equal UTase activity in the presence of ATP and α-ketoglutarate, but neither cation supports UTase activity in the absence of these effectors. Table 2 also shows that UTase activity is inhibited by glutamine whereas other data (not shown) demonstrate no effect of glutamine on UR activity.

All attempts to separate the UR and UTase activities have failed. An example of their copurification is illustrated in Figure 2.

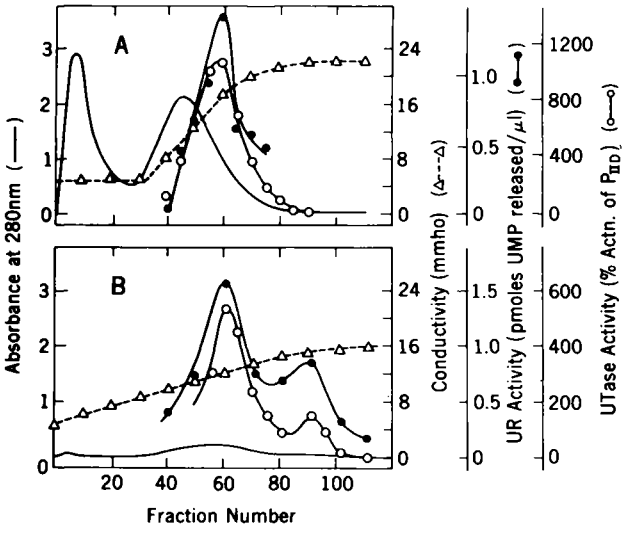

Figure 2. Chromatography of UR-UTase activities on sepharose-C_5-NH_2. (A) An extract containing UR-UTase activities was applied on a sepharose-C_5-NH_2 column (8.5 x 1.5 cm). After the unabsorbed protein was eluted, a KCl gradient up to 0.4 M was applied. (B) Fractions from experiment A containing UR-UTase activities were pooled, and reapplied to the same column as in A, only eluted with a shallower KCl gradient. See ref. **(15)**.

A partially purified extract containing UR-UTase activities was chromatographed on a hydrophobic column (Sepharose-C_6-NH_2). Even when eluted with a very shallow KCl gradient both activities

cochromatograph (Figure 2B). The facts that both activities copurify through a variety of procedures, are stabilized by high ionic strength buffers (14), and in the presence of Mg^{++} require ATP and α-ketoglutarate for activity (Table 2) indicate that both activities might be catalyzed by a single enzyme or enzyme complex.

Discussion

Figure 3 summarizes current knowledge of the complex system that regulates glutamine synthetase activity in E. coli. The system consists of two opposing sets of reactions (cascades) that lead on the one hand to inactivation (adenylylation) of glutamine synthetase and on the other hand to its activation (deadenylylation). The inactivation cascade (Fig. 3A) is initiated by the action of UR enzyme which catalyzes the deuridylylation of P_{II}·UMP (i.e., P_{IID}). Deuridylylation leads to an unmodified form of P_{II}, which together with ATase promotes the adenylylation of glutamine synthetase, thus converting it from a Mg^{++}-dependent form of high catalytic potential and a pH optimum of 8.0, to a Mn^{++}-dependent form of low catalytic potential and a pH optimum of 6.9. The activation cascade (Fig. 3B) is initiated by the action of UTase, which catalyzes uridylylation of P_{II}. The P_{II}·UMP, thus formed, together with ATase promotes deadenylylation of glutamine synthetase, converting it back to the Mg^{++}-dependent form of high catalytic potential. The activities of these two opposing cascade systems are finely modulated by the concentrations of various metabolites, including UTP, ATP, α-KG, Pi, glutamine and probably other compounds as yet unidentified. The catalytic potential of glutamine synthetase is determined by its state of adenylylation (i.e., the average number of adenylylated subunits per mole of enzyme). When glutamine synthetase is incubated with ATase, P_{IIA}, P_{IID} and the effectors shown in Figure 3, it assumes a dynamic steady state of adenylylation in which the rates of adenylylation and deadenylylation are equal (16); moreover, the actual state of

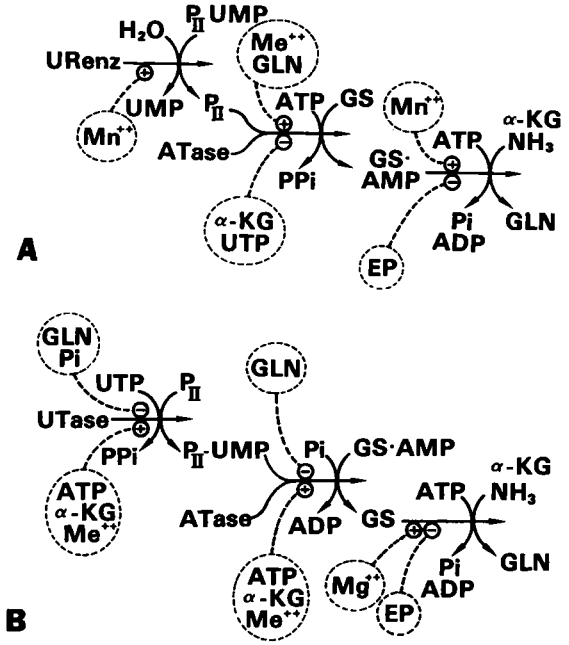

Figure 3. Cascades involved in the regulation of glutamine synthetase activity: (A) inactivation (adenylylation) of glutamine synthetase; (B) activation (deadenylylation) of glutamine synthetase. EP refers to end products of glutamine metabolism.

adenylylation ultimately obtained is determined by the relative concentrations of P_{IIA}, P_{IID} and the various effectors. Since for any given steady state the rates of adenylylation and deadenylylation of glutamine synthetase (GS) are equal, it follows from theoretical considerations that the final state of adenylylation, $\bar{n}$, is a function of the magnitude of the specific rate constants for adenylylation (k_1) and deadenylylation (k_2) and the mole fraction of P_{IIA}, $(P_{IIA})_f$, according to the equation:

$$\bar{n} = \frac{12\ (P_{IIA})_f}{k_2 + k_1\ (P_{IIA})_f - k_2\ (P_{IIA})_f}$$

Figure 4 shows how the value of $\bar{n}$ varies as a function of the $(P_{IIA})_f$ and the ratio of k_1/k_2.

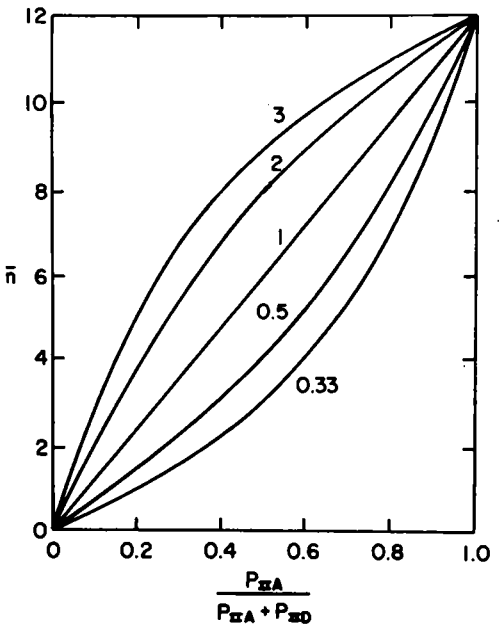

Figure 4. Dependence of the steady state level of adenylylation on the mole fraction of P_{IIA} and the relative specific rate constants for adenylylation and deadenylylation. Data are calculated values derived from equation in the text (where $(P_{IIA})_f = P_{IIA}/P_{IIA} + P_{IID}$). It is assumed that ATase is present in excess compared to P_{II} and that its activity is determined by the specific rate constants for adenylylation (k_1) and deadenylylation (k_2), and the proportions of P_{IIA} and P_{IID} present. Numbers on curves indicate the ratio, k_1/k_2.

Note that when $k_1 = k_2$, $\bar{n}$ is a linear function of the mole fraction of P_{IIA}, but that nonlinear functions are obtained if the ratio of k_1/k_2 is varied as occurs, in the response to the differential effects of metabolites on the ATase catalyzed adenylylation and deadenylylation reactions. Additional flexibility in control derives from the fact that the mole fraction of P_{IIA} can vary independently in response to changing concentrations of metabolites that affect the rates of the uridylylation and deuridylylation reactions (Fig. 3).

Cascade systems offer other advantages in the regulation of certain cellular functions. Because they consist of a series of reactions in which one catalyst acts upon another, they can amplify the response of the target enzyme to primary effectors acting on the initial enzyme in the series. This could be important if the changes in effector concentrations are small compared to the concentration of the target enzyme and in situations as described here in which the multiple protein catalysts are not present in comparable concentrations as they are in organized multienzyme complexes.

In addition, cascade systems increase the number and types of allosteric effectors that can affect the activity of the ultimate target enzyme, since each enzyme in the cascade can be independently regulated. This may be important in the regulation of enzymes such as glutamine synthetase that occupy a central position in metabolism and therefore need to receive a massive input of regulatory information from diverse cellular functions. In such cases physical and steric limitations may preclude the existence on a single enzyme of a sufficient number of allosteric sites to accommodate the required number of regulatory effectors. This may account for the fact that the direct regulation of glutamine synthetase by eight different end products of glutamine metabolism is supplemented by a cascade system in which regulation of the UR-UTase and ATase activities is mediated by six additional effectors, including divalent cations (Fig. 3). In addition 3-phosphoglycerate, fructose-6-P, P-enolpyruvate, CoA, and fructose-P_2 have been shown to inhibit the adenylylation of glutamine synthetase (17).

Finally, another advantage of cascade systems is obtained when more than one step in the cascade is subject to control by the same effector. Increased sensitivity of the system to negative control is obtained when two steps are inhibited by the same ligand. Thus, when a given concentration of a single metabolite

inhibits each of two steps by 50%, the overall inhibition will be 75%. Moreover, if a metabolite stimulates two separate steps in a cascade, the net effect is to increase the apparent reaction order with respect to that effector; therefore, under appropriate conditions activation of the last enzyme in the cascade will be a sigmoidal function of the metabolite concentration. This advantage of cascade systems appears to be realized in the glutamine synthetase cascade, since as is shown in Fig. 3, two steps, (the uridylylation of P_{II} and the adenylylation of glutamine synthetase) are inhibited by glutamine and are stimulated by both α-ketoglutarate and ATP.

In the last analysis, the elaborate cascade system illustrated in Figure 3 serves as a physiological computer which is programed to sense fluctuations in the concentrations of numerous metabolites and to integrate their effects so as to modulate the activity of glutamine synthetase to meet changing metabolic demands.

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Structure and Biosynthesis of an Acidic Glycoprotein in a Bacterial Cell Envelope

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INTRODUCTION

Although glycoproteins are universally distributed in animals and plants, their occurrence in microorganisms is less well-documented. Some eucaryotic microorganisms are known to produce glycoproteins and in particular several yeast glycoproteins have been described, some that are enzymes[1, 2] and others that appear to be envelope or extracellular proteins[3, 4]. Mannose is the major sugar in many of these yeast glycoproteins. In contrast, there are fewer than ten reports of glycoproteins in bacteria. Everse and Kaplan[5] have described glycoprotein enzymes from Bacillus subtilis and there are reports of poorly-characterised glycoproteins in the envelopes of Escherichia coli[6] and Pseudomonas aeruginosa[7]. The bacterial glycoprotein that has been studied in most detail is that from the envelope of the marine pseudomonad BAL 31[8], but this may be a viral-specific component that results from infection with bacteriophage PM 2. N-Acetylglucosamine is the major sugar in all these bacterial glycoproteins; the only exception to this is the fucose-containing glycoprotein of a corynebacterium whose biosynthesis has been studied by Strobel and his co-workers[9].

There have been three recent reports of glycoproteins that contain phosphodiester linkages in their glycan moieties. These 'phospho-

glycoproteins' have been found in Hansenula holstii[10], Cladosporium werneckii[11] and in Penicillium charlesii[12]. They represent an interesting new class of macromolecules whose function is not yet known.

We report here studies on the biosynthesis and preliminary characterisation of a glycoprotein from B. licheniformis that is rich in N-acetylglucosamine and phosphate and therefore appears to be a phosphoglycoprotein.

METHODS

Growth of Cells and Preparation of Membrane

B. licheniformis ATCC 9945 was grown to exponential phase and membranes were prepared by treatment of the cells with lysozyme in the absence of an osmotic stabiliser as previously described[13]. Membranes were suspended at about 50 mg dry wt/ml in 0.05M Tris-HCl, pH 8.0 containing 5mM ethanethiol, and were stored frozen until required.

Preparation of radioactive Glycoprotein

Large scale enzyme reactions for preparation of the phosphoglycoprotein were carried out as follows: 1.0 ml membrane suspension was incubated with 0.1 ml $MgCl_2$ (0.8 M), 0.1 ml UDP-N-acetylglucosamine (U-^{14}C; 2.98 x 10^6 cpm/μmole; 10mM) and tris buffer in a total volume of 1.5 ml at 30^o for 1 h. The reaction was stopped by addition of 0.5 ml butan-1-ol and the mixture applied as a band on the origin of a preparative paper chromatogram (Whatmann 3MM). The chromatogram was developed for 18 h in solvent system A, and the material that remained at the origin was extracted into water at 60^o for 3 h. More than 95% of the radioactivity from the origin was recovered in this way.

Chromatography and Electrophoresis

Paper chromatography was carried out on Whatman No. 3MM paper or No. 1 paper in the following solvents:- A propan-1-ol-ammonia (0.88 sp. gr.)-water (6 : 3 : 1 by volume). B ethylacetate-pyridine-acetic acid-water (5 : 5 : 1 : 3 by volume). Paper electrophoresis was carried out on Whatman No. 1 paper in 0.1M pyridinium acetate pH 6.5 at 40 volts/cm for $1\frac{1}{2}$ h.

Polyacrylamide gel electrophoresis in 0.1% sodium dodecylsulphate (SDS) was accomplished as described by Weber and Osborn[14]. Gels were stained for protein with Coomassie blue, and for carbohydrate material by the periodate-Schiff reagent of Segrest and Jackson[15].

Phosphate-containing compounds were detected on paper chromatograms by the method of Hanes and Isherwood[16]. Reducing sugars were stained with silver nitrate[17]. Quantitative Estimations and radioactivity measurements were carried out as previously described[18].

Preparation of polyisoprenol monophosphate

Polyisoprenols were phosphorylated as described by Popjak et al[19] and purified by column chromatography on DEAE cellulose acetate in methanolic ammonium acetate[20]

Radioactive substrates

UDP-N-(acetyl^{14}C)-acetylglucosamine was prepared as previously described[21]. UDP-N-acetyl (U^{14}C)glucosamine was purchased from

the Radiochemical Centre, Amersham, Bucks. U.K.

RESULTS

During a study of peptidoglycan biosynthesis in a membrane preparation from B. licheniformis the incorporation of radioactivity from [^{14}C]UDP-N-acetylglucosamine into polymeric material was measured in the presence and absence of UDP-N-acetylmuramyl pentapeptide (Park nucleotide), the other substrate required for peptidoglycan synthesis. Table 1 shows the results of such an experiment.

Table 1 - Incorporation of ^{14}C-UDP-N-acetylglucosamine into macromolecular material.

	With Park nucleotide	no addition
cpm incorporated into polymer	10693	7664
cpm incorporated into lipid	1105	10837

Reaction mixtures contained 0.1 ml membrane suspension, UDP-N-[acetyl-^{14}C] acetylglucosamine (0.1 μmol, 2.98 x 10^5 cpm), $MgCl_2$ (8 μmol) in a total volume of 0.13 ml. The mixture was incubated for 1 h at 30^{o} and then the reaction mixture was applied in a 2.5 cm band to the origin of a paper chromatogram (Whatman no. 3MM paper). The paper was developed in Solvent System A for 18 h. Polymeric material remained at the origin, while lipids migrated with an R_f of 0.85.

It was found that even in the absence of Park nucleotide, when peptidoglycan synthesis is impossible, a large amount of radioactivity was incorporated into high molecular weight material, and also into lipid.

In order to ascertain whether the radioactivity remained entirely in glucosamine residues in the product, the above experiment was repeated using uniformly labelled ^{14}C-N-acetylglucosamine and the baseline area from the chromatogram, and the lipid region, were cut out and hydrolysed on the paper in 2M-HCl at 100° for 3 h. The hydrolysate was chromatographed on Whatman no. 1 paper in Solvent system B for 18 h. All the radioactivity co-chromatographed with standard samples of glucosamine and N-acetylglucosamine, for both polymer and lipid.

Properties of the polymer

It was found that the material containing N-acetylglucosamine that remained at the origin of the chromatogram of reaction mixtures in Solvent A could be completely eluted from the paper into water at 60° in 3 h. Material was isolated in this way from large-scale incubation mixtures as described in 'Methods'. Column chromatography of the material was carried out on Sephadex G75 in both 10 mM-tris-HCl, pH 8.0 containing 1 mM-mercaptoethanol and in the same buffer containing 0.1% sodium dodecyl sulphate (SDS). In both cases the bulk of the radioactivity was excluded from the gel and the small amount of included material was UDP-N-acetylglucosamine. The excluded material contained protein as indicated by the Lowry method and absorption at 280 nm. Analytical polyacrylamide gel electrophoresis in 0.1% SDS revealed two diffuse protein bands, one at the top of the gel and the other with R_f of about 0.75. The R_f 0.75 band contained all the radioactivity and stained positively with periodate-Schiff.

Radioactive polymer that had been extracted from large scale incubation mixtures as described was further purified by preparative SDS polyacrylamide gel electrophoresis, under the same conditions as described for analytical electrophoresis, on a 2.5 cm x 6.5 cm

cylindrical gel. The fast running protein fraction (R_f 0.65 - 0.85), which contained all the radioactivity, was eluted from the gel into water, dialysed, and passed through a column of Sephadex G75, from which the excluded peak containing all the radioactivity was retained. Double-diffusion against DEAE-dextran in 1.0% agarose gel at pH 7.3 revealed two sharp precipitin lines, indicating that the purified material contained two anionic components. This partially purified labelled material was used for further chemical studies.

Acid hydrolysis in 2N HCl, 3 h at 100°, followed by paper chromatography in solvent system B and staining with silver nitrate reagent, revealed the presence of glucose and glucosamine (with a trace of N-acetylglucosamine). Paper electrophoresis of the same hydrolysate revealed a non-reducing component that stained positively for phosphate and amino groups but was unidentified. This component was also detected by paper chromatography in solvent A, in which it displayed an $R_{GlcNH_2\text{-}6\text{-}P} = 2.24$.

Table 2 shows the amino acid composition of the glycoprotein. It contained only small amounts of the aromatic amino acids and negligible amounts of amino acids containing sulphur.

Table 2 - Amino acid composition of glycoprotein

Lysine	1.35	Proline	0.30	iLeucine	0.35
Histidine	0.17	Glycine	0.91	Leucine	0.44
Arginine	1.70	Alanine	1.61	Tyrosine	0.09
Aspartic acid	1.0	half-cystine	0	Phenylalanine	0.13
Threonine	0.35	Valine	0.44	Tryptophan	0
Serine	0.52	D.a.p.	trace	Glucosamine	0.44
Glutamic acid	1.22	Methionine	0.13		

The analysis was carried out on an 18 h hydrolysate. Aspartic acid was set arbitrarily at 1.0.

Determination of N-terminal amino acids by dansylation gave two major AAs, one of which was the dansyl derivative of glycine. The other has not been identified.

The preliminary evidence suggested that the material containing N-acetylglucosamine was an acidic glycoprotein. In order to examine this hypothesis the polymer was subjected to digestion with pronase (1 mg/ml) for 24 h at pH 7.0 in Tris-HCl containing 1mM-$CaCl_2$, 37°. After 24 h a further 1 mg/ml pronase was added and incubation was continued for a further 24 h. The digestion mixture was subjected to paper electrophoresis in pyridinium acetate at pH 6.5. This yielded a small, sharply defined fraction that had a slight positive charge, was radioactive and stained with ninhydrin, together with a more diffuse, very acidic peptide fraction that contained most of the radioactivity. These peptides were eluted into water and chromatographed on a column of Sephadex G25. The acidic electrophoresis fraction yielded two peaks of radioactivity, a small one (G1A) which was excluded from the resin and a much larger one (G2A) which was included. The basic electrophoresis fraction yielded a single peak on G25 (G3B) which had approximately the same apparent molecular weight as G2A. The release of these radioactive glycopeptides from the high molecular weight material by treatment with the proteolytic enzyme confirmed that the product of the biosynthetic reaction was a glycoprotein.

Biosynthesis of the polymer

The chromatographic properties of the N-acetylglucosamine-containing lipid that was formed during biosynthesis of the polymer, and its extreme lability to acid (it had a half-life of 15 min at 100° in 0.01M-HCl in 50% MeOH) suggested that it might be a polyprenol phosphate

derivative of the type known to participate in the biosynthesis of many bacterial polysaccharides[22]. In order to test this hypothesis, the effect of added C_{55}-polyprenol monophosphate on lipid and polymer synthesis was examined. Added polyprenol monophosphate stimulated lipid synthesis, but only in the presence of the nonionic surfactant Triton X-100 (Fig. 1).

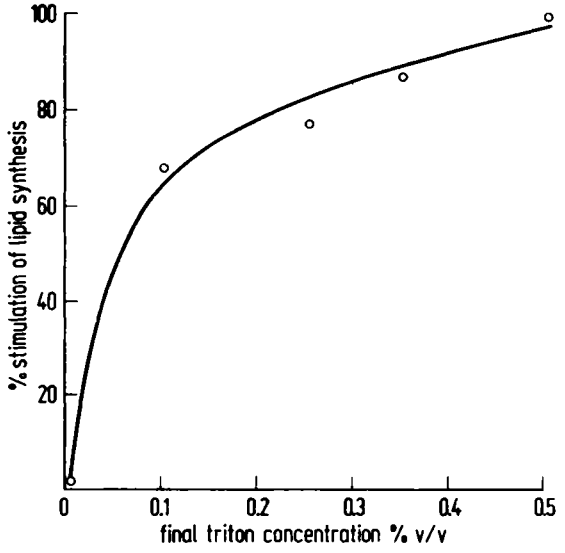

Fig. 1. The dependence of stimulation of polyprenol phosphate upon Triton X-100.

Reaction mixtures contained 0.1 ml membrane suspension, $MgCl_2$ (5 μmol), UDP-N-[^{14}C-acetyl]acetyl glucosamine (0.1 μmol, 2.98 x 10^5 cpm), and Triton X100 at the indicated concentration. The total volume was 0.13 ml. C_{55}-polyprenol phosphate (1n mol) was added to each incubation tube first in chloroform:methanol 2:1 v/v, then the solvent was evaporated off under reduced pressure before addition of the other reagents and mixing. Reaction was terminated by the addition of 0.05 ml n-butanol and lipid synthesis was measured as described for Table 1. Incubations for 1 h at 30°.

In the presence of Triton X-100 C_{55} -polyprenol monophosphate stimulated incorporation of label from UDP-N-acetyl glucosamine into the lipid as shown in Fig. 2.

The effect of a number of different polyprenol monophosphate preparations on lipid and polymer synthesis is shown in Table 3. Only the phosphate of the all-trans C_{45} isoprenol, solanesol failed to stimulate both lipid and polymer synthesis.

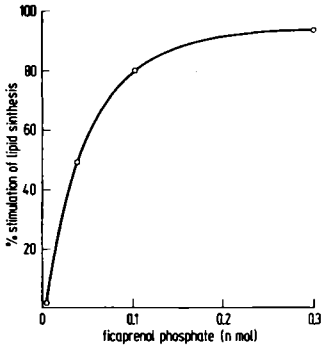

Fig. 2. Stimulation of lipid synthesis by C_{55}-polyprenol monophosphate.

Incubation mixtures were as described for Fig. 1. All incubations contained Triton X-100 at a final concentration of 0.3% v/v. Incubations were carried out at 30° for 1 h and lipid synthesis was assayed as described for Table 1.

Table 3 - Effect of polyprenol monophosphates on lipid and polymer synthesis

	no added lipid	heveaprenol phosphate	bactoprenol phosphate	solanesol phosphate
cpm in polymer	8144	13263	11652	8102
cpm in lipid	6060	12297	12235	6091

Incubation mixtures were as described for Fig. 1. Approx. 3 nmol of the indicated polyprenol phosphate was added.

This evidence suggested that the lipid was a polyprenol monophosphate or pyrophosphate N-acetylglucosamine that might be intermediate in the biosynthesis of the polymer. In order to obtain further information about the synthesis of the lipid the effects of UMP and UDP on its formation were examined. It was found that UMP strongly inhibited both polymer and lipid synthesis, while UDP inhibited the synthesis of polymer but not of lipid. This result suggested that lipid synthesis

involves the transfer to polyprenol monophosphate of N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine, with the release of UMP.

UDP-N-acetylglucosamine + lipid-P $\rightleftharpoons$ UMP + lipid-P-P-N-acetylglucosamine

UMP would inhibit this reaction by a mass-action effect. Transfer of either N-acetylglucosamine or its 1-phosphate from lipid to polymer could subsequently occur.

DISCUSSION

Our preliminary results show that in the membrane of B. licheniformis there are enzymes that catalyse the incorporation of N-acetylglucosamine, N-acetylglucosamine 1-phosphate, or both, into an endogenous glycoprotein acceptor. It is not yet known whether the product of this cell-free biosynthetic system represents the cellular end-product although work is in progress to characterise the glycoprotein of the cell.

The amino acid analysis of the glycoprotein does not indicate a large excess of acidic AA residue in the protein moiety and the polyanionic nature of the glycoprotein may therefore be attributable to the glycan part, and in particular to phosphate groups in the glycan. The high acidity of the phosphoglycoprotein and its high glycan content make measurements of its molecular weight by conventional physical techniques difficult.

The role of lipid intermediates in the biosynthesis of glycoproteins in yeasts[10] and in some mammalian systems[23, 24, 25] has received considerable attention recently, but work has been hindered by difficulties in characterising the protein acceptor and the end products in these experiments. The B. licheniformis system described here