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Contents

The Pathology of Marginal Renal Function.
By H. FAVRE, Geneva/Switzerland, and
N. S. BRICKER, Los Angeles, CA/USA.
With 3 Figures 1

Human Thyroxine Binding Globulin (TBG).
By G. HOCMAN, Bratislava/Czechoslovakia . . 45

Structure and Function of Phospholipase A_2.
By H. M. VERHEIJ, A. J. SLOTBOOM, and
G. H. DE HAAS, Utrecht/The Netherlands.
With 15 Figures 91

Author Index 205

Subject Index 223

Indexed in Current Contents

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The Pathology of Marginal Renal Function

HERVÉ FAVRE* and NEAL S. BRICKER**

Contents

1 Introduction 2

2 Historical Perspective and the Development of an Experimental Model 3

3 The Level of Functional Organization of the Chronically Diseased Kidney: Homogeneity of Glomerulo-Tubular Balance 6
3.1 Clearance Ratios in Stage II Animals 6
3.2 Glucose Titration Studies 9
3.3 Micropuncture Studies 12
3.4 Summary 12

4 The Response of the Surviving Nephrons to the Changing Requirements for Solute and Water Excretion 13

5 The Magnification Phenomenon 15

6 The Concept of "Biologic Control Systems" 16

7 The Regulation of Specific Solutes in CRD 18
7.1 Sodium 18
7.1.1 The Location of the Detector Element 19
7.1.2 The Effector Element 20
7.2 Potassium Transport by the Remaining Nephrons 25
7.3 Phosphate Transport by the Surviving Nephrons 28

8 Acid Base Regulation: The Adaptation in Ammoniagenesis and the Excretion of Titratable Acid 31
8.1 Bicarbonate Reabsorption 32
8.2 Hydrogen Excretion 33
8.2.1 Ammoniagenesis 33
8.2.2 Titratable Acid 34

9 Conclusion 34

References 35

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1 Introduction

When the renal mass is reduced either experimentally or in the course of spontaneously occurring chronic renal disease (CRD), the remaining nephrons perform the excretory functions of the kidney essential to survival sufficiently well to preserve the basic integrity of body fluids. They do this by maintaining external balance for many of the key solutes and water of the extracellular fluid on an ongoing basis despite random and unpredictable variations in the rates of accession of these substances. The purpose of this review is to discuss the mechanisms by which this contribution to homeostasis can occur in the face of the profound obstacles imposed by the presence of CRD. It is obvious that as the nephron population diminishes progressively without a commensurate decrease in the amounts of those substances that require excretion by the kidneys, each remaining nephron must assume an ever-increasing share of the total excretory burden.

The response of the surviving nephrons in CRD will be addressed first in terms of their ability to function as an integrated group serving to defend the biologic integrity of the organism. Emphasis will be given to how well the composite group of surviving nephrons maintains its organizational composure as nephron destruction and the extent of anatomic distortion of the renal parenchyma progress. In these comments, a statement and explanation of the "Intact Nephron Hypothesis" will be provided.

The second major area of consideration will deal with the adaptations, both general and specific, that occur as CRD advances. In this portion of the discussion attention will be focused on the fact that an organized (i.e., homogeneous) pattern of function among the residual nephrons in the chronically diseased kidney is not sufficient in itself to maintain homeostasis on an ongoing basis. The course of CRD is characterized by a fall in glomerular filtration rate (GFR) from normal towards zero. To preserve life requires that continuous *solute-specific* adaptations occur in the remaining nephrons each time new nephron loss occurs. The execution of the adaptations, in turn, requires the availability of a means of monitoring the rates of acquisition of the individual solutes to be excreted and of transmitting "information" to the remaining nephrons that will modulate transtubular transport rates (reabsorption or secretion) so as to affect the required excretion rates of each substance into the urine. To control the regulation of many different excretory rates simultaneously, it would seem likely that a series of *biologic control systems* must exist for individual constituents of the extracellular fluid (ECF). A theoretical discussion of the nature of the adaptations and of a prototypic control system will be considered in this manuscript.

2 Historical Perspective and the Development of an Experimental Model

Until approximately a decade ago it was widely believed that the kidney afflicted with any form of advancing CRD lost its responsivity to the homeostatic needs of the patient. The surviving nephrons were thought to undergo progressive functional deterioration and ultimately to be reduced to a heterogeneous and disorganized group with disparate and unpredictable abilities to contribute to the maintenance of life and well-being. Indeed, it was generally accepted that no diseased kidney functioned like another and no nephron in a given diseased kidney necessarily functioned like any other nephron in the same kidney (*Oliver* 1939; *Platt* 1952; *Steele* et al. 1968; *Gottschalk* 1971). Coupled with this view was the belief that any approach to treatment of CRD based on established principles of normal renal physiology was futile.

When the foregoing concepts were subjected to critical examination, serious problems became apparent. For example, patients do maintain external balance for sodium, potassium, magnesium, and a number of other solutes until the end stage of CRD (*Bricker* et al. 1965, 1971). Moreover, a very small percentage of the original nephron population, perhaps as few as 20 000–30 000 nephrons residing in a severely scarred and damaged kidney can often maintain life in a patient without major dietary alterations or the use of chronic hemodialysis. Finally, these accomplishments seem to occur in all forms of CRD and thus are largely independent of the nature of the structural changes. However, patients or animals with advanced uremia do exhibit abnormalities and limitations in renal function that could lend support to the view that the pathologic changes in the scarred and damaged renal parenchyma impair numerous functional systems in the surviving nephrons (*Bricker* et al. 1964). For example, chronically uremic patients cannot concentrate their urine, nor can they dilute it normally (*Bricker* et al. 1959; *Kleeman* et al. 1961; *Holliday* et al. 1967; *Tannen* 1969; *Harrington* and *Cohen* 1973). Acidification is impaired by virtue of decreased ammoniagenesis, and alkalinization of the urine may be defective (*Puchett* and *Goldberg* 1969; *MacLean* and *Hayslett* 1980). Chronically uremic patients cannot conserve sodium maximally on a low salt diet (*Polak* 1971), and they may have a reduced capacity to reabsorb glucose and bicarbonate (*Morrin* et al. 1962b; *Shankel* et al. 1967). To establish the validity of the view that "so goes structure, so goes function," however, other explanations for the functional changes had to be excluded. One of these was the possibility that the accumulation of potentially toxic materials in the blood in chronic uremia affects various transport systems adversely. Another explanation is that many of the changes in nephron function are either due directly or are related in some manner to adapta-

tions that basically serve to enhance the excretory ability of the surviving nephrons.

Resolution of this dilemma required the design of an experimental approach that in a sense could outmaneuver nature. The approach that was employed, and that made it possible to examine and quantify the functional integrity of the surviving nephron of the chronically diseased kidney, is described below.

The experimental design which allowed clarification of this issue, as is so often the case, turned out to be simple. Techniques were developed for producing chronic renal lesions in *only one* of the two kidneys of experimental animals, leaving the contralateral kidney of each animal intact and with its full complement of nephrons. One such form of unilateral renal disease is the so-called remnant kidney which is produced by ligating second- and third-order branches of the renal artery of one kidney so as to infarct approximately 75%–80% of its renal parenchyma, leaving the residual nephrons in the uninfarcted renal parenchyma intact. Other lesions that have been induced unilaterally include pyelonephritis and various forms of immunologic glomerulonephritis (*Bricker* et al. 1960a, c; *Dorhout Mees* 1966; *Lubowitz* et al. 1969; *Wagnild* et al. 1974).

With the lesion confined to one kidney, the composite (i.e., bilateral) nephron population must by definition exceed 50% of the original number. Thus, compositional changes in body fluids are minimized and any impact of high levels of "toxic" materials in the blood on the functional systems of the nephrons of the diseased organ is virtually eliminated as a variable. Moreover, because of the large number of functioning nephrons, the necessity for major adaptation in the residual nephrons of either the normal or the diseased kidney is obviated. Hence, the second variable is largely eliminated. The advantages of the model go beyond the elimination of these two variables, however. The reasons for this are as follows:

1. Theoretically, each functioning nephron, regardless of the kidney in which it resides, shares the same fraction of the total excretory requirements for the various solutes and water.

2. The nephrons of the diseased kidney are perfused by the same blood that perfuses the nephrons of the intact organ and thus are exposed to the same concentration of any humoral modulators of tubular transport.

3. Any other extrarenal events capable of modulating hemodynamic or transport functions of the nephrons such as blood pressure elevation, or changes of serum protein concentration, and hematocrit, also should exercise closely comparable effects on the nephrons of the diseased and intact kidneys.

4. The composition of the glomerular filtrate should be identical in the nephrons of both kidneys.

Thus, the animal with unilateral renal disease and a contralateral intact kidney offers a unique opportunity to evaluate the intrinsic functional capabilities and functional organization of the nephrons of the diseased organ and thus to quantify the impact on function of the pathologic stigmata of the underlying renal lesion. This opportunity is made possible by the ability to study many different functional systems in the diseased organ and to compare the data with those obtained simultaneously on the same functional systems in the contralateral kidney of the same animal.

This experimental model has additional utility. Sequential studies may be performed in individual animals employing the following protocol. After dividing the urinary bladder (generally of the dog) into two permanent hemibladders, each of which drains urine from a separate kidney, data are obtained in one or more studies before the induction of unilateral renal disease. These observations on the two normal kidneys are referred to as the stage I studies. Thereafter a renal lesion is induced in one of the two kidneys and, after allowing an appropriate period of time for recuperation (generally at least a week), stage II studies are performed. These may be limited to a single clearance study or serial experiments performed over periods of many months. In each experiment the function or functions of the diseased organ are compared with the data from the contralateral kidney.

At the completion of the stage II studies the *nondiseased* kidney is removed surgically. The animal now is left with only surviving nephrons of the diseased kidney, and the chemical and other stigmata of uremia evolve rapidly. Stage III studies are now performed. Again the number of experiments may be limited to a single set of measurements, or series studies may be performed on the diseased organ for periods as long as 2 years. In the stage III studies not only is the diseased kidney solely responsible for the total renal contribution to life preservation, but its nephrons must also accomplish their functions in a uremic milieu. By comparing the patterns of function that are observed in the diseased kidney in the stage II experiments with the changes in the same functions that take place with time and under varying experimental conditions in stage III, the nature of the adaptation in a specific functional system (as well as any nonadaptive functional changes) may be quantified and characterized. As will be indicated subsequently, techniques designed to reverse specific adaptations also may be evaluated in serial studies in stage III animals (*Schmidt* et al. 1974).

3 The Level of Functional Organization of the Chronically Diseased Kidney: Homogeneity of Glomerulo-Tubular Balance

Glomerulo-tubular (G-T) balance will be used in the present discussion in its broadest sense to indicate the relationship between GFR and a tubular function of the appended nephron regardless of the segment or segments of the tubule in which the tubular function takes place (*Wesson* 1973). The degree of homogeneity of G-T balance for the composite population of nephrons of any kidney, normal or diseased, is reflected by the frequency distribution curve of the individual values for G-T balance for all the individual nephrons tested (either the total population or a representative group) in the kidney under study. In essence, homogeneity of G-T balance exists if the ratios between single nephron glomerular filtration rate (SNGFR) and the rate of tubular transport of the reference material are closely comparable in all the nephrons of the kidney, irrespective of the absolute values for SNGFR in the nephrons tested.

Three experimental techniques have been employed in the evaluation of homogeneity of G-T balance in the chronically diseased kidney. The first involves the use of clearance techniques in stage II animals wherein the ratio of GFR to the value for a tubular function in the diseased kidney is compared to the value simultaneously measured in the intact organ. The second technique, also a clearance procedure, utilizes the method known as the glucose titration test. The third technique involves the study of SNGFR and tubular transport of a reference solute in a group of nephrons studied individually on a diseased kidney using micropuncture techniques. Each of these approaches will be considered below.

3.1 Clearance Ratios in Stage II Animals

A summary of the results of several hundred experiments performed on dogs, sheep, and rats with unilateral or predominantly unilateral disease is shown in Fig. 1. In the upper panel on the left the individual blocks represent values for glomerular filtration rate in the two normal kidneys in stage I. The mean values, as expected, are equal. The upper panel on the right depicts the changes in GFR after induction of unilateral renal disease. The mean value for GFR in the intact kidneys is approximately 10% higher than in the same kidneys in stage I. GFR in the diseased organs is markedly decreased. The bottom panel depicts the ratios between GFR and values for several different tubular transport systems in the two kidneys. In stage I the ratios of GFR are closely comparable in the two normal kidneys. The same ratios were restudied in stage II. Despite the compensatory

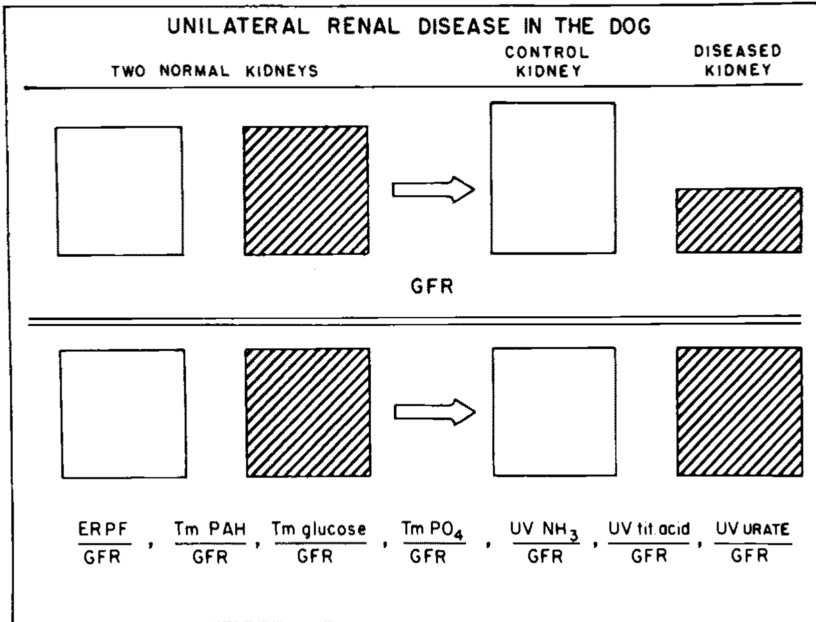

Fig. 1. Functional relationships between the two kidneys of the dog. The changes in glomerular filtration rate (GFR) in both kidneys after induction of unilateral renal disease are shown in the *upper row of boxes.* In the *lower row of boxes,* values for various tubular functions expressed as a function of the simultaneously determined GFR are equal in the two kidneys before the induction of the disease; they remain equal bilaterally after induction of unilateral disease. *Tm,* the maximal rate of transport of PAH, glucose, and phosphate. *UV,* the rate of excretion of NH_3, titratable acid, and urate. *ERPF,* effective renal plasma flow (from *Bricker* et al. 1965)

increase in GFR in the intact kidney and the variable fall in GFR in the diseased kidney, all the ratios remain equal in the two sets of organs. This equality of G-T balance between diseased and contralateral control kidneys is independent of the nature and the severity of the underlying lesion. Moreover, the same equality of clearance ratios between the two kidneys has been found in *patients* with unilateral or predominantly unilateral renal diseases for a series of solutes that are transported in different segments of the nephron, which strengthens the evidence for homogeneity of G-T balance in the nephrons of the diseased organ. The fact that the ratios remain equal for solutes that are reabsorbed and solutes that are secreted lends further support to the evidence favoring homogeneity of G-T balance in diseased organs (*Reiss* et al. 1961; *Rieselbach* et al. 1964; *Bricker* 1969; *Schultze* et al. 1971). Finally, the fact that the clearance ratios of the two kidneys remain equal in a patient or an animal in which the degree of involvement is unequal in the two kidneys adds further credence to the view that G-T balance is preserved in CRD.

The significance of equal clearance ratios in a diseased and contralateral normal kidneys may be examined in a more explicit fashion by a mathematical analysis of the clearance data; such an analysis is shown in Table 1. The maximal rate of ammonia excretion has been chosen as the tubular transport marker for several reasons: (1) Ammonia is secreted in both proximal and distal segments of the nephron; (2) the synthesis of ammonia within the tubular epithelial cells requires continuous delivery of substrate to the tubular cell interior; (3) it requires appropriate spatial orientation of key subcellular organelles; and (4) the trapping of NH_3 as NH_4 in the tubular fluid and the delivery of the NH_4 into the final urine depends upon finely modulated transport of hydrogen ions across the luminal cell membrane (*Pitts* 1974).

Table 1. Mathematical analysis of equal clearance ratios in a stage II dog. Adapted from *Bricker* et al. (1971)

Diseased kidney		*Normal kidney*
1. $\dfrac{U_{NH_4} V}{U_{in} V \; P_{in}}$	=	$\dfrac{U_{NH_4} V}{U_{in} V \; P_{in}}$
2. $\dfrac{U_{NH_4} \text{ (mol/ml)}}{U_{in} \text{ (mol/ml)}}$	=	$\dfrac{U_{NH_4} \text{ (mol/ml)}}{U_{in} \text{ (mol/ml)}}$
3. $\dfrac{\text{moles } NH_4}{\text{mol inulin}}$	=	$\dfrac{\text{moles } NH_4}{\text{mol inulin}}$

[a] $U_{NH_4} V$ = absolute rate of ammonia excretion in mol/min; $U_{in} V$ = inulin clearance in ml/min; U_{NH_4} = urinary concentration of ammonium; U_{in} = plasma concentration of inulin in mol/ml

The data in Table 1 are from a stage II animal. Equation 1 indicates that the ratio of ammonia excretion to GFR is equal in the diseased and normal kidneys of the same acidotic dog. The V terms on each side of the equation are common to the numerator and denominator and may be canceled; plasma inulin concentration (P_{in}), which has the same value for both kidneys, may also be canceled. In Eq. (2), the equal clearance ratios result in an equality between the ammonia–inulin concentration ratios in the urine of the respective kidneys. Once again, however, there is a common term that can be canceled (i.e., ml). The final expression, shown in Eq. (3), establishes the fact that the ratio of ammonia to inulin (both in

moles) is identical in the urine of the diseased and intact kidneys (*Morrin* et al. 1962a; *Bricker* et al. 1971). Thus, *for any given number of inulin molecules filtered (i.e., for any given volume of glomerular filtrate), the tubules of the diseased kidney secrete exactly the same number of molecules of ammonia as do the tubules of the normal kidney* (*Bricker* et al. 1960b; *Bricker* 1969).

Expressed in terms of G-T balance, the equality of clearance ratios between the two kidneys of any animal or patient (whether there is unilateral renal disease, predominantly unilateral renal disease, or bilateral renal disease) establishes the fact that the relationship between the mean rate of tubular transport of the reference marker and the mean rate of SNGFR is exactly the same in the nephrons of the left as in those of the right kidney.

It is theoretically possible to obtain equal clearance ratios in a stage II animal due to a highly fortuitous combination of two equally balanced groups of abnormal nephrons, each of which offsets the effects of the other on whole kidney clearance ratios. One group would have values for SNGFR depressed out of proportion to impairment of tubular function; the other would have the reverse abnormality. However, the greater the number of species subjected to clearance ratio studies, the more forms of renal disease that are examined, the greater the spectrum and severity of pathologic changes, the greater the number of tubular transport markers examined, and the greater the number of sites in the tubule in which the transport takes place, the less likely it is that there would be equal clearance ratios. There would have to be a highly consistent balance between "hypoglomerular and hypotubular" nephrons. The glucose titration test and micropuncture studies approach this possibility in a rigorous manner.

3.2 Glucose Titration Studies

The glucose titration technique consists of elevating the concentration of glucose in the serum (by intravenous infusion) in a stepwise fashion from the fasting level to values sufficiently high to exceed the maximum capacity of the tubules to reabsorb glucose [i.e., the maximal tubular reabsorption capacity for glucose ($Tm_{glucose}$)]. Because the concentration of glucose is identical at any moment in time in the filtrates of all functioning glomeruli, the *amount* of glucose filtered by each nephron will be determined by its value for SNGFR. The $Tm_{glucose}$ in any nephron, therefore, will depend upon the balance between SNGFR and the glucose reabsorptive capacity of the appended proximal tubule. In the normal kidney virtually all of the filtered glucose is reabsorbed until the Tm is approached. However, some glucose does escape reabsorption and enter the urine before the Tm level is completely reached. The blood sugar level at which

glycosuria begins is denoted the "threshold." The glucose that enters the urine between the threshold blood sugar level and the Tm blood sugar level accounts for a bend of "splay" in the glucose titration curve, which plots the filtered load of glucose against glucose reabsorption (*Kurtzman* and *Pillay* 1973).

A normal glucose titration curve is shown in Fig. 2. The splay zone, which is small in area, resembles the transition zone between first-order and zero-order kinetics observed in a standard Michaelis-Menton plot of an enzyme-substrate reaction (*Bricker* et al. 1960c).

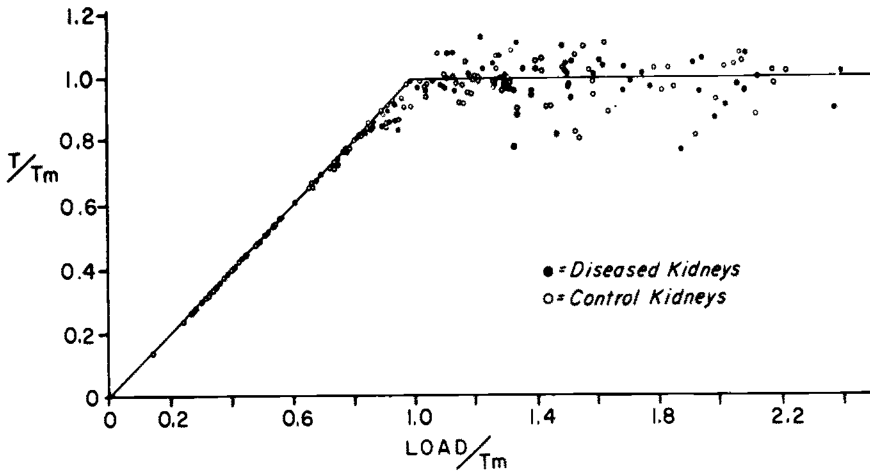

Fig. 2. A mass plot of the data obtained in 12 animals undergoing glucose titration experiments. *T/Tm,* the observed rate of glucose reabsorption expressed as a fraction of the Tm for the same kidney. *Load/Tm,* the filtered load expressed as a fraction of the Tm (from *Bricker* et al. 1960c)

The glucose titration test seems almost ideally suited to detect the presence of any major degree of heterogeneity of G-T balance among the nephrons of the diseased kidney. Because the titration curve for the whole kidney does represent the composite of the individual titration curves for all of its functioning nephrons, any nephrons in which tubular function (i.e., glucose reabsorption) is reduced out of proportion to glomerular function would excrete glucose into the urine at low blood sugar levels. Conversely, nephrons in which SNGFR is reduced disproportionately to glucose reabsorptive capacity would require higher blood levels of glucose to saturate their glucose transport system than the rest of the nephrons. Either category of nephrons, if present in detectable numbers, would alter the appearance of the whole kidney titration curve, leading to an increase in the splay zone. The coexistence of both types of nephrons in the same kidney

would, rather than having an offsetting effect, have an additive effect, i.e, exaggerate the splay zone.

Glucose titration studies have been performed both in stage II rats and dogs with different forms of unilateral renal disease. The results have shown consistently that the splay in the titration curve for the diseased kidney is not greater than that for the contralateral intact kidney and that in neither organs is the titration curve abnormal or the splay zone increased (*Bricker* et al. 1960a, c; *Kawamuara* et al. 1977).

Glucose titration studies have also been performed in patients with bilateral CRD of varying etiologies. No increase in splay in the titration curves was demonstratable in patients with GFRs over 15 ml/min (*Rieselbach* et al. 1967). In the group with GFRs between 10 and 15 ml/min there was a modest increase in the splay, but only in patients with GFR below 10 ml/min was the degree of splay increased substantially. However, even the existence of an increase in splay in far advanced chronic renal disease does not necessarily establish the existence of inhomogeneity of G-T balance among the surviving nephrons (*Shankel* et al. 1967). Thus, when the intact kidney of the stage II rat is removed, the degree of splay in the glucose titration curve of the diseased organ increases markedly, although there is no increase in the severity of underlying disease process (*Shankel* et al. 1967). Conversely, when stage III dogs with an exaggerated splay were subjected to a graded reduction in sodium intake, which was proportional to the decrease in their GFR, the splay zone decreased to normal (*Schmidt* and *Danovitch* 1979). Finally, in normal rats subjected to marked ECF volume expansion with a resultant decrease in proximal fluid reabsorption, the splay zone in the glucose titration curve increases to a degree comparable to that observed in far advanced uremia (*Robson* et al. 1972).

The examination of urine glucose concentrations at serum glucose levels below the threshold is also relevant to the question of whether a tubular glomerulus contributes to urine formation in CRD. It has been noted that the urine remains free of glucose in the diseased kidney of stage II animals until the blood sugar concentration approaches the Tm level. Were there even a small number of nephrons with relatively normal values for SNGFR but "impotent" tubules with respect to their capacity for glucose transport, glycosuria should occur either before or shortly after glucose infusion begins and certainly at blood glucose concentrations well below those required to initiate glycosuria in the contralateral normal organ. The urine is also free of glucose under normal blood sugar levels in moderately advanced bilateral renal disease in man and experimental animals.

3.3 Micropuncture Studies

Micropuncture studies from a variety of laboratories have also allowed investigators to deal with the question of the homogeneity of G-T balance in the nephrons of the chronically diseased kidney with both glomerular and nonglomerular lesions. Regardless of the nature or severity of the lesion, when fractional fluid reabsorption is plotted against the percentage length of proximal tubule at which the tubular fluid is sampled, the function is closely comparable to that obtained from nephrons of normal kidneys (*Mazumdar* et al. 1975). When *absolute* sodium reabsorption along the proximal tubules is plotted against SNGFR for the same nephrons, similar evidence for homogeneity of G-T balance is found (*Lubowitz* et al. 1966; *Allison* et al. 1974; *Maddox* et al. 1975). Finally, when single nephron glucose reabsorption is plotted against SNGFR (or single nephron filtered load of glucose) in rats with experimental glomerulonephritis, homogeneity of G-T balance appears to be preserved (*Kawamura* 1977). It is of considerable interest that homogeneity of G-T balance in the chronically diseased kidney persists despite an increase in values for SNGFR among the constituent nephrons.

3.4 Summary

In summary, the level of G-T balance and the degree of its homogeneity using a number of different tubular transport systems as the indexes of tubular function are either identical to or are closely comparable in the diseased and in the contralateral nondiseased, or less diseased, kidney in the same host when both organs are studied simultaneously. G-T balance also has been found to be relatively homogeneous throughout the nephron population of the chronically diseased kidney of man and animals by use of the glucose titration technique which has special attributes for this type of analysis of whole kidney function. Finally, study of single nephrons from chronically diseased kidneys using micropuncture techniques has provided additional and compelling support for the view that the processes of disease do not disrupt the homogeneity of G-T balance among the surviving nephrons, even though values for SNGFR and the absolute rate of tubular transport for the reference solutes may vary widely from one nephron to the next in the same kidney.

4 The Response of the Surviving Nephrons to the Changing Requirements for Solute and Water Excretion

The fact that the residual nephrons in CRD appear to function as an organized group with an unexpected degree of homogeneity of G-T balance does not in itself explain how these nephrons can continue to maintain external balance for many key solutes as their numbers diminish progressively. It is evident that if the load of any given solute (either ingested or produced metabolically) that requires renal excretion remains constant, each wave of nephron loss increases the excretory task of each surviving nephron. In essence, therefore, for external balance to be preserved for multiple solutes, the level of G-T balance must be reset repeatedly for every solute under renal regulation. For a solute that is excreted by filtration and partial tubular reabsorption, tubular reabsorption must decrease every time GFR falls, if the total amount of the solute delivered into the urine is to be prevented from falling. For a solute that is excreted by filtration and tubular excretion, the rate of secretion per nephron must increase with each successive fall in GFR. Finally, for a solute that is filtered and both reabsorbed and secreted, the balance between the two tubular transport processes must be readjusted continuously so as to permit the required increase in absolute excretion rate per nephron as the number of nephrons falls.

The patterns by which solute and water excretion per nephron increases as GFR falls may be divided into three general types.

No Regulation. For some solutes, of which urea and creatinine are the principal examples, the excretion rate is controlled primarily by the filtration rate and there is little or no active tubular transport in health (*Smith* 1951). For urea, a variable fraction of the filtered load is excreted (*Shannon* and *Smith* 1935; *Chasis* and *Smith* 1938), but the amount reabsorbed (i.e., the nonexcreted amount) does not appear to be modulated in any precise fashion by a transport system geared to the need to maintain constancy of serum content or concentration. The fraction of the filtered load of urea that is excreted is determined in large measure by concurrent rate of fractional water excretion, and since the latter increases in advancing chronic renal disease, so does the former. For creatinine, there is no net reabsorption and although some tubule secretion occurs at elevated plasma levels (*Shannon* 1935), the secretory mechanism again is not finely attuned to the need to maintain excretion rates equal to acquisition rates. Thus, nephron loss cannot lead to adaptive changes in tubular reabsorption or secretion that are regulatory in nature. For these solutes, each successive loss of nephrons (and associated decrement in GFR) results in a

temporary period of retention in the blood and a consequent rise in the serum concentration. (This assumes that the rate of acquisition of the solute stays constant and the volume of distribution unchanged.) Once GFR stabilizes, balance will be reachieved, but only after the serum level rises sufficiently to increase the filtered load per residual nephron to such an extent that the rate of excretion again equals the rate of acquisition. In general, for each 50% reduction in GFR, the serum levels of urea and creatinine double (*Kopple* and *Coburn* 1974).

Regulation with Limitation. The solutes that fall into this group are filtered and actively reabsorbed or secreted by the tubules. Each time nephrons are lost and there is a fall in GFR, no matter how small, retention of the solute will occur and serum levels will rise as in the case of urea and creatinine. However, as opposed to urea and creatinine, the retention of the solute sets a series of corrective events in motion that culminate in changes in the rate of tubular reabsorption or secretion. The effect of these changes will be to increase excretion rate per nephron in each of the remaining nephrons. The increments must be precise, first, providing for the excretion of the solute retained and second, maintaining a new rate if, following the loss of nephrons, excretion per nephron is to be high enough, to permit the continued preservation of external balance. The regulation is qualified by the term "with limitation" because the adaptation operates with maximum effectiveness through only part of the natural history of chronic renal disease. Two of the solutes which have been studied in considerable depth and which fall into the category of "regulation with limitation" are phosphate and uric acid. Serum phosphate levels tend to remain normal until GFR is reduced by approximately 70%–75% (*Goldman* and *Bassett* 1954). Urate levels may be elevated earlier in the course of chronic renal disease, but the elevation is not progressive until relatively late in the course of the disease (*Brochner-Mortenson* 1938). Once the limitation of the adaptation is reached, each further reduction of the number of functioning nephrons will result in an additional permanent elevation of serum levels, unless the rate of acquisition of the solute is diminished [e.g., by restricting *the protein intake or administering phosphate binding gels in the case of phosphate* (*Slatopolsky* et al. 1968b; *Massry* et al. 1973) or by reducing the rate of metabolic production of urate using allopurinol (*Danovitch* et al. 1972)].

Complete Regulation. For some solutes the adaptive increase in excretion rates per nephron continues to provide for the maintenance of normal serum concentration virutally until the nephron population is exhausted. Two of the most important solutes in this group are sodium (*Bricker* 1967) and potassium (*Platt* 1950; *Kleemann* et al. 1966; *Schultze* et al. 1971). A

third solute, magnesium (*Coburn* et al. 1969), is often regulated with sufficient precision to maintain normal serum levels until GFR reaches very low values. Other solutes, including zinc, also appear to fall into this category. The precise modulation of excretion rates per nephron must take into account not only the diminishing number of excretory units but also the random variations in the rates of acquisition of specific solutes. As has been emphasized already and will be developed further, the regulation of each solute would appear to require a "solute specific" biologic control system.

5 The Magnification Phenomenon

The magnification phenomenon defines a truly extraordinary and essential characteristic of the adaptations in solute excretion that occur in CRD. The definition is as follows: "For any given perturbation of body fluids occasioned by the entry of any given amount of solutes into the extracellular fluid, the excretory response per nephron must increase as GFR decreases" (*Bricker* et al. 1978). The remarkable nature of this phenomenon can be readily illustrated. In a normal person with a GFR of 120 ml/min, the ingestion of 120 mEq sodium in the course of 24 h requires that each nephron excrete an average of only 1 of every 200 sodium ions filtered during the 24-h period. In striking contrast, the ingestion of the same amount of sodium by a patient with far advanced chronic renal disease in whom the GFR has fallen to 2 ml/min is attended by the excretion of about 30% of the filtered sodium. An identical perturbation (i.e., 120 mEq Na) thus leads to a sodium excretion rate per nephron (expressed as fractional excretion of sodium) more than 60 times greater in the uremic patient than in the normal person. To extend the example, if the normal person ingests 60 mEq of sodium on one day and 120 mEq on the following day, fractional excretion of sodium will change from 0.25% to 0.50%, an increment in excretion of 1 sodium ion of every 400 filtered. The same change in sodium intake over the same time interval in the patient with the GFR of 2 ml/min will result in an increment of fractional sodium excretion from 16% to 32%. Once again the average individual nephron response in the uremic patient is more than 60 times greater than that in the normal subject, although it must be presumed that the perturbation of the ECF produced by the ingestion of the same amount is indistinguishable in the two subjects (*Slatopolsky* et al. 1968a).

The magnified end organ excretory response illustrated vividly in the case of sodium (*Fine* et al. 1976b) is by no means limited to the sodium ion. The magnification phenomenon applies to all regulated solutes that

fall into the category "regulation with limitation" and "complete regulation." For example, in the hypothetical patient with a GFR of 2 ml/min whose intake of sodium rises from 60 to 120 mEq in 2 days, the intake of potassium could fall from (for example) 80–40 mEq during the same time period. On the initial potassium intake (assuming a serum potassium concentration of 4.5 mEq/liter), the excretion of potassium must be approximately six times greater than the amount filtered. On the following day, a sodium excretion rises from 16% to 32% of the amount filtered, potassium excretion must fall by 50% (*Schultze* et al. 1971). If all of the solutes regulated completely or with limitation are added to this exposition, the accomplishments of the residual nephrons assume dimensions that become difficult to comprehend, let alone explain.

6 The Concept of "Biologic Control Systems"

As the body of knowledge about the nature of the adaptations in solute excretion in CRD has grown, so also has the belief that the surviving nephrons do not operate in isolation. Rather, there are compelling reasons to postulate the existence of a series of control systems which assist the nephrons in the maintenance of external balance for at least certain major constituents of body fluids. In an overall sense, a prototypic biologic control system would contain a "detector element" which is capable of monitoring changes in some facet of the ECF induced by the addition or loss of the specific solute being regulated. Activation of the detector element then will lead to modulation of the rate of transtubular transport of the specific solute by the residual nephrons. The system by which the detector element communicates with the effector element end organ of the nephron may, for at least some solutes, be humoral in nature. The integrated activity of a biologic control system must operate to reverse the initial translocation from the steady state and thus to restore body fluid composition to the preperturbation level. Presumably this occurs by virtue of an oscillating pattern of operation, whereby the level of inhibition or stimulation of net tubular transport (either reabsorption or secretion) is increased and decreased in an alternating fashion in search of the null point. If sodium is the prototypic solute, the following model may be presented for the biologic control system. It will be assumed that at time zero the patient is in a steady state condition with respect to ECF volume. Shortly thereafter, a meal is ingested which contains a finite amount of sodium chloride. The sodium and its anion (chloride) will be absorbed across the gastrointestinal (GI) tract and enter the ECF, and the initial effect may be to increase the concentration of sodium in the plasma.

However, this change is neither consistent nor long lived, for activation of the thirst mechanism will lead to an increased intake of water, a slight osmotic gradient will lead to the movement of water into the ECF out of the intracellular fluid, and the release of antidiuretic hormone will result in a reduced rate of water excretion. The overall effect is to restore the plasma sodium concentration to the control value. Operationally, therefore, the entry of the sodium chloride into the ECF occurs isosmotically. *It is this isosmotic expansion of ECF that presumably constitutes the perturbation* that activates the detector limb of the biologic control system. Either the ECF volume expansion per se or some derivative of the expansion is sensed by the detector element of the system. Precisely where the detector element resides is unknown, but there is growing evidence that a (or the) major location is intrathoracic and possibly within the wall of the left atrium (*Gilmore* 1968; *Epstein* et al. 1972, 1975; *Begin* et al. 1976). Activation of the detector element will lead to an increase in the level of activity of "natriuretic forces" which will decrease fractional and absolute sodium reabsorption by the residual nephrons, thus increasing the rate of excretion of sodium per residual nephron (*Epstein* et al. 1978). The augmented rate of sodium excretion will continue until the initial expansion of ECF volume is reversed. It would seem likely that the intensity of the natriuretic forces would diminish as the initial steady state value for ECF volume is approached, but it is possibly that there could be some "overshoot" resulting in modest contraction of the ECF volume from the initial level. Where this is the case, natriuretic forces would be reduced below the control level of activity and sodium retention would follow, resulting in a slight expansion (real or apparent) of the ECF volume. Deactivation of the natriuretic forces would then follow once again. With this sequence there would perforce be an oscillating pattern of control wherein oscillations would begin only after most of the ingested sodium is excreted and would become progressively smaller as the preset value for ECF volume is approached.

This model of a biologic control system for sodium is "detector oriented." Although it would not necessarily require an element that would sense the number of surviving nephrons or the magnitude of the residual glomerular filtration rate, the magnification phenomenon does dictate that the overall sensitivity of the system to any given perturbation of extracellular fluid volume, as judged by the end organ response, increases progressively as GFR falls.

Though selected for presentation, the biologic control system for sodium may not, in fact, be prototypic; for it is the only one that detects changes in volume. Whatever other solute control systems exist, they very likely detect changes in the *concentration* of their solute in the ECF (*Schmidt* and *Bricker* 1973). However, in each instance the control system

(1) is presumably "detector oriented", (2) responds to nephron loss by progressively magnifying the response to the gain or loss of fixed amounts of the solute; (3) maintains specificity for the solute which it regulates; (4) may well contain a humoral "messenger" that modifies transtubular transport of the solute; and finally, (5) possesses the ability to override, if necessary, the effects of any other solute control system that could theoretically interfere with the required degree of modulation of the specific solute being regulated.

7 The Regulation of Specific Solutes in CRD

7.1 Sodium

The difference in the end organ response of the normal subject and a patient with a GFR of 2 ml/min to the ingestion of the same amount of sodium illustrates the magnitude of the adaptation phenomenon and in particular of the magnification. However, the adaptation actually begins with the first wave of nephron destruction and continues throughout the natural history of CRD. In Fig. 3, values for fractional sodium excretion are plotted against GFR through the entire course of chronic renal disease.

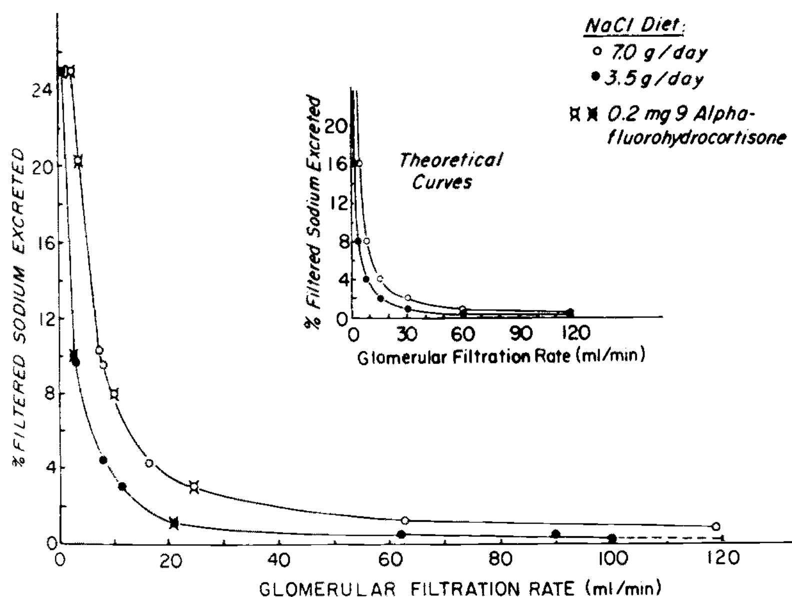

Fig. 3. The relationship between steady-state GFR and the fraction of filtered sodium excreted on 3.5- and 7.0-g salt diets (from *Slatopolsky* et al. 1968a)

The two curves, one representing the response to a 60 mEq per day sodium intake, the other to a sodium intake of 120 mEq a day, are smooth, continuous, and at each GFR appropriate for the maintenance of external balance and a normal serum sodium concentration (*Slatopolsky* et al. 1968a).

7.1.1 The Location of the Detector Element

Some evidence, mostly phenomenologic, points to the upper portions of the body and presumably the thorax as the location of the detector element. For example, when a normal subject is immersed in a tank of water to the neck, a translocation of ECF takes place from the lower extremities into the central circulation. A modest natriuresis follows (*Epstein* et al. 1972). When patients with varying degrees of severity of CRD are "water immersed," the same internal translocation of ECF occurs. However, the lower the steady state GFR, the greater is the natriuresis, and values for fractional sodium excretion in patients with advanced CRD may increase by more than 15% (*Bricker* 1978). The inverse relationship between the magnitude of the rise in fractional sodium excretion and the GFR serve further to highlight the magnification phenomenon (*Schultze* et al. 1969). However, it also seems to lend credence to an intrathoracic location for the detector element inasmuch as the extreme degrees of natriuresis take place in the face of contraction of ECF volume in the lower extremities. Another experiment leads to similar conclusions. When patients with CRD are subjected to ECF volume expansion of approximately 1.5 liters, the natriuretic response (as measured by fractional sodium excretion) occurs, and once again the magnitude is inversely related to steady state GFR (*Schultze* et al. 1969; *Wilkinson* et al. 1972; *Schultze* and *Berger* 1973). If, at the height of the natriuretic response, the resistance to venous return from the lower extremities is increased by inflating tourniquets around both thighs to pressure slightly below the diastolic blood pressure, the natriuresis is aborted and values for fractional sodium excretion return to the preinfusion level (*Slatopolsky* et al. 1968a). Presumably, the latter maneuver results in sequestration of fluid in the lower extremities and a relative decrease in central blood volume.

Up to now it has not been possible to determine further the nature of the detector element, precisely what is detected, exactly where the element (or elements) is located, and whether the magnification phenomenon owes its existence to progressive enhancement of the sensitivity of the detector element or to progressive enhancement of the sensitivity of elements in the control system beyond the detector element.

7.1.2 The Effector Element

The natriuretic forces serving to maintain sodium balance and volume homeostasis in health presumably are operative in progressive CRD. Brief comments will be made about several of the factors currently believed to participate in the regulation of sodium excretion.

7.1.2.1 Increase in GFR. In various models of experimental CRD, SNGFR increases in stage III to values two or more times that of the control values (*Bank* and *Aynedjian* 1966; *Deen* et al. 1974; *Weber* et al. 1975). Such an increase, when it occurs, should certainly play a supportive role in enhancing the natriuretic capability of the involved nephrons. However, it presumably does not explain the adaptation in sodium excretion with time in CRD, nor does it explain the magnification phenomenon. For example, it has been shown in the stage II dog on a constant intake that reduction of GFR toward the stage III value produced by compression of the renal artery does not lead to progressive sodium retention; rather, external balance of sodium is preserved (*Schultze* et al. 1969). Moreover, in experimental models with immunologic glomerulonephritis, values for SNGFR are either normal or reduced (*Rocha* et al. 1973; *Lubowitz* et al. 1974). Nevertheless, balance studies have demonstrated the ability of such animals to maintain external sodium balance as long as they are not nephrotic (*Godon* 1972). A final argument against a prepotent role for increases in SNGFR in the continuing ability to maintain external sodium balance in CRD is implicit in Fig. 3. To account for the magnification phenomenon on the basis of SNGFR, one would have to evoke a rise in values that would in some way parallel a hyperbole shown in Fig. 3; moreover, the lower the total GFR, the greater is the increased fractional sodium excretion associated with a modest change in dietary salt intake. Hence, one also would have to postulate that the lower the GFR, the greater is the acute increased fractional sodium excretion associated with a modest change in dietary salt intake. Hence, one also would have to postulate that the lower the GFR, the greater is the acute increment in SNGFR to permit survival on a varying intake of salt.

7.1.2.2 Redistribution of Glomerular Plasma Flow. Another possibility, not too dissimilar to that based on rising values for SNGFR, is that there is a shift in the distribution of blood flow and of glomerular filtration rate between superficial and deep nephrons, a shift that would enable any given number of nephrons to increase its rate of fractional excretion (*Del Greco* et al. 1969; *Carriere* et al. 1973). The arguments presented in the foregoing paragraph about SNGFR all appear to be applicable to the redistribution hypothesis. These arguments will not be redeveloped in the present context.

7.1.2.3 "Physical Factors". A number of events that influence the intrarenal peritubular environment are known to modify fluid reabsorption in the proximal tubule. These include changes in peritubular capillary oncotic pressure (*Davidman* et al. 1972), changes in peritubular capillary hydrostatic pressure (*Martino* and *Earley* 1968), and changes in hematocrit (*Bahlmann* et al. 1967). For the deep nephrons, the rate of blood flow through vasa recta and local sodium radiance also may theoretically influence the rate of sodium excretion into the urine.

It seems most unlikely to the authors that physical factors could account for the type of regulation depicted in Fig. 3 and could explain the magnification phenomenon. Were this the case, one or more of the physical factors would have to show the change that parallels the natriuretic response to a fixed sodium load, and the change would have to progress throughout the entire course of CRD. However, not all patients with CRD are hypertensive, nor is there any consistent relationship between the level of the blood pressure and the ability to maintain external sodium balance on an ad libitum sodium intake. Although the filtration fraction does fall in the transition from stage II to stage III in the dog, this fall is independent of the intake of sodium chloride and does not bear any consistent relationship to the ability of the experimental animal to maintain external sodium balance (*Schultze* et al. 1969).

Most of the information about the influence of physical factors on sodium reabsorption relates to the proximal convoluted tubule of superficial nephrons. It is not at all clear that modification of proximal sodium reabsorption can, under any circumstances, lead to subtle modulation of sodium excretion rates (*Howards* et al. 1968; *Knox* 1973; *Sonnenberg* 1973; *Stein* et al. 1973, 1974). But, to whatever degree inhibition of proximal reabsorption contributes to the natriuresis per nephron, no correlation could be found between fractional sodium reabsorption in superficial proximal tubules of stage III rats and sodium intake (*Weber* et al. 1975). Moreover, in both nephrotic uremic patients and nephrotic uremic rats, the profound hypoalbuminemia should lead to marked inhibition of fluid reabsorption in the proximal tubule. Yet, in both groups external sodium balance is not preserved and sodium retention occurs (*Godon* 1972; *Bourgoignie* et al. 1974). This represents one of the rare examples where external sodium balance is not preserved in advancing CRD. On the other hand, when the nephrotic patients and rats were given salt poor albumin intravenously so as to elevate their serum albumin levels, a striking natriuresis followed despite the presumption that fractional sodium reabsorption increased (*Luetscher* et al. 1950; *Allison* et al. 1975). Little is known about the role of physical factors in the inner medulla in the fine modulation of sodium excretion. There are data to indicate that in the transition from stage II to stage III total blood flow and presumably medullary blood

flow goes up strikingly in the remnant kidney of the dog (*Kaufman* et al. 1975). However, to account for the pattern shown in Fig. 3 and for the magnification phenomenon, it would be necessary to demonstrate a change in the influence of the intermedullary physical factors that corresponds to the established changes in sodium excretion in the course of CRD. To date neither the evidence for this nor an acceptable theoretical model has been produced.

7.1.2.4 Aldosterone Activity. Aldosterone levels may be elevated in patients with advanced CRD (*Cope* and *Person* 1963; *Hayslett* et al. 1969; *Schrier* and *Regal* 1972; *Berl* et al. 1978). Moreover, if the values for SNGFR are markedly increased, absolute reabsorption in those portions of the nephrons that are influenced by aldosterone may be increased at the same time that absolute sodium excretion and fractional excretion rates are increased. However, when SNGFR is normal or low, it is difficult to invoke increased levels of a sodium-retaining hormone in explanation of patterns of sodium excretion depicted in Fig. 3. Moreover, the natriuretic response that characterizes the transition from stage II to stage III and dogs with a unilateral remnant kidney took place in animals in which mineralocorticoid hormone activity was maintained at supernormal levels through the administration of fluorocortisol. It also took place in animals that were adrenalectomized and given maintenance doses of desoxycorticosterone (*Schultze* et al. 1969).

7.1.2.5 Prostaglandins. The role of prostaglandins in modulating sodium transport is currently under study in a number of laboratories. At the present time the data are conflicting (*Papanicolaou* et al. 1975; *Tobian* and *O'Donnell* 1976; *Kaye* et al. 1978; *Dunn* 1979), but none of the available observations supports the possibility that prostaglandins represent the key modulators of sodium excretion in CRD and none can account for the patterns shown in Fig. 3 or for the magnification phenomenon. A supportive role of prostaglandin cannot, however, be dismissed at this time.

7.1.2.6 Natriuretic Hormone. Although a natriuretic hormone has not yet been isolated in pure form, chemically defined, or synthesized in the laboratory, there is a large, growing, and impressive body of evidence supporting the existence of such a hormone. Moreover, an impressive case may be made in favor of this putative hormone representing the major modulator for sodium excretion in advancing CRD (*Brickler* 1967) by its behavior in normal individuals (*Brown* et al. 1972; *Buckalew* and *Lancaster* 1972; *Favre* et al. 1975, 1979; *Gonick* and *Saldanha* 1975; *Favre* 1978a, b; *Clarkson* et al. 1979). Some of the evidence of a circulating inhibitor of sodium transport in uremia [i.e., natriuretic hormone (NH)] as well as

some of the biologic properties of NH will be reviewed briefly. A low molecular weight substance (less than 1000 daltons) has been found in urine and serum of patients and dogs with chronic uremia who are on an average salt diet and who demonstrate the typical natriuresis per nephron seen in CRD (*Bourgoignie* et al. 1972, 1974). Detection of the biologic activity involves the use of one of several bioassay techniques. These include inhibiton of transcellular sodium transport by the isolated frog skin or toad bladder (*Bourgoignie* et al. 1971; *Kaplan* et al. 1974), inhibition of sodium-potassium-activated ATPase activity (*Hillgard* et al. 1976), increase in absolute and fractional sodium excretion in the unanesthetized stage III rat (*Bourgoignie* et al. 1974), increase in the water-loaded stage I rat (*Sealey* et al. 1969; *Brown* et al. 1972; *Favre* et al. 1979), inhibition of active sodium transport in the isolated perfused cortical collecting tubule of the rabbit (*Fine* et al. 1976a), and inhibition of sodium efflux by the Modin-Darby canine kidney (MDCK) strain of tubular epithelial cells grown in tissue culture and originally obtained from the intact dog kidney (*Licht*, unpublished data).

As indicated, the active inhibitor may be obtained using either urine or serum as the source. It also has been obtained from kidney homogenates and hypothalamic preparation (*Clarkson* et al. 1974; *Gonick* and *Saldanha* 1975; *Louis* and *Favre* 1980a). The initial step of isolation and purification involves the use of gel filtration techniques (usually Sephadex G-25 or biogel P-2). The biologic activity has been found by most investigators in the fraction of eluate that appears immediately after the peak containing the majority of the inorganic salts, which include sodium, chloride, and calcium as well as urea and creatinine. A higher molecular weight fraction has also been observed by some investigators (*Sealey* et al. 1969; *Buckalew* and *Lancaster* 1971; *Buckalew* 1972; *Clarkson* et al. 1976; *Godon* 1978), but the relationship between the two inhibitors of sodium transport has not yet been clarified. The possibility exists that the larger substance is a precursor and the smaller is the active hormone (*Gruber* and *Buckalew* 1978).

Some of the biologic properties of NH are as follows: It is active when added to the "blood side" of anurian membranes or the peritubular capillary surface of the isolated perfused renal tubule (*Bourgoignie* et al. 1974; *Kramer* et al. 1974; *Favre* et al. 1975; *Fine* et al. 1976a). NH increases the intracellular sodium content of isolated epithelial cells from the toad bladder and decreases the rate of pyruvate oxidation by the same cells (*Kaplan* et al. 1974). NH also has no effect on systemic or renal hemodynamics in the rat (*Favre* et al. 1979). In both the assays involving the unanesthetized stage I and III rats and the MDCK epithelial cells, a dose-response relationship may be demonstrated (*Favre* et al. 1979; *Licht*, unpublished data). In the former assay, increments in fractional excretion of sodium as great as

15% may be produced using a concentrated fraction of NH contained in a total volume of 200 μl (*Licht,* unpublished data).

In uremic dogs in which the adaptive natriuresis per nephron is reversed by proportional reduction of sodium intake, no NH activity may be demonstrated using the standard stage III rat bioassay (*Schmidt* et al. 1974). In uremic man with concomitant high rates of protein excretion, hypoalbuminemia, and avid sodium retention with values for fractional excretion of sodium of less than 1%, no activity has been demonstrated (*Bourgoignie* et al. 1974). Activity, however, is present in the urine of normal dogs fed a high salt diet and given a potent mineralocorticoid hormone after the animals have "escaped" (*Favre* et al. 1975). In the latter experiments, the incidence of positive bioassays correlated with the degree of sodium retention prior to escape (*Favre* et al. 1975). Activity is present in normal man during the natriuresis of water immersion (*Epstein* et al. 1978). It has been found in nonuremic patients with aldosterone secreting tumors before surgery and the activity has disappeared in the same patients after removal of the tumors (*Kramer* et al. 1977; *Favre* 1978a). Activity has been found in the kidney tissue extracts from rats acutely expanded by isotonic saline in amounts depending on the previous sodium intake (*Louis* and *Favre* 1980a), but it is not detectable in rats expanded by albumin solution in which the natriuretic syndrome failed to appear (*Stein* et al. 1973; *Louis* and *Favre* 1980b). The gel filtration eluate containing the biologic activity has been subjected to a number of additional purification techniques and considerable purification has been effected with several of these, particularly those involving high performance liquid chromatography (*Buckalew* and *Gruber* 1978; *Licht,* unpublished data).

The active fraction has either chemical and/or biologic properties which differentiated it from prostaglandins, vasopressin, parathyroid hormone (or fragments thereof), angiotensin, and kallikrein.

The presence of the active material in both blood and urine of uremic patients and animals with an adaptive natriuresis per nephron suggests that it is produced in increased quantity in uremia rather than being retained in the blood by virtue of failure of excretion. Its existence in increased activity (demonstrable in bioassay) in both animals and patients, who, for various reasons, are undergoing high rates of sodium excretion per nephron suggests that it is not a nonspecific concomitant of the uremic state. The foregoing observations, coupled with the fact that the activity is not readily demonstrable in the presence of chronic uremia when the adaptive natriuresis per nephron is absent (e.g., proportional reduction of sodium in the dog and nephrotic syndrome in uremic man), support a physiologic role for NH in the modulation of sodium excretion. The demonstration that it inhibits transcellular sodium transport in the toad bladder, which is an analogue of the distal portions of the nephron, and that it inhibits

sodium reabsorption in the isolated perfused cortical collecting tubule supports the possibility that NH acts "downstream" in the nephron and thus would be a candidate for the "fine modulator" of sodium excretion. The fact that it acts only from the peritubular capillary surface, that it increases intracellular sodium content, and that there is a fall in oxidated phosphorylation that accompanies the decrease in sodium transport supports the view that NH acts on some key step or steps in the active transport system for sodium either on the carrier mechanism, the energy source for the carrier or perhaps the coupling between the two.

The fact that the increased rate of production of NH is associated with the increased values for fractional excretion of sodium in CRD would favor its role in supporting the adaptation and in providing at least a partial explanation for the pattern of sodium excretion shown in Fig. 3. Evidence obtained by infusing NH preparations directly into the renal artery of stage I and stage III rats has shown a marked increase in end organ sensitivity in the nephrons of the latter group, and this observation could help to explain the magnification phenomenon (*Fine* et al. 1976b).

The ultimate determination of the role of the natriuretic hormone in the modulation of sodium excretion in health, in the adaptive changes in sodium excretion in progressive CRD, and in the explanation of the magnification phenomenon represents an area of importance and one that is the subject of considerable interest in many laboratories at the present time.

7.2 Potassium Transport by the Remaining Nephrons

The regulation of the potassium concentration of the ECF must be accomplished in advancing CRD. The range of potassium concentration as consistent with life and well-being is small (ca. 2–7 mEq/liter), and the total amount of potassium entering the ECF daily through the diet equals or exceeds the total amount of potassium contained in the ECF in a 70-kg adult person. Thus the adaptive kaliuresis per nephron that occurs with advancing nephron loss must be hihgly developed and highly sensitive (*Berlyne* 1971).

In a healthy person the daily rate of ingestion of potassium, and thus the amount requiring renal excretion, is approximately 10%–15% of the amount filtered each 24 h. Approximately half of the filtered potassium is reabsorbed by the end of the accessible portion of the proximal convoluted tubule. Additional reabsorption continues in the ascending limb of the loop of Henle, and by the time tubule fluid reaches the distal tubule, a very small fraction of the filtered load (approximately 10%) remains unreabsorbed (*Beck* et al. 1973; *Wright* 1977). Thus, virtually all of the potas-

sium delivered into the urine, at least in superficial nephrons, derives from secretion beyond the last accessible portion of the distal tubule (*Berliner* and *Kennedy* 1948). Some reabsorption may occur in the collecting tubule, but the precise role of this reabsorptive process in the modulation of the excretion rates of potassium remains unknown.

The continuing ability to maintain external balance and normal serum potassium concentrations until very late in the course of CRD is due principally to the increasing rates of kaliuresis per nephron, although there is some increase in the contribution of fecal excretion of potassium in uremia. The amount of potassium reaching the distal tubule in the uremic rat has not been found to be increased over normal (*Bank* and *Aynedjian* 1973); thus, in uremia as in health virtually all the potassium delivered into the urine is secreted "downstream." On a potassium intake of 60–80 mEq per day, a patient with a GFR of 5 ml/min and a serum potassium of 4.5 mEq/liter must excrete 123% of the total amount of potassium filtered. At a GFR of 2 ml/min the total amount of potassium filtered in 24 h is only 13 mEq. Hence, to deliver 40 mEq of potassium into the urine requires a secretory rate that is 300% greater than the amount filtered.

The distal segments of the nephron must therefore play the principal role in the maintenance of external potassium balance in CRD (*Schon* et al. 1974). An impressive demonstration of the magnitude of this role has recently been obtained from observations on cortical collecting tubules removed from uremic rabbits maintained on high versus low potassium diets (*Fine* et al. 1979). The isolated perfused tubules from the animals on the high potassium intake secreted more than six times the rate observed in the same nephron segment in normal rabbits and several times the amount secreted by nephron segments from uremic rabbits on a normal potassium intake. The fact that these high rates of secretion persisted in tubular segments removed from the uremic animal as well as from the kidney of that animal suggests that the potassium secretory adaptation, whatever its genesis, contains a "memory."

None of the known factors thought to control potassium excretion in health accounts for the remarkable adaptive capacity for potassium secretion in CRD nor for the magnification phenomenon for potassium (*Schultze* et al. 1971). A nonaldosterone-mediated increase in Na-K-ATPase activity in the outer medulla has been found to accompany this adaptive kaliuresis per nephron (*Finkelstein* and *Hayslett* 1974), and a similar increase has been observed in the kidneys of normal rats maintained on a large potassium intake (*Silva* et al. 1973; *Epstein* 1975). The change in ATPase activity, on the other hand, can be prevented by reducing the potassium intake in proportion to the decrement in GFR, an event which serves also to prevent the adaptive kaliuresis per nephron (*Silva* et al. 1973). Na-K-ATPase activity also has been found to be increased in the

colon of uremic rats in association with an enhanced rate of GI excretion of potassium (*Fisher* et al. 1974; *Basti* et al. 1975). Thus, the possibility that the Na-K-ATPase system participates in the adaptation in potassium excretion in CRD is not without a certain amount of experimental support. But, it is not clear whether the relationship is a primary one or whether the increased ATPase activity is a supportive or an associated phenomenon. For example, in the previously cited studies of the isolated perfused uremic rabbit cortical collecting tubules, no correlation could be found between the degree of augmentation of potassium secretion and the level of Na-K-ATPase activity.

Other factors which could play a role, from permissive to regulatory, in the adaptive kaliuresis include an increase in distal delivery of sodium in the tubular fluid (*Kleeman* et al. 1966), hyperaldosteronism (*Schmidt* et al. 1975), an increased intraluminal electronegativity in the collecting duct (*Hauley* et al. 1980), and an increased potassium activity gradient from tubular epithelial cell water (in the collecting tubules) to tubular fluid (*Giebisch* 1971).

A number of these factors may probably be ruled out as the major modulating event in the potassium adaptation and magnification phenomenon. External potassium balance is maintained accurately in chronically uremic dogs and rats in the face of changing rates of intake and excretion of sodium as well as other solutes, including phosphorus and ammonium (*Schultze* et al. 1971). Balance is maintained in the uremic dog that is either adrenalectomized and given a low fixed dose of mineralocorticoid hormone or given a super maximal dose of mineralocorticoid hormone without adrenalectomy. The adaptation occurs within 24 h of the conversion of a stage II dog to a stage III animal and appears to be independent of any changes of serum potassium concentration, although values for intracellular activity have not been measured in uremic animals (*Schultze* et al. 1971). Thus, an increase in potassium excretion rate per nephron from an amount approximating 10% of the filtered load to an amount in excess of 200%–300% of the filtered load occurs within 24 h of the initiation of uremia. This increase cannot be explained on the basis of the concurrent patterns of excretion of sodium or any other solutes, at the level of mineralocorticoid hormone activity, or on the concentration of serum potassium. Moreover, the kaliuresis per nephron persists when SNGFR is reduced in the surviving nephrons by constricting the renal artery experimentally (*Espinel* 1975b). The composite group of data raises the possibility that a modulator of potassium secretion, as yet not defined, may not only exist but may also be the principal determinant of the high rates of potassium secretion in the surviving nephrons in advanced CRD. If such a modulator plays this role in uremia, then it is quite likely that it is present in health and participates in the regulation of potassium excretion

and the maintenance of potassium balance in normal states. Although no concerted effort has yet been launched to find a "kaliuretic hormone," there appears to be sufficient evidence of a phenomenologic nature to warrant such a search.

7.3 Phosphate Transport by the Surviving Nephrons

Normal phosphatemia will prevail in advanced CRD with an unrestricted intake of elemental phosphorus only if the total increment in phosphate excretion (i.e., the aggregate of the single nephron increments) offsets precisely the reduction of the total excretion resulting from the loss of nephrons. In practice, normal phosphatemia does prevail irrespective of the form of CRD through at least 75% of the natural history of the disease (*Bricker* et al. 1972). A considerable amount of information has been accumulated about biologic control systems regulating phosphate excretion in health, and although there are still areas of uncertainty and some areas of conflict, the body of knowledge about the adaptation in phosphate excretion that occurs in CRD is substantial and growing.

The amount of phosphorus ingested in the diet each 24 h varies from culture to culture and from society to society, but a value of 1 g per day is probably an accepted representative figure.

Of this amount, approximately 70% is absorbed across the GI tract and enters the ECF. The remaining 30% is excreted in the stool. Following each phosphate-containing meal there is a finite elevation of serum phosphate levels and, although the precise physical chemical mechanisms are not completely known, the elevation of serum phosphate results in a reciprocal fall in the concentration of ionized calcium in the serum. In both normal and uremic dogs given an oral load of phosphorus of 500 mg there is a 0.1 mg percent fall in ionized calcium for every 1 mg percent rise in serum phosphate. The effect of the drop in ionized calcium on the parathyroid glands is to increase the rate of release of parathyroid hormones (PTH) and thus to increase the concentration of PTH reaching the receptor site in the renal tubule (*Kaplan* et al. 1978). With a normal GFR and an amount of phosphate requiring excretion equal to 700 mg/day, the PTH must inhibit 15% of the filtered phosphate from being reabsorbed. Thus, 85% of the filtered load of phosphate ions will be returned to the ECF and 15% will be excreted. This is a value for the full 24 h and does not reflect the postprandial increments in fractional excretion. At the end of the 24 h the full 700 mg of ingested and absorbed phosphorus has been excreted and the serum phosphate concentration, serum calcium concentration, and serum PTH levels are restored to their previous control levels (*Bricker* et al. 1972).

Adaptation in phosphate excretion begins, as with other solutes, with the loss of nephrons (*Massry* et al. 1973). If the intake in absorption of phosphorus is unchanged, nephron loss will lead to retention of that amount of phosphate that was being excreted by the destroyed units. There thus will follow an elevation in serum phosphate concentration which is greater than would have occurred if the full population of nephrons continued to function. The reciprocal fall in ionized calcium will also be greater, and the stimulation of PTH secretion will lead to higher levels of circulating hormones. Due to the latter, the level of inhibition of phosphate reabsorption per surviving tubule will be increased (*Bank* et al. 1978) and the total rate of phosphate excretion will rise sufficiently to restore serum phosphate levels and ionized calcium levels to normal (*Goldman* and *Bassett* 1954; *Slatopolsky* et al. 1968b). As the calcium levels rise, the stimulus to PTH secretion will diminish; however, values for PTH cannot return to the control level, for if this were to happen, the rate of phosphaturia per nephron would diminish to its control level, retention would reoccur, ionized calcium values would fall, and the augmented stimulus to PTH secretion would be renewed. Consequently, following each wave of nephron destruction, PTH levels will rise and then will remain at the new level, although very likely the existing as oscillations as well as pre- versus postprandial swings (*Reiss* et al. 1970) probably persist.

The adaptation must serve more than the elimination of the phosphate retained immediately following nephron destruction. Owing to the decrease in the GFR and the number of nephrons, the mean value for 24-h phosphate excretion per nephron must remain elevated, if the amount of phosphorus entering the ECF daily is not reduced in proportion to the fall in GFR.

Within the framework of the foregoing theoretical formulation an explanation may be presented for the magnification phenomenon for phosphate. When an acute load of phosphorus (600 mg) was administered to a group of uremic dogs and to a matched group of normal animals, the serum phosphate concentration rose by approximately 2 mg percent more in the uremic group, although peak values occurred at approximately 2.5 h after the phosphate load in both groups. Ionized calcium fell in an identical fashion in the two groups of animals (i.e., 1 0.1/10 mg percent fall with each 1 mg percent rise in serum phosphate concentration). Thus, serum PTH levels which were already markedly elevated in the uremic dogs prior to the acute load rose further, and fractional phosphate reabsorption, which already was reduced in the uremic dogs, was further reduced. The filtered load of phosphate (GFR times serum phosphate concentration) exceeded the Tm for phosphate through most of the 5-h period of observation following the phosphate loading. The augmentation of phosphate excretion per nephron thus was following the same oral load of phosphate

and despite the existence of identical fasting serum phosphate concentrations. Indeed, the calculated peak rate of phosphate excretion per nephron (expressed as an increase over the preloading control value) was over 300% greater in the uremic than in the normal dogs (*Kaplan* et al. 1978).

There are certain observations that raised the question as to whether PTH is the principal mediator of the adaptive increase in phosphate excretion per nephron in CRD (*Sherwood* et al. 1968; *Fotino* 1977). Although it has been shown that PTH levels rise throughout the course of CRD in both patients and dogs (*Arnaud* 1973; *Reiss* et al. 1969; *Kaye* 1974; *Slatopolsky* et al. 1971), most of the observations have been performed using antibodies that measure the carboxy terminal end of the PTH molecule and its fragments (*Arnaud* et al. 1974). There are data indicating that the rise in the carboxy terminal species correlates with the fall in GFR and that the relationship between the values obtained and the biologic activity of the circulating material measured may be variable and/or inconsistent (*Martin* et al. 1978; *Massry* et al. 1979). To date, not enough data using an aminoterminal antibody are available, although information of this nature is currently being collected.

One point in favor of the validity of the hypothetical formulation is that the rise in PTH levels using the carboxy terminal antibody is largely if not completely suppressed by preventing the adaptive phosphaturia from occurring in the course of CRD. Thus, if phosphate intake and absorption into the ECF are reduced in proportion to the decrement in GFR, there is no requirement for an adaptive phosphaturia per nephron in the residual nephron, and neither the adaptation nor the rise in PTH levels occurs (*Slatopolsky* et al. 1972; *Slatopolsky* and *Bricker* 1973; *Kaplan* et al. 1979).

Another observation which could question the theoretical formulation presented here is found in a recent study demonstrating that uremic dogs that are parathyroidectomized appeared to maintain external phosphorus balance without the intervention of parathyroid hormones (*Swenson* et al. 1975). This observation requires confirmation and extension, and experiments are currently in progress in an effort to accomplish both (*Slatopolsky* et al. 1978b).

This theory also omits the potential role of vitamin D metabolites in the pathogenesis of secondary hyperparathyroidism, and conceivably in adaptation the most active vitamin D metabolite is 1-25 dihydroxy D_3. A precursor of this hormone is 25 hydroxy D_3, and its conversion to the 1-25 form occurs in the kidney. Thus, if 1-25 dihydroxy D_3 activity were to diminish early in the course of CRD and values were to fall progressively with time, it is conceivable that a defect in the enteric absorption of calcium could be the primary stimulus to increased secretion rates of PTH rather than the mechanism based on transient periods of phosphate reten-

tion in the serum. At this time several laboratories are in the process of defining the relationship between GFR and the circulating levels of 1-25 dihydroxy D_3. The available data suggest that the values are not low in early or moderately advanced disease, and data on far advanced disease are as yet quite limited (*Slatopolsky* et al. 1975, 1978a; *Colodro* et al. 1978). However , at whatever point vitamin deficiency serves to retard GI absorption of calcium, the development of and/or rate of progression of secondary hyperparathyroidism would be augmented. Moreover, reduced biologic activity of vitamin D and its metabolites on the GI tract may be assumed with a reasonable degree of certainty to account in large measure for any osteomalacic component of uremic osteodystrophy.

It is possible that a decrease in the one hydroxylation step of 25 hydroxy D_3 could influence the adaptation in phosphate excretion in CRD through additional mechanisms, i.e., a direct effect of D_3 and/or a biologically active metabolite on the renal regulation of phosphate excretion (*Bonjour* et al. 1977). However, if the vitamin or its metabolites does influence the transtubular movement directly or through some vitamin-D-PTH interdependence, the role of the vitamin-hormone in the adaptation could be very substantial (*Popovtzer* et al. 1974). There also is uncertainty as to the effect of 1-25 dihydroxy D_3 and other vitamin metabolites, particularly 24-25 hydroxy D_3, on the rate of PTH secretion (*Maser* et al. 1975). If one or another of these substances represents a component part of a feedback loop, once again the vitamin hormones would assure a role in the adaptation and conceivably in the magnification phenomenon of a higher order than that attributed to them in the present discussion.

8 Acid-Base Regulation: The Adaptation in Ammoniagenesis and the Excretion of Titratable Acid

The requirements involved in the regulation of acid base homeostasis in CRD have dimensions that differ strikingly from that of any of the other key solutes of the ECF. From a physical and chemical point of view, the hydrogen ion is a unique particle among the solutes of body fluids. It is highly reactive, consisting of a single proton, and has a very high ratio of charge to density, with an exceedingly high affinity for combining with other molecules that are negatively charged. It is likely that the hydrogen ion exists only transiently as a free proton in body fluids. Rather, it is presumed to attach itself to water molecules to form hydronium ions or H_3O with a positive charge. The concentration of hydrogen ions (or hydronium ions) under normal circumstances in the ECF is extremely small, both in relation to that of other key solutes of the ECF and in rela-

tion to the rate of acquisition of new hydrogen ions and over 3 million sodium ions for every hydrogen ion circulation in the ECF. Moreover, for every hydrogen ion normally present in the ECF, approximately 500 000 new hydrogen ions, generated principally from metabolism of protein, enter the ECF in the course of 24 h. If less than 140 thousandths of this daily load were to remain free in solution, death would ensue rapidly. Obviously, a highly effective control system must exist in the face of this striking imbalance between the rate of acquisition of hydrogen ions and the circulating concentration which is consistent with survival. The changes that take place in the regulatory system in progressive renal disease must, and do, maintain the hydrogen ion concentration of the ECF within the rather narrow limits compatible with life.

The hydrogen ions that enter the ECF are buffered primarily by bicarbonate. Under steady state circumstances, the same number of hydrogen ions is excreted daily in the urine by virtue of a process which serves to restore the same number of bicarbonate ions that were consumed in the initial buffering process. The process whereby both of these events are accomplished involves the secretion of hydrogen ions from the tubular epithelial cells into the tubular fluid and their combination with the two principal nonvolatile buffers, ammonia and phosphate. The hydrogen that is secreted derives from carbonic acid, and the residual bicarbonate is returned across the contraluminal membrane to the venous circulation.

The other major charge of kidneys in the maintenance of acid-base homeostasis is the reabsorption of all of the bicarbonate that is filtered, an amount which is in excess of 4,500 mEq per day in a normal person.

In the course of CRD the rate of hydrogen ion accession is no less than in a normal subject, unless the protein intake is diminished; this usually takes place relatively late in the course of the disease (*Elkington* 1957). Thus, in order to prevent progressive acidemia as nephron destruction proceeds in CRD, the residual nephrons must adapt by excreting more hydrogen in the form of ammonium and dihydrogen monosodium phosphate while simultaneously increasing the rate of bicarbonate production. The nephrons must also continue to reabsorb all or virtually all of the filtered bicarbonate, for the loss of bicarbonate into the urine would serve to offset compensatory adaptive increments in net hydrogen ion excretion per nephron and de novo bicarbonate excretion per nephron.

8.1 Bicarbonate Reabsorption

Although there is a body of opinion that holds that bicarbonate loss into the urine due to inhibition of proximal tubular bicarbonate reabsorption is a major factor in the pathogenesis of the metabolic acidosis of chronic

renal disease, the bulk of the evidence would seem to oppose this point of view (*Morrin* et al. 1962b; *Espinel* 1975a). In the physiologic state, proximal bicarbonate reabsorption is diminished by expansion of the ECF (*Purkenson* et al. 1969; *Slatopolsky* et al. 1970; *Herbert* et al. 1972) as well as by high circulating parathormone levels (*Crumb* et al. 1974), both conditions that are met in CRD. By extrapolation it often is assumed and actually supported by experiments in man and rats (*Slatopolsky* et al. 1970; *Lubowitz* et al. 1971) that in uremia the kidneys are no longer able to reabsorb the total amount of the filtered bicarbonate. However, recent observations in uremic dogs have clearly demonstrated that the capacity to reabsorb the bicarbonate is increased despite the presence of high fractional excretion rates for sodium, potassium, and phosphorus (*Schmidt* et al. 1976). Superimposed ECF expansion decreases in both control and uremic dogs the proximal tubular bicarbonate reabsorption, but *for any given degree of ECF expansion, the uremic animal reabsorbs more bicarbonate than its control* (*Arruda* et al. 1976). Explanations for the fact that in uremia the capacity for reabsorbing bicarbonate is increased remain unclear, since it could not be accounted for by the effects of known determinants of bicarbonate reabsorption. In addition, under steady-state conditions in all forms of CRD, the pH of the urine tends to remain between 4.5 and 5.5 (*Morrin* et al. 1962a). For these pH values, because the pK of the carbonic acid bicarbonate buffer system operationally is approximately 6.1 in urine and the pCO_2 is rarely in excess of 40 to 60 mmHg, the urine must, by virtue of the law of mass action, be virtually free of bicarbonate. Thus, factors other than diminution in bicarbonate reabsorption appears to be responsible for the degree of acidosis that does develop in the course of CRD.

8.2 Hydrogen Excretion

The amount of hydrogen a kidney could eliminate depends on its capacity to produce ammonia and on the amount of titrable acid (most is phosphate) available in the urine.

8.2.1 Ammoniagenesis

In health 60% or more of net hydrogen excretion is accounted for by the excretion of ammonia, the production of which is stimulated by the exogenous and/or endogenous acid load. The capacity to produce adequate amounts of ammonia both in steady state and in response to systemic acidosis is preserved for GFR values equal to or above 20%–30% of normal (*Schoolwerth* et al. 1975; *Welbourne* et al. 1972). This implies an adaptive

increase in the ammoniagenesis per nephron as GFR decreases. Because no changes in enzymes or substrates and no increase in the production rate of ammonia per milligram DNA are associated with the increased production of ammonia per nephron in the rat, this compensatory phenomenon has to be explained on the basis of the formation of additional ammonia-producing cells due to hyperplasia (*Benyajati* and *Goldstein* 1978; *MacLean* and *Hayslett* 1980).

As GFR decreases further, although the ammoniagenesis per nephron may still increase, the total 24-h rates of ammonium excretion may be reduced to levels below 10 mEq and acidosis develops (*Schwartz* et al. 1959). Both the increased ammoniagenesis per nephron and its limitation are supported by experimental evidence from the studies of stage II acid-loaded dogs reduced to stage III (*Morrin* et al. 1962b).

8.2.2 Titratable Acid

The other form in which hydrogen ions are excreted in the urine is titratable acid (most of which is buffered phosphate). In the discussion of the adaptation in phosphate excretion, it was noted that external phosphate balance is maintained through approximately 75% of the course of CRD, even when the load of dietary phosphorus is undiminished. Phosphate balance is also maintained during the last 25% of the course, although it occurs at the expense of hyperphosphatemia (*Bricker* et al. 1972). Nevertheless, the 24-h excretion rates of phosphate under steady-state conditions remain equal to the rate of entry of phosphate into the extracellular fluid. Given the fact that the pK of phosphate is approximately 6.9 and that the urine pH in CRD averages close to 4.9, 99 of every 100 phosphate ions excreted enter the urine in the dihydrogen monosodium form. Thus, the contribution of titratable acid to hydrogen ion excretion and bicarbonate synthesis in progressive renal disease will depend upon phosphate intake, and values may well be equal to those observed in normal individuals. The adaptive phosphaturia per nephron observed in progressive renal disease, therefore, is associated with an adaptive increase in hydrogen excretion per nephron.

9 Conclusion

The residual nephrons respond to the progressive nephron loss by an organized functional adaptation which permits them to maintain homeostasis. They do this by magnifying their response to the information provided by the control systems for the major key solutes (i.e., sodium, phosphorus, and possibly potassium), by increasing secondary to hyper-

plasia the number of specific cells (i.e., ammonium), or by unknown mechanisms (i.e., bicarbonate). As discussed, all the physiologic mechanisms of intrarenal transports apply to the residual nephrons which behave like normal nephrons facing an overload of solute and water.

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Human Thyroxine Binding Globulin (TBG)

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Contents

1 History 46

2 Isolation 47
2. 1 Tata 47
2. 2 Seal and Doe 48
2. 3 Andreoli and Salvatore 48
2. 4 Giorgio and Tabachnik 49
2. 5 Marshall and Pensky 49
2. 6 Hamada and Sterling 50
2. 7 Korcek and Tabachnik 51
2. 8 Nilsson and Peterson 52
2. 9 Kagedal and Kallberg 52
2.10 Present Attempts 53

3 Properties 55

4 Molecule 61
4.1 Structure 61
4.2 Microheterogeneity 63
4.3 Subunits 64

5 Interactions 65
5.1 Experimental Examinations 65
5.2 Theoretical Analyses 71

6 Metabolism 72
6.1 Biosynthesis 72
6.2 Degradation 76

7 Determination 78
7.1 Immunoassay 78
7.2 Other Methods 80

8 Physiologic Role 81

9 Future 84

References 85

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1 History[1]

Since its discovery by *Gordon* et al. (1952) the human thyroxine-binding α-globulin (TBG) has been a subject of extensive research. Being the main carrier of l-thyroxine (T4) in blood, the physiologic importance of TBG was considered to be great. Moreover, as the binding properties of TBG in plasma are in close relationship to the function of the thyroid gland, a number of tests measuring various binding functions of thyroxine-binding proteins have been devised to evaluate the function of the thyroid gland.

The aim of the present review is to survey the most important attempts to purify human TBG and to summarize the available data (as obtained from pure TBG preparations) on its nature. However, the review does not include the thyroxine-binding pre-albumin (TBPA) or any other T4-binding protein. The present article summarizes examples of the relevant literature published toward the end of 1979. It is not the aim of the paper to give a comprehensive account of all papers concerning human TBG; rather, it aims at illustrating the recent development and at anticipating results which could reasonably be expected in this field in the near future. In the past there were several attempts to summarize the results on this topic in the form of review articles (e.g., *Lecureuil* et al. 1977; *Hocman* 1978a–c; *Robbins* et al. 1978).

The contemporary research on TBG seems to cluster around four main issues:

1. Attempts to achieve further purification of TBG;
2. Further elucidation of the structure of TBG, its subunits, its heterogeneity, and its interaction with T4 and 3,5,3′-l-triiodothyronine (T3) from the point of view of physical chemistry;
3. The biosynthesis and metabolic fate of TBG; and
4. Analytic determination of TBG in blood for clinical and research purposes.

The basic principle of purification of TBG is the addition of a tracer dose of either ^{131}I- or ^{125}I-l-thyroxine to blood plasma, serum, or any other starting material such as Cohn fraction IV-5. The radioactive T4 is is preferentially bound to TBG, because T4 is the ligand with the highest affinity to this protein. The fractionation of the above material is carried out by usual physicochemical methods and the fraction with the highest ratio of radioactivity to protein content is collected. The control of the TBG entity after each step of fractionation is done usually by paper or acrylamide gel electrophoresis. During the process of purification, suitable experimental conditions for avoiding the denaturation of TBG, customary in protein chemistry, should be maintained.

1 All statistical values throughout the paper are means ± standard deviations, unless mentioned otherwise.

By means of electrophoresis of plasma, it was shown by *Freinkel* et al. (1955) that human TBG was concentrated in Cohn fractions IV-4, IV-6, and IV-9, all of them containing large amounts of α-globulins. These fractions are therefore considered suitable as a starting material for further purification of TBG. The isolation of TBG from such Cohn fractions was attempted by ion exchange chromatography on Dowex 1, but this procedure caused a marked diminution of the T4-binding capacity of TBG (*Ingbar* et al. 1957). It seems that every attempt to purify TBG, i.e., to separate TBG from its "natural environment" of other proteins in plasma, led invariably to a – sometimes considerable – loss of its binding capacity.

2 Isolation

2.1 Tata

The first serious attempt to purify human TBG was carried out by *Tata* (1961a, b). The proteins of fraction IV-9, labeled with ^{131}I-T4, were subjected to preparative electrophoresis on cellulose at pH 8.6 in barbiturate buffer to prevent the binding of T4 to the TBPA fraction. The protein concentration of the fractions was determined by measuring the absorbancy at 280 nm; the T4 content was estimated by the measurement of radioactivity. The fractions which had the highest ^{131}I to protein ratio (specific radioactivity) were isolated, dialyzed three times, and freeze dried. The above procedure was repeated twice with the freeze-dried protein fractions.

The proteins obtained in this way were subjected to gel chromatography on Sephadex G 75, then further chromatographed on dieethylaminoethanol (DEAE)-cellulose, equilibrated with 0.05 *M* NaH_2PO_4, and eluted with a gradient of sodium chloride up to 0.1 *M* NaCl and 0.05 *M* NaH_2PO_4. The isolated, dialyzed, and freeze-dried T4-binding proteins obtained in this step were purified further by starch gel electrophoresis in glycine buffer, pH 9.0. The zone containing TBG was eluted, dialyzed, freeze dried, and the remains of starch were separated by repeated DEAE-cellulose chromatography as described previously.

A shorter but less efficient method is described by the same author (*Tata* 1961b). In this method the Cohn fraction IV-9 of proteins labeled with radioactive T4 are chromatographed on DEAE-cellulose in 0.05 *M* NaH_2PO_4. The proteins containing the highest specific activity were further purified by repeated (twice) electrophoresis on a cellulose column in barbiturate buffer, pH 8.6. The fraction of proteins was then chromatographed on DEAE cellulose with the salt gradient elution as described previously.

The protein fraction obtained in the first, more thorough procedure seemed to be homogeneous according to the results of zone electrophoresis on paper, cellulose acetate, agar gel, and cellulose column. Electrophoresis on starch, however, revealed a marked heterogeneity of the proteins. The relative purification of TBG, calculated from the specific radioactivity ratio for Cohn fraction IV-9 and the final protein, showed that the purified TBG had a specific radioactivity approximately 50 times higher than that of the starting material.

2.2 Seal and Doe

Seal and *Doe* (1962a, b, 1964) introduced a procedure for isolating TBG, TBPA and corticosteroid-binding protein from human plasma. Blood plasma (pH 7.0 by addition of bicarbonate) was dialyzed against water and applied to a column of DEAE Sephadex A 50 equilibrated with 0.02 *M* phosphate buffer with a pH of 5.9. The adsorbed TBG was eluted by the same phosphate buffer containing 0.3 *M* sodium chloride and was then precipitated by addition of ammonium sulphate. The precipitate was separated, dissolved in water, and fractionated by gel filtration on Sephadex G 200. The most retarded peak, containing albumin and TBG, was isolated, concentrated by ultrafiltration, equilibrated with 0.01 *M* phosphate buffer (pH 6.8), and finally fractionated on hydroxylapatite. TBG was eluted with 0.04 *M* phosphate, pH 6.8. This protein was free of contaminants as judged by polyacrylamide electrophoresis, immunoelectrophoresis, and ultracentrifugation. However, the behavior of TBG in the ultracentrifuge proved to be concentration dependent. The authors suggest a reversible dissociation of the protein. According to this work it is possible to purify TBG 8000 times in comparison with TBG in normal human plasma.

2.3 Andreoli and Salvatore

By conventional chromatography on DEAE cellulose saturated with Cohn fraction IV-4 *Andreoli* et al. (1964) and *Salvatore* et al. (1966) obtained a globulin fraction (TBG) not contaminated by other T4-binding globulins. However, their protein fraction contained only 33 times more TBG than did normal serum.

2.4 Giorgio and Tabachnik

In 1968 *Giorgio* and *Tabachnik* isolated TBG by ammonium sulfate precipitation (30% to 50% saturation) of blood plasma to remove as much albumin as possible. The precipitated proteins were dialyzed against deionized water, thus precipitating the euglobulins. The solution containing TBG was concentrated by freeze drying, labeled with ^{131}I-T4, and subjected to preparative zone electrophoresis on cellulose column (Tris-maleate buffer, pH 8.8, 0.073 mA, 150–175 V, 5–6 days at 4°C). After electrophoresis, the separated fractions were eluted from the column with the same buffer, and the TBG-containing fraction was collected, freeze dried, and subjected to repeated electrophoresis under the above conditions.

The freeze-dried TBG-containing fraction from the second electrophoresis was then chromatographed on a DEAE-cellulose column (phosphate buffer 0.01 *M*, pH 7.8 at 4°C) and eluted with an NaCl gradient (up to 0.2 *M*). TBG was eluted at approximately 0.12 *M* NaCl. The fraction was further purified by gel chromatography on Sephadex G 150 or G 200 (eluting buffer 0.2 *M* NaCl plus 0.01 *M* phosphate, pH 7.8 at 4°C) and the TBG-containing fraction was finally purified by preparative electrophoresis on polyacrylamide gel (Tris-HCl buffer, 0.38 *M*, pH 8.9 in resolving gel, and Tris-HCl buffer, 0.052 *M*, pH 8.1 as elution buffer, at 0°C, 50 mA for 6 to 10 h, 300 to 800 V). The TBG was then 4300 times more concentrated than in plasma and seemed to be homogeneous according to analytic disc electrophoresis, starch gel electrophoresis, immunoelectrophoresis, and ultracentrifugal analysis.

2.5 Marshall and Pensky

Marshall and *Pensky* published two methods for the purification of TBG. The first (*Marshall* and *Pensky* 1969) precipitates human serum with ammonium sulfate at 40% saturation; the supernatant was then dialyzed against water and chromatographed on Dowex 2 x 10 anion exchange resin at pH 7.3. The main band of unabsorbed protein emerging at void volume and containing TBG was saturated with ammonium sulfate to 60% saturation. The mixture was filtered and the filtrate brought to 65% saturation by ammonium sulfate. The precipitate was dissolved in water, dialyzed, and chromatographed on DEAE-cellulose at pH 7.3 with a linear concentration gradient of sodium chloride (0–0.15 *M*). The main radioactive fraction of TBG labeled with ^{125}I-T4 was pooled, concentrated, and dialyzed against 0.06 *M* Tris chloride buffer, pH 8.6. The sample was finally chromatographed on DEAE-Sephadex A 50 in the above buffer and eluted with a linear concentration gradient of sodium chloride (limit:

0.2 *M*); then the samples from the ascending part of the radioactive peak were pooled and concentrated by pressure dialysis.

The authors claim that this preparation is free of all traces of contaminating albumin and is homogeneous as shown by disc electrophoresis and immunoelectrophoresis.

The second method (*Pensky* and *Marshall* 1969) utilizes bioselective adsorption (affinity) chromatography on agarose gel containing covalently bound *l*-thyroxine.

Pooled human serum was stirred with the suspension of T4, bound to agarose (Sepharose 4 B) overnight, and decanted, and the suspension was washed four times with 0.1 *M* $NaHCO_3$, pH 8.6. The washed suspension was then packed into a chromatographic column and washed with the above $NaHCO_3$ solution until no proteins were eluted. The proteins retained on the column were then eluted with 0.002 *M* KOH, pH 9.3. The elution pattern showed two distinct peaks, the first containing a single thyroxine-binding protein which had the same mobility as TBG in normal serum, or, the same as the highly purified TBG obtained by the first method of the same authors.

The same procedure repeated with agarose (to which no T4 was bound) yielded no stainable protein in the TBG region as established by disc electrophoresis.

In further purification, the thyroxine-binding protein, eluted from the T4-Sepharose column in the first peak, was concentrated by pressure dialysis, then dialyzed against 0.06 *M* Tris-chloride buffer, pH 8.6, and subjected to DEAE-Sephadex column chromatography as in the first method. The purified TBG produced a single band on analytic polyacrylamide gel electrophoresis and by electrophoresis on cellulose acetate. The calculated yield of TBG was 18%–37%.

2.6 Hamada and Sterling

In 1970 *Hamada* et al., followed by *Sterling* et al. (1971), published a method for preparation of TBG from Cohn fractions IV-4 and IV-5,6. These fractions, together with a tracer amount of ^{125}I-T4, were subjected to column chromatography on carboxymethyl cellulose (in 0.075 *M* acetate buffer, pH 5.0). The fractions containing the ^{125}I-T4 were concentrated by ultrafiltration and chromatographed further on Sephadex G 200 (in 0.05 *M* NaCl). The radioactive peak, again concentrated by ultrafiltration and dialyzed against a Tris HCl buffer (0.05 *M*, pH 8.0), was then chromatographed on DEAE-Sephadex A 50 in the above Tris buffer and eluted with a salt gradient (0.10–0.18 *M* NaCl). The peak containing TBG was then subjected to the "double gel" preparative polyacrylamide gel

electrophoresis with acid and alkaline pH gel layers devised for better separation of albumin from TBG. The pH of the upper gel was 4.0, that of the lower, 8.6 (concentration of the gel: 7.5%). During the first electrophoresis (24 mA for 30 min, then 60 mA for 30 min, and finally 800 V for 75 min) a pH gradient appeared in both the upper and lower gels. At pH 4.5 the negatively charged TBG enters the upper gel, while the other proteins are excluded into the upper electrode buffer. This buffer was then removed and replaced with Tris-barbiturate buffer (pH 7.4), and a second electrophoresis was carried out at 50 mA for 18–20 h. The TBG fraction eluted from the "double gel" electrophoresis column was finally purified by usual preparative polyacrylamide gel electrophoresis in Tris-glycine (0.052 *M*, pH 8.9) upper buffer and Tris-HCl (0.1 *M*, pH 8.1) lower buffer at 50 mA for 15–17 h. This step resulted in a homogeneous (by disc and paper electrophoresis, immunoelectrophoresis, gel chromatography on Sephadex G 200, and ultracentrifugation) protein, TBG.

2.7 Korcek and Tabachnik

The method of *Korcek* and *Tabachnik* (1974) uses the first two steps introduced by the second method of *Pensky* and *Marshall* (1969). In a comprehensive, three-step method of isolation, the first step involves bioselective adsorption (affinity) chromatography of whole human blood plasma on thyroxine-Sepharose 4 B, with results rather similar to those of *Pensky* and *Marshall* (1969). It should be noted that, according to *Korcek* and *Tabachnik* (1974), a large number of proteins other than TBG were found in the sample after the bioselective adsorption (affinity) chromatography step. The second step consists again, as in the previous method, of chromatography on DEAE-Sephadex A 50 from which the sample is eluted by a linear concentration gradient (0.06 *M* – 0.2 *M* NaCl). After this step the sample still contains proteins other than TBG, as is shown by analytic polyacrylamide gel electrophoresis.

In the third step the sample from the DEAE-Sephadex chromatography containing TBG is subjected to preparative polyacrylamide gel electrophoresis as described by *Giorgio* and *Tabachnik* (1968). This step resulted in a TBG preparation virtually free (by analytic polyacrylamide electrophoresis) of any contaminating proteins. The yields of TBG by this method ranged from 20% to 27%, assuming a starting concentration of TBG in plasma of 1.5 mg per 100 ml.

2.8 Nilsson and Peterson

In the method of *Nilsson* and *Peterson* (1975) the human plasma to which ^{125}I-T4 has been added was adjusted by ammonium sulfate up to 40% saturation. A majority of plasma proteins precipitated, but albumin, TBG, and TBPA remained in the solution. The supernatant was diluted with an equal volume of 0.02 *M* Tris-HCl buffer, pH 7.4, concentrated by ultrafiltration, and then dialyzed exhaustively against 0.02 *M* Tris-HCl (pH 7.4) containing 0.2 *M* NaCl. The sample was then subjected to ion exchange chromatography on DEAE-Sephadex A 50 in the same buffer. Elution was performed by an NaCl gradient (0.2–0.6 *M*) at pH 7.4. The TBG peak, indicated by the ^{125}I radioactivity, was eluted at a concentration of approximately 0.26 *M* NaCl. The fraction containing TBG was then dialyzed against 0.06 *M* Tris-HCl buffer (pH 8.6) containing 0.1 *M* NaCl, concentrated by ultrafiltration, and subjected to a second ion exchange chromatography on DEAE-Sephadex in the above buffer. The peak containing TBG appeared at the NaCl concentration of 0.32 *M* and was further purified by gel chromatography on Sephadex G 200 (in 0.02 *M* Tris-HCl, pH 7.4, containing 0.15 *M* NaCl). TBG appeared in the last, third peak by elution with the same buffer. This sample was concentrated by ultrafiltration and subjected to zone electrophoresis in barbital buffer at pH 8.6. The zone containing TBG was isolated and finally purified by preparative polyacrylamide gel electrophoresis in 0.1 *M* borate buffer, pH 8.9. The prepared TBG appeared to be homogeneous according to immunoelectrophoresis (against polyvalent antihuman serum protein serum).

The purified TBG was freed of endogenously and exogenously bound thyroid hormones by dialysis against a saturated solution of ANS (8-anilino-1-naphtalene sulphonic acid) in 0.05 *M* phosphate buffer (pH 7.5) containing 0.15 *M* NaCl. After the exhaustive dialysis the sample contained no radioactivity, indicating that all bound thyroid hormones were substituted by ANS. Subsequently, the ANS was removed by dialyzing the ANS-containing TBG against large volumes of phosphate buffer containing 2% serum albumin. At the end of dialysis no significant ANS fluorescence was detected in the TBG preparation.

2.9 Kagedal and Kallberg

Since human pregnancy serum reportedly contains large amounts of TBG, it is considered a suitable source for isolation of the protein. *Kagedal* and *Kallberg* (1977) described a method for obtaining pure TBG from pooled human sera collected during the third trimester of pregnancy. From serum diluted 2:1 with water the gross impurities were adsorbed batchwise onto

the same volume of gravity-settled hydroxylapatite. After centrifugation, the TBG contained in the supernatant was adsorbed batchwise on epoxy-activated Sepharose 6 B coupled with T4. From this material the TBG was desorbed by elution with 2 m*M* ANS. The obtained crude TBG (approximately 80% pure, 1200-fold purification, 23% yield) was concentrated by ultrafiltration. The resulting TBG was further adsorbed as a glycoprotein on Concanavaline-A(Con-A)-Sepharose. This step separated the nonglycoproteinic impurities. The purified TBG was desorbed from the Con-A-Sepharose by washing with alpha-methylmannoside. This preparation of TBG appeared to be pure by polyac 'lamide gel electrophoresis and immunologically. Its T4-binding ability was tested by equilibrium dialysis. This kind of TBG binds 1.1 mol T4 per mole protein, assuming its molecular weight to be 58 000 daltons, and its association constant is 2.0×10^9 liter per mole.

2.10 Present Attempts

The present methods of preparation of TBG are essentially combinations of the steps presented in the last three methods, i.e., affinity chromatography, gel filtration, and either ion exchange chromatography or electrophoresis (*Robbins* 1976; *Horn* and *Gärtner* 1979). Moreover, since the chemical entity of a highly purified TBG is at present already available, the research concerning its isolation has shifted already to the isolation of parts of the protein, e.g., its carbohydrate content (*Zinn* et al. 1978a, b).

Horn et al. (1979) presented a comprehensive and precise method for the isolation of human TBG. The first step consists of bioselective adsorption (affinity) chromatography on CH-Sepharose 4 B to which T3 was attached by means of a six-carbon-long spacer. This T3-Sepharose was mixed (60 min, 4°C) with 2 liters of human plasma, the solute filtered away, and the excess proteins removed (cold 0.05 *M* barbiturate buffer, pH 9.0). The TBG was then eluted with a solution of T3 at pH 9.1 at 30°C.

This TBG solution was then subjected to ion exchange chromatography on QAE-Sephadex A 50 (0.15 *M* Tris NaCl buffer, pH 8.6). The TBG-containing fraction was eluted by raising the concentration of salt (NaCl) to 0.225 *M*. The third step consisted of affinity chromatography on Con-A-Sepharose (0.05 *M* Tris, pH 7.4) for the separation of glycoprotein entities. TBG, as a glycoprotein, remained bound to the Con-A-Sepharose and was subsequently eluted with 0.02 *M* alpha-methyl-D-mannoside in Tris and NaCl solution (0.17 *M*, pH 8.6). In the next step the TBG-enriched fraction was subjected to another ion exchange chromatography on DEAE-Sephadex A 50 (linear salt gradient 0.15 to 0.20 *M* NaCl) in 0.05 *M* Tris, pH 8.6, and the TBG solution was concentrated by means of a subsequent

Con-A-Sepharose chromatography and QAE-Sephadex A 50 ion exchange chromatography as described above. This preparation of TBG was dialyzed against water and chromatographed again on hydroxylapatite (BioGel HTP, natriumphosphate gradient 0.03 to 0.08 *M*, pH 6.8); the TBG was then dialyzed again against water and stored frozen at –60°C.

Horn and *Gärtner* (1979) recently reported an improvement and simplification of their procedure for the isolation of TBG from human serum. TBG was adsorbed onto an epoxy-activated Sepharose 6 B with covalently bound T3. The protein was eluted by a solution containing 10 mg T4 and 100 ml of serum albumin. The albumin was then removed by Con-A-Sepharose affinity chromatography. The overall yield of TBG is about 40%.

From the point of view of relative simplicity and the relatively high yield of TBG as well as the combination of the three most efficient methods – i.e., bioselective adsorption (affinity) chromatography, ion exchange chromatography utilizing elution by salt concentration gradient, and the preparative polyacrylamide gel electrophoresis – the method of *Korcek* and *Tabachnik* (1974) seems at present to be the most suitable for the purification of TBG from human plasma. It should be noted, however, that a fourth step, namely the bioselective adsorption (affinity) chromatography on Con-A-Sepharose used by the last-mentioned authors, is also important in achieving a thoroughly purified TBG. This step uses the glycoprotein nature of TBG to separate it from other proteins of nonglycoproteinic entities.

Since thyroxine has a high tendency to become bound by various unspecific compounds, the bioselective adsorption (affinity) chromatography step on T4-bound Sepharose isolates from the blood plasma not only TBG but also TBPA, albumin, and probably also a number of other proteins, although the TBG is bound preferentially to the T4-containing chromatography column. Therefore, this first step in the above purification procedure alone is not sufficient to produce isolated TBG.

Since the published characteristics of different recent preprations of TBG obtained in various laboratories by the above three- or four-step methods agree within narrow limits with one another, the preparation of TBG could be considered now to be virtually free of contaminants, i.e., as a well-defined human plasma protein. Today, human TBG is even commercially available (UCB – CHRISTIAENS, Bioproducts, Peptide Department, Rue de Berkendael 68, B–1060 Brussels, Belgium) for laboratory purposes only, of course.

3 Properties

Many reports dealing with the properties of human TBG involve studies of this protein in its "natural environment," i.e., blood plasma. For example, many investigators studied the electrophoretic mobilities of blood plasma proteins labeled with radioactive T4 on a wide variety of supporting media under different conditions of pH, buffer composition, and concentration as well as different conditions of electric current, or they studied the competitive interaction of T4 and a number of other compounds with their binding proteins in blood. Since it is next to impossible to deal with the whole of this literature without selection, in the present review we will deal only with those properties of TBG which were derived from the study of isolated, purified preparations of human TBG.

All authors claim to have isolated an individual, pure protein, TBG. However, the physical and chemical properties of these protein fractions differ to a large extent. For comparison these properties are listed in Tables 1, 2, and 3.

The ultracentrifugal analysis of the TBG obtained by *Tata* (1961a, b) showed two major protein components, the sedimentation constants of which were about 3.5 and 7.2. The author suggests that TBG is present in the fraction with the sedimentation constant of 3.5. This preparation of TBG is essentially free of TBPA; it has a molecular weight of 40 000–50 000 daltons and is present in human serum in a concentration of approximately 1–2 mg TBG in 100 ml serum.

The mobilities of isolated TBG (*Tata* 1961b) studied by paper electrophoresis (pH 8.6 and 4.5, ionic strength 0.05–0.10) alone and in mixture with whole human serum are presented in Table 4. The author concluded that his preparation of TBG had a high carbohydrate content but a low concentration of lipids and tryptophan.

The molecular weight of the preparation isolated by *Korcek* and *Tabachnik* (1974) was estimated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, giving a value of approximately 65 000 daltons. The "slow TBG" (STBG) moved during electrophoresis behind the normal band of TBG (see also Sect. 4.2) and appeared after repeated use of the bioselective adsorption (affinity) chromatography column in a similar way as in the isolation of electrophoretically STBG according to the second method of *Pensky* and *Marshall* (1969); its molecular weight had a value of 57 000 ± 1000 daltons. On ultracentrifugal analysis of sedimentation equilibrium, a value of 60 700 ± 1800 daltons was calculated for the molecular weight of the normal TBG preparation isolated by *Korcek* and *Tabachnik* (1974).

Table 1. Physical constants of TBG. [a]

Parameter	1	2	3	4	5	6	7
Molecular weight (daltons)	59 000	58 000	54 000	63 000–65 000	36 500	65 000 ± 450	54 000–55 000
Sedimentation constant S_W^{20}	3.6	3.92	3.91	3.4	3.0	3.4	3.95
Extinction, $E^{1\%}_{1\ cm,\ 280\ nm}$	8.43	8.9		6.9	7.25		
Ex_{280}/Ex_{260}	1.64				1.35		
Diffusion constant D_W^{20}					8.05		6.2
K_{assoc}, $n = 1$ M^{-1}		4.8×10^8 – -2×10^9		[b] 2.3×10^{10} [b] 1.7×10^{10}	8.1×10^8 – -1.17×10^{10}		
Isoelectric point (pH)					3.8		
Stokes' radius (angström)					28.8		37
Frictional ratio					1.31		1.49
Partial specific volume (ml/g)							0.718
Molar extinction coefficient, 280 nm							47 500

[a] Parameters are listed according to authors: (1) *Seal* and *Doe* 1964; (2) *Giorgio* and *Tabachnik* 1968; (3) *Marshall* and *Pensky* 1969; (4) *Pensky* and *Marshall* 1969; (5) *Hamada* et al. 1970; (6) *Korcek* and *Tabachnik* 1974; and (7) *Nilsson* and *Peterson* 1975.

[b] At different temperatures.

Table 2. Amino acid composition of TBG (mole/mol TBG). [a]

Amino acid	1	2	3	4	5	6
Lysine	12	25	18	22.49	29	28
Histidine	11	10	7	13.94	12	11
Arginine	22	16	4	15.96	17	6
Aspartic acid	25	38	26	41.64	45	36
Threonine	18	25	20	25.69	28	25
Serine	19	34	24	31.64	49	29
Glutaminic acid	34	52	30	62.91	61	42
Proline	27	30	16	29.48	20	15
Glycine	25	25	23	32.37	29	19
Alanine	27	31	32	34.80	30	28
1/2 Cystine	5	8	3	10.63	8	5
Valine	12	23	21	26.75	25	27
Methionine	3	5	6	10.56	8	12
Isoleucine	8	9	12	18.54	17	18
Leucine	43	44	28	46.81	37	38
Tyrosine	7	10	4	11.62	15	9
Phenylalanine	14	18	12	22.49	17	22
Tryptophan	4	4	3	N[b]	4	4

[a] Given according to authors: (1) *Seal* and *Doe* 1964; (2) *Giorgio* and *Tabachnik* 1968; (3) *Sterling* et al. 1971; (4) *Korcek* and *Tabachnik* 1974; (5) *Nilsson* and *Peterson* 1975; and (6) *Gershengorn* et al. 1977.

[b] N, not determined.

Table 3. Carbohydrate composition of TBG (moles/mol TBG). [a]

Carbohydrate	1	2	3	4	5	6
Hexose	56					
Hexosamine	15					
Fucose	19		0	0	1	1
Sialic acid	9	5	4	6.23	0	10
Neutral hexose		24				
Glucosamine		12	11	12.37	7	22
Galactosamine		3	0	4.47	0	0
Xylose			1	0		
Mannose			5	12.17	7	6
Galactose			6	5.84	7	13
Glucose			2	2.2		6

[a] According to authors as in Table 2.

Table 4. Electrophoretic mobility of human TBG (according to *Tata* 1961b). [a]

TBG	pH	Mobility $\times 10^{-5}$ cm^2 V^{-1} s^{-1}
Alone	8.6	4.9
Mixed with whole serum	8.6	4.7
Alone	4.5	1.8
Mixed with whole serum	4.5	1.7

[a] TBG, thyroxine-binding α-globulin.

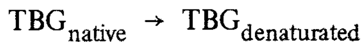
The yield of TBG isolated by the above method was about 27%. The same authors found measurable amounts of various fatty acids associated with their TBG preparation. It is interesting that in the isolation of TBG, *Korcek* and *Tabachnik* (1974) found no difference between the bioselective adsorption (affinity) chromatography columns, where the T4 was coupled directly to the Sepharose, and where, between the Sepharose and T4, different "arms" (ethylene diamine or 3,3′-diaminodipropylamine) were attached.

Fullerton (1974) achieved the crystallization of TBG prepared by *Sterling* et al. (1971). To 3 mg TBG 0.13 ml distilled water, 0.04 ml ethanol, and 0.01 ml 2 *M* NaOH were added. Crystals (approximately 20-μm long) appeared after 20 min at room temperature. After 3 weeks at 2°C the crycrystals reached a size of 60 μm and in a further 3 months reached maximal size (100 × 70 × 40 μm). The pH of the medium was 8.5.

TBG was also crystallized in the presence of T4. The solution of T4 consisted of 2.7 mg T4 dissolved in distilled water (0.6 ml), to which 0.3 ml 2 *M* NaOH was added. Three mg TBG and 0.04 ml of the above T4 solution were kept for 5 min at room temperature to achieve the binding of T4; then 0.08 ml distilled water and 0.04 ml ethanol were added. Crystals (approximately 50-μm long) grew overnight at room temperature. The crystals appeared to be morphologically identical with those of native TBG. After the crystals were placed into the cold (+2°C), they grew to a maximal size of approximately 75 μm. Crystals were not obtained in the absence of protein. The presence of bound iodine in the crystals of TBG crystallized in the presence of T4 may prove particularly useful as a heavy atom in X-ray crystallographic studies.

The kinetics of thermal denaturation of TBG were measured in the following experiment (*Takemura* et al. 1971): Whole human serum was heated for some time (5–60 min) at a certain temperature (52.5–60°C) and the T4-binding capacity of TBG was then measured by means of paper

electrophoresis with addition of T4 and ^{131}I-T4. The process of denaturation could be considered to be a simple monomolecular reaction



$$TBG_{native} \rightarrow TBG_{denaturated}$$

where the denaturated TBG does not bind T4. From the decrease of the binding capacity in heated samples of TBG in comparison with native TBG in serum, the rate constant of the reaction was calculated ($k_{52.5}$ = 0.00458; k_{60} = 0.10801). The changes of the rate constant with the temperature are related to the activation energy of denaturation of TBG, which, according to Arrhenius' equation, seems to be E = 89 867.2 ± 33.4 cal per mole of TBG.

For studies of the metabolism of TBG, radioiodinated preparations of this protein may prove particularly useful. *Refetoff* et al. (1975) used purified human TBG or desialylated STBG which were labeled by radioiodine; their properties were compared with those of the native TBG in whole human serum. The authors found that the liver rapidly and selectively clears the STBG injected into the bloodstream. The half life of STBG in rabbit is approximately 3 min. Radioactivity from labeled TBG and/or STBG was found in urine and bile. The T4-binding capacity of both TBG and STBG was close to 1 mol T4 per mole protein. The binding affinity of purified TBG was identical to that of the TBG in native serum; however, in the case of STBG is was lower. The polyacrylamide gel electrophoretic mobility of purified TBG was identical with that of native TBG, but under the same conditions STBG migrated in a broader band more cathodically placed than TBG. In general, radioiodinated TBG behaves in many respects in a manner similar to that of native TBG.

Inherited TBG abnormalities in man may be due to mutations at a single X-chromosome-linked locus controlling the synthesis of TBG. After injecting ^{131}I- or ^{125}I-labeled purified TBG into normal subjects, *Refetoff* et al. (1976) measured the half-life ($t_{1/2}$) of TBG, which is 5.3 ± 0.4 days; its distribution space is 7.2 ± 1.0 liters; total daily degradation is 0.211 ± 0.053 μmol per day. The total daily degradation of TBG was proportional to the serum concentration of this protein. They found different values for $t_{1/2}$ for TBG in patients with altered thyroid status, e.g., 3.6 days in patients with thyrotoxicosis. The authors studied and described pedigrees of families with altered TBG concentration in blood (absence, low, and high TBG). In subjects with changed concentration of TBG in serum due to X-chromosome-linked abnormalities, the TBG is identical to that in normal person electrophoretically, immunologically, and with respect to its affinity for T4 and its response to heat denaturation. The changes in TBG concentration did not seem to affect in a significant way the euthy-

roid status and the concentration of free T4 in blood, which were close to normal values.

Cavalieri et al. (1975) prepared TBG according to a slight modification of the method of *Pensky* and *Marshall* (1969), using T3-substituted Sepharose for affinity chromatography. The purified TBG was iodinated by ^{125}I, separated from free iodide by gel chromatography and Sephadex G 75, and finally purified by preparative polyacrylamide gel electrophoresis. After injecting the ^{125}I-TBG to normal and hypothyroid patients, the authors established the half-time of the disappearance from plasma as 5.0 ± 1.2 days in normals and 6.1 ± 3.2 days in hypothyroids. The TBG turnover rate was 17.8 ± 2.1 mg per day in healthy and 23.2 ± 8.5 mg per day in hypothyroid patients. The radioiodinated TBG retained the electrophoretic and immunologic characteristics of the unlabeled TBG save for a partial loss of T4-binding activity.

The preparation of TBG obtained by *Horn* et al. (1977), which represented a 20% yield of its content in the blood serum, had a molecular weight of approximately 57 000 daltons, but the molecular weight of its protein component alone was about 48 500 daltons. The amino acid composition of this preparation seems to be in fairly good agreement with the other preparations, i.e., those of *Korcek* and *Tabachnik* (1974) and *Gershengorn* et al. (1977b) (see Table 2). The preparation of TBG obtained by *Horn* et al. (1977) proved to be free of contaminants as judged by disc electrophoresis at polyacrylamide gel concentrations of 5%, 7.5%, and 10%, in glycine-acetate buffer (pH 8.9), and Tris-barbiturate buffer (pH 7.0).

In the reports of *Gershengorn* et al. (1977a, b) the authors used TBG, prepared according to the second method of *Pensky* and *Marshall* (1969), for characterization of its properties and subunit structure. This preparation of TBG showed one single protein band even in overloaded analytic polyacrylamide disc electrophoresis columns, as well as in electrophoresis in 0.1% SDS. Six preparations of TBG contained from 0.09–0.64 mol T4 per mole TBG; the TBG used for obtaining the present results contained 0.19 mol T4 per mole TBG and was able to bind an additional 0.85 mol T4. Its molecular weight (by equilibrium sedimentation) was 54 000 daltons in water (0.1 *M* KCl, 0.05 *M* phosphate, pH 7.5) and 52 000 daltons in 6 *M* guanidine (0.1 *M* KCl, 0.05 *M* phosphate, pH 6.5). Its extinction $E^{1\%}_{1\,cm}$ at 280 nm, corrected for the absorbancy of T4, was 6.17, and its partial specific volume was 0.724 ml per gram in water and 0.735 ml per gram in guanidine. The authors found 374 amino acid residues per mole TBG; carboxypeptidase A digestion suggested that the carboxy terminal amino acid of this TBG is leucine.

4 Molecule

4.1 Structure

The size and shape of the TBG molecule as well as its structure have been investigated by a number of authors. The preparation of TBG obtained by *Gershengorn* et al. (1977a, b) prepared according to the second method of *Pensky* and *Marshall* (1969) is stable in diluted alkali; however, it undergoes minor but irreversible structural alteration in very dilute acid, which greatly alters its ability to bind T4. The ultraviolet circular dichroism (CD) spectra at pH 8.3 showed that 24% of total peptide groups are ordered in alpha-helix structure and 27.7% in beta structure and that 48.3% should be considered unordered. The binding of T4 to the molecule caused certain changes in the CD spectrum in the near-ultraviolet region.

The relaxation time of TBG was measured by fluorescence polarization after covalent labeling of the protein with dansyl (5-dimethylaminonaphtalene-1-sulphonyl). Above 50°C the authors observed a time-dependent increase in polarization. They concluded that TBG is stable up to about 50°C but that above this temperature aggregation of the thermally denaturated TBG may occur. The measurement of the relaxation time suggests that TBG is a compact and symmetric molecule.

Gershengorn et al. (1977a) claim that the molecule of TBG consists of 374 amino acids. About one-half of the peptide groups are equally distributed in the alpha helical and beta structures. The carbohydrate content of this TBG is approximately 15% by weight. By means of isoelectric focusing four major bands of TBG protein were found; this microheterogeneity is attributable to different contents of N-acetylneuraminic acid in them (see Sect. 4.2). After desialylation with neuraminidase only one band of protein was found (*Horn* and *Gärtner* 1979).

The molecular transitions of human TBG in guanidinium chloride solutions were described in an excellent paper by *Johnson* et al. (1980). The TBG molecule, made up from a single polypeptide chain, has a compact, symmetric structure in neutral and alkaline solutions. Acidification below pH 5 results in irreversible loss of its hormone-binding ability and in minor structural changes. The molecule of TBG is easily denaturated by mechanical agitation, by even mild heating (*Takemura* et al. 1971), and during storage in isolated form.

Johnson et al. (1980) found that solutions of guanidinium chloride strongly enhance the denaturation process. The measurement of circular dichroism spectra of native TBG showed a relatively highly structured molecule (at pH 8.3 the percentages of structures were: alpha helix, 48%; beta

structure, 19%; and random coil, 33%) which is altered by acidification (at pH 3.4 the percentages of structures were: alpha helix, 35%; beta structure, 28%; and random coil, 36%). The addition of guanidinium chloride (2, 4, and 6 *M*) results in a corresponding decrease of alpha helix structure (28%, 14%, and 17%, respectively) as well as the beta structure (23%, 15%, and 16%, respecitively). The amount of unordered, random coil increases accordingly (49%, 71%, and 67%). It should be noted that the mean length of the alpha helical structure is rather short (4.5 residues per alpha helix segment) and is even more shortened by addition of guanidinium chloride (to 3.0 residues per alpha helix segment).

The fluorescence polarization of either native TBG or dansyl derivatives of TBG (DNS, 5-dimethylamino-1-naphtalenesulphonyl chloride) were measured either in the presence of guanidinium chloride or in its absence. The results of the changes of tryptophanyl fluorescence revealed a subsequent transition of the protein molecule to different conformational structures. The molecular transition of TBG in about 2 *M* guanidinium solutions results in irreversible loss of the binding site for T4. At neutral pH and above 2 *M* guanidinium concentration, further structural changes of the TBG molecule occurred, which led to a series of several new molecular species (*Johnson* et al. 1980). The increase in the polarization of the DNS-TBG complex could be considered as a sign of an association of the unfolded form of TBG. A conformational transition of the molecule precedes the self-association reaction.

These transition reactions, evoked by the presence of a denaturating agent, guanidinium chloride, are rather fast (the first-order rate constants being in the order of $5 \cdot 10^{-3}$ sec^{-1}). Upon removal of guanidinium chloride TBG does not refold to the original native structure. The authors, logically enough, conclude that the native form of TBG in serum does not represent its most stable form.

Zinn et al. (1978a) described the structure and composition of the carbohydrate part of a TBG preparation obtained by the affinity chromatography procedure. The carbohydrate composition of TBG (14.6% by weight) consisted of mannose, galactose, N-acetylglucosamine, and N-acetylneuramidinic acid in molar ratios 11:9:16:10 per mole glycoprotein. No fucose or N-acetylgalactosamine was found. The presence of glucose in various preparations of TBG is considered to represent a contamination (*Zinn* et al. 1978a). According to the authors, TBG contains four N-glycosidically linked oligosaccharide chains, and their probable structures are presented in an excellent second article (*Zinn* et al. 1978b). These four chains are of three different kinds of branched carbohydrate entities, probably to a great extent similar to one another in structure. The above three kinds of branched oligosaccharide chains are contained in the TBG molecule in the molar ratios of 1:2:1. The authors propose that the asialo-TBG

binds to the membranes of liver cells; however, native TBG did not show this kind of interaction.

4.2 Microheterogeneity

The TBG purified by the second method of *Pensky* and *Marshall* (1969) is homogeneous by conventional criteria but shows a marked microheterogeneity when subjected to isoelectric focusing (*Marshall* et al. 1973). In polyacrylamide gels in a pH gradient from pH 3 to 6, at least nine stainable protein bands were found. All these bands appeared to bind T4. Completely desialylated TBG showed approximately the same phenomenon. The authors demonstrated that all protein bands are immunologically identical.

In contrast to the findings of *Marshall* et al. (1973), *Korcek* and *Tabachnik* (1974) did not find any multiple bands of TBG after polyacrylamide gel electrophoresis of TBG first treated with 8 *M* urea.

The microheterogeneity of TBG has been associated with different metabolic states. *Henze* et al. (1979) studied desialylated TBG preparations. From a preparation of TBG, the N-acetylneuraminic acid was removed either to a certain degree or completely by means of treatment with neuraminidase from *Clostridium perfringens.* These desialylated preparations of TBG were analyzed by means of isoelectric focusing. The partially desialylated preparation of TBG revealed four protein bands (in the pH region of 4.2–4.6); the completely desialylated protein represented one band at pH 6.2. The association constants of all these preparations with T4 as well as their antibody binding were nearly identical. The authors conclude that the progressive desialylation in vitro causes an increasing microheterogeneity, with bands migrating to more basic regions with the decrease of N-acetylneuraminic acid content of the protein and finally resulting in a single band of completely desialylated TBG at pH 6.2. This microheterogeneity is obviously caused by different degrees of desialylation of TBG.

Since the microheterogeneity of TBG varies with different metabolic states, *Gärtner* et al. (1979) studied this phenomenon in relation to different nonthyroidal metabolic states. Examination of pooled normal human sera by means of isoelectric focusing and subsequent immunofixation of different TBG entities by monospecific TBG antiserum revealed three major (pI 4.55, 4.45, and 4.35) and one minor (pI 4.25) protein bands. This typical distribution pattern of microheterogeneous TBG was found in hypo- and hyperthyroidism as well as in genetic TBG deficiency.

However, in pregnancy (increased glycoprotein synthesis under the influence of estrogens) a further band of TBG (pI 4.15) appeared and the

"minor" band (at pI 4.25) became more intense. On the other hand, in liver disease characterized by diminished glycoprotein degradation, an increase in the pI 4.55 band and diminution of the pI 4.25 band was observed.

The healthy, normal newborns showed a pattern closely related to that of normal human adults; premature infants showed a pattern of four double bands which were normalized 6 months later. In genetic TBG deficiency the pattern of microheterogeneity was comparable to that of normals.

4.3 Subunits

By means of the method of *Nilsson* and *Peterson* (1975) a total of 110 amino acid residues per molecule of TBG were found, and consequently a molecular weight of 12 235 daltons was obtained for the protein. This TBG seems to contain 7.5% carbohydrate which yielded a molecular weight of 13 317 daltons. Since the molecular composition suggested a molecular weight of approximately 13 000 daltons and ultracentrifugation gave a value of approximately 54 000 daltons, the authors investigated the possible subunit structure of TBG. Electrophoresis in SDS indicated that subunits do not dissociate in SDS. In a further test TBG, fully reduced and alkylated, was subjected to gel chromatography in 6 *M* guanidine hydrochloride on Sepharose 6 B. Three peaks emerged suggesting substances with molecular weights of 52 000, 25 000, and 13 000 daltons, respectively. According to these authors, prolonged exposure of TBG to 6 *M* guanidine hydrochloride causes a partial dissociation of the molecule. The TBG seems to be composed of polypeptide chains of similar molecular weights which are held together by noncovalent interactions only (*Nilsson* and *Peterson* 1975). Electrophoresis on starch gel in 8 *M* urea at pH 2.7 suggested a stabilizing effect upon "monomeric" TBG and demonstrated that the two half cystines in the globulin subunit form an interchain bridge (disulfide bridge). Repeatedly frozen and thawed preparations of TBG subjected to gel chromatography on Sephadex G 200 at pH 8.0 yielded two peaks: the first consisted presumably of larger aggregates, the second was immunologically identical with TBG but had a molecular weight of 26 800 daltons. The gel chromatography of this material (reduced and alkylated TBG) on Sepharose 6 B in 6 *M* guanidine hydrochloride revealed two peaks with molecular weights of approximately 28 000 (60% of the material) and 14 000 (40%) daltons. From these data *Nilsson* and *Peterson* (1975) conclude that the isolated component represented half molecules of TBG composed of two subunits of identical size.

On the other hand, in the report of *Gershengorn* et al. (1977a, b) the authors used TBG prepared according to the second method of *Pensky* and *Marshall* (1969) for characterization of its properties and subunit structure. They concluded that TBG consists of a single polypeptide chain. Four lines of chemical and physical evidence were presented for this conclusion:

1. Quantitative –COOH terminal amino acid analysis by hydrolysis with carboxypeptidase A revealed 1 mol leucine liberated per mole TBG and lesser amounts of serine and alanine (0.89 Leu; 0.46 Ser; 0.59 Ala, after 120 min of hydrolysis; carboxypeptidase B failed to release any amino acids).
2. Peptide mapping revealed six different arginine-containing peptides and a total of 27–30 arginine- and lysine-containing peptides. Altogether 6 arginine- and 34 lysine- and arginine-containing peptides should be expected after tryptic digestion, if TBG were a single polypeptide chain, and only one-quarter of this number would be found if TBG were composed of four identical subunits.
3. After reduction, alkylation, and exposure to detergents no TBG subunits were observed by gel electrophoresis.
4. Exposure of native or reduced and alkylated TBG to 6 *M* guanidine hydrochloride for prolonged periods (7 days) and examination by gel filtration and equilibrium sedimentation failed to demonstrate any subunits.

These data suggest that TBG indeed consists of a single polypeptide chain. In a comprehensive article *Robbins* et al. (1978) discuss the structure of TBG and support the view that TBG is not a polymeric protein but consists of a single polypeptide chain. They found 1 mol N-terminal alanine per mole TBG and a unique sequence for the first 15 amino acids. Moreover, the relaxation time of the TBG molecule (49 ns) is almost the same as for a sphere of the same weight and partial specific volume. This means that TBG is very probably a compact molecule with no evidence for loosely attached subunits. The single polypeptide chain of TBG is easily denatured by acid, heat, or guanidine. At present, most authors agree that TBG consists of a single polypeptide chain which is not composed of subunits.

5 Interactions

5.1 Experimental Examinations

Tata (1963) was the first to suggest that the interaction of T4 with TBG is a phenomenon of reversible, simple binding, probably governed by electrostatic forces. TBG binds T4 with such a high affinity that only about

one-thousandth of the hormone is not protein bound. There is a high degree of specificity in the binding of thyroid hormones and their chemical derivatives by TBG.

Seal and *Doe* (1962a, b, 1964) established the glycoprotein nature of TBG and also found that the T4 binding is reversibly inhibited by calcium ions. The depressed T4 binding can be restored by treatment with 0.01 *M* ethylenediaminetetraacetic acid (EDTA) followed by gel filtration on Sephadex G 25. The protein appears to have a single sulfhydryl group, low sulphur and high proline content, and one binding site for thyroxine per molecule. The TBG contains approximately 32% carbohydrates and has a rather high fucose content.

Salvatore et al. (1966) suggested that T4 quenches the intrinsic ultraviolet fluorescence of its binding proteins. The measurement of fluorescence quenching showed that the process of binding of T4 to a protein is extremely rapid, e.g., the interaction of T4 with albumin is complete in 150 ms.

The final TBG product isolated by *Giorgio* and *Tabachnik* (1968) was found to bind a maximum of 0.25 ± 0.05 mol T4 per mole protein. If it is assumed that TBG binds 1 mol T4 per mole of protein, then the purified TBG exhibited only 25% of its theoretical binding capacity. In the presence of whole serum no significant increase in binding capacity was observed. However, the TBG showed a high affinity for T4, with a binding constant of the order of $10^9\ M^{-1}$. The authors claim that the reduced binding capacity may result from the removal during purification of a second component or cofactor which is required for the maintenance of full T4-binding capacity. Alternatively, a change in conformation of TBG as a consequence of the removal of a protective substance such as a protein or, perhaps, of T4 itself during the purification may result in the loss of binding capacity.

Competitive binding experiments with purified TBG (*Tabachnik* et al. 1971) in which ^{125}I-T4 bound to TBG was displaced by various (di- and tri-) peptides containing T4 showed that covalently bound T4 is capable of interacting with TBG as strongly as the free T4. A similar study using various analogues of T4 for displacement of the hormone from TBG (*Hao* and *Tabachnik* 1971) showed that (1) all four halogens, either iodides or bromides, are required for optimal binding, (2) a free phenolic group is also necessary; and (3) the alanine side chain of T4 is also intimately involved in the binding reaction. On the other hand, neither a free amino nor a carboxyl group appeared to be essential for the binding.

The preparation of TBG purified by *Hamada* et al. (1970) and *Sterling* et al. (1971) seemed to be better than the one isolated by *Giorgio* and *Tabachnik* (1968). The former preparation was also able to bind 0.7 mol T4 per mole TBG, which, assuming one single binding site for T4 on TBG,

represents 70% of the binding capacity of TBG. The binding capacity of purified TBG was 14 740 μg T4 p gram TBG; this represents a 5 000-fold purification in comparison with the proteins of whole human serum. By competitive binding studies of T4 employing TBG and TBPA, the association constant was estimated to be of the order of 10^{10} M^{-1}.

The solutions of TBG in water and/or buffer gradually lost their binding ability, probably because of denaturation. Also, the removal of T4 from the TBG preparation resulted in loss of the binding capacity. The properties of the TBG isolated by *Marshall* and *Pensky* according to their first method (1969) showed a close relationship with the TBG preparation obtained by *Giorgio* and *Tabachnik* (1968). The final yield of TBG by this method was approximately 10%–20%, assuming the concentration of TBG in serum to be 2 mg per 100 ml.

The properties of the preparation of TBG, isolated according to the second method of *Pensky* and *Marshall* (1969) as well as its interactions with thyroid hormones are described in a series of articles (*Marshall* and *Pensky* 1971; *Green* et al. 1972a–c; *Marshall* et al. 1972, 1973).

This preparation of TBG again bound T4 in a molar ratio of approximately 1:1. The binding of T4 to TBG is maximal in the range of pH between 6.4 and 10.4; below pH 6.4 the binding declines and has nearly disappeared at pH 4.2. After the treatment of TBG with 8 *M* urea the protein does not bind T4 and shows an electrophoretic mobility slower than that of native TBG. After the removal of urea by dialysis, five or six protein bands were detectable by polyacrylamide gel electrophoresis, but only one of them showed the same mobility and ability to bind T4 as the original TBG (*Marshall* and *Pensky;* 1971; *Marshall* et al. 1973).

The native, intrinsic fluorescence of TBG is quenched by binding of thyroxine (*Green* et al. 1972a). Since the fluorescence maximum of TBG is at approximately 340 nm and the absorption maximum of T4 is at about 320 nm, this fluorescent-molecule–quencher pair is especially suitable for the study of their interactions by means of fluorescence quenching. The authors established by this method that one mole TBG binds 0.85 ± 0.06 mol T4 or 0.91 ± 0.06 mol T3 (3,5,3′-triiodothyronine). The fluorescence data also suggested some thermodynamic parameters of the interaction of T4 and TBG. The binding reaction is accompanied by a small change of enthalpy (–0.210 kcal/mole) but a larger change of entropy (+ 46 cal/degree · mole). These data suggest a typical hydrophobic interaction between T4 and TBG. The association constant of this interaction is 2.3×10^{10} M^{-1} at 23°C, and 1.7×10^{10} M^{-1} at 37°C as measured by ultrafiltration.

The same authors (*Green* et al. 1972b, c) investigated the binding site of TBG by observing its fluorescence emission in the presence of a T4-binding competitor dye, 1,8-anilinonaphtalene sulphonic acid (ANS).

They (1) found a conformational change of the TBG molecule affecting the binding site below pH 6.4; (2) demonstrated a thermally dependent transition of TBG structure (decrease of the slope of the quenching curve above 40°C and the irreversibility of this curve when temperature is lowered from 65°C); and (3) proved that the ionic strength of the solvent above 0.15 influences neither the fluorescence of the bound ANS nor the binding of T4 to TBG.

Using the preparation of TBG obtained by the second method of *Pensky* and *Marshall* (1969), *Schussler* (1972) studied the conformational requirements for the binding of T3 to TBG. Due to the restricted rotation at the ether bond, there are two existing conformations for T3: one with the 3′ iodine distal to the alpha ring and the other with the 3′ iodine proximal to the alpha ring. The molecule of T3 with the distal orientation of the 3′ iodine in the T3 was found to be the more effective one in displacing T3 and T4 from TBG.

When the column for bioselective adsorption (affinity) chromatography was used several times, the preparation of TBG obtained from such a column moves more slowly during electrophoresis on polyacrylamide gel (*Marshall* et al. 1972). The authors suggest that this kind of STBG is a partially desialylated normal TBG. STBG binds T4 with a molar ratio of 1:1, but its affinity for T4 is approximately ten times less then that of the normal TBG.

The binding of T4 and T3 to TBG isolated by *Nilsson* and *Peterson* (1975), examined by means of equilibrium dialysis under physiologic conditions, gave for both iodothyronines the same number of binding sites per molecule (0.95), and the corresponding association constants were $6.3 \times 10^9 M^{-1}$ for T4 and $5.4 \times 10^8 M^{-1}$ for T3. At high pH values (approximately 10) TBG showed a high association constant, the maximal binding being at a pH of about 8. On lowering the pH, TBG progressively lost its affinity for the hormones. Below pH 3 no interaction was detected. The tryptophyl fluorescence of TBG was greater at lower pH values and was suddenly diminished at higher pH values. This suggest a conformational change of the TBG molecule at a pH of about 6. The binding of T4 to TBG affects the pK value of its phenolic group, too, by displacing its value by about 1 pH unit in comparison with the pK of free T4. Similar equilibrium dialysis experiments with half molecules of TBG suggested that half molecules as well as the native protein have a single thyroid hormone-binding site, with a association constant of approximately $1 \times 10^7 M^{-1}$.

Korcek and *Tabachnik* (1976) investigated the interaction of T3 and T4 with purified TBG by means of equilibrium dialysis at different temperatures and pH values. They confirmed that T4 is bound to TBG by one single binding site. The apparent association constant (K, $moles^{-1}$) calculated from Scatchard's plots at different temperatures are as follows (for

T4): 5°C, $K = 4.73 \times 10^{10}$; 25°C, $K = 1.55 \times 10^{10}$; and 37°C, $K = 9.08 \times 10^9$ (all values at pH 7.4,. The data for T3 seem to be more complex and could be calculated by assuming two different classes of binding sites ($n_{1,2}$ = numbers of binding sites obtained from Scatchard's plots). At pH 7.4 the values are:

1. at 5°C, $K_1 = 3.35 \times 10^9$, $n_1 = 1.04$, $K_2 = 0.69 \times 10^8$, and $n_2 = 1.40$;
2. at 25°C, $K_1 = 6.5 \times 10^8$, $n_1 = 1.04$, $K_2 = 0.43 \times 10^8$, and $n_2 = 0.77$;
3. at 37°C, $K_1 = 4.32 \times 10^8$, $n_1 = 1.02$, and $n_2K_2 = 0.056 \times 10^8\ mol^{-1}$.

The thermodynamic values of this interaction calculated from the above constants for T4 at 37°C and 5°C, (pH 7.4) are $\Delta G^\circ_{37} = -14.1$ kcal per mol; $\Delta H^\circ = -8.96$ kcal per mol; and $\Delta S^\circ = +16.7\ cal \cdot grad^{-1} \cdot mol^{-1}$. For T3 under the same conditions $\Delta G^\circ_{37} = -12.3\ kcal \cdot mol^{-1}$, $\Delta H^\circ = -11.9\ kcal \cdot mol^{-1}$, and $\Delta S^\circ = +1.4\ cal \cdot grad^{-1} \cdot mol^{-1}$.

The quenching of fluorescence of TBG by T4 indicated that 0.86 mol T4 is bound to 1 mol TBG. The dependence of the binding upon pH showed that for both T3 and T4 the maximal binding occurs in the physiologic range of pH 6.8 to 7.7.

Korcek and *Tabachnik* (1976) recommend the addition of ovalbumin to the sample of TBG during equilibrium dialysis. Ovalbumin, while interacting with thyroid hormones very weakly, protects the binding ability of TBG, which was found to decrease during the dialysis procedure. They also recommend the storage of the solution of TBG at + 4°C, possibly with the addition of 0.02% sodium azide. Under these conditions of storage no loss of T4-binding activity was observed for a period of about 8 weeks. The storage at – 20°C in a frozen state or one single freezing and thawing of the TBG solution resulted in losses of binding ability up to 20%. The authors also caution against vigorous stroking of TBG solution. At + 5°C shaking of a TBG solution (200 strokes per min) for 48 h resulted in almost complete loss of its T4-binding ability. Apparently, TBG is rather sensitive to surface denaturation.

The binding of T4 to TBG probably differs from the binding of T3 to the protein. Besides hydrophobic forces, steric and allosteric effects and different charges may contribute to these differences. The different ionization state of phenolic –OH group in T3 and T4 at physiologic pH may also be of importance. These differences are emphasized by different values of changes of entropy for the interaction of T3 and T4.

The binding of analogues of thyroid hormones to TBG was studied by *Snyder* et al. (1976). The authors used diluted (1:100) human serum or purified TBG (*Pensky* and *Marshall* 1969) and measured the binding of T4 and its analogues to T4-binding proteins by means of equilibrium dialysis (20 h at 37°C) in 0.035 *M* barbital buffer, pH 7.5. They found two classes of binding sites, the first (with an affinity constant of $2.5 \times 10^9\ M^{-1}$) belonging to TBG and a second one (affinity constant less than $10^6\ M^{-1}$)

belonging to albumin. The investigation of the ability of various analogues of T4 to become bound by TBG or to replace the already bound ^{125}I-T4 from its binding showed that the authors' results essentially confirm those of *Hao* and *Tabachnik* (1971).

Horn et al. (1977) examined the binding of T3 and T4 by their preparation of TBG and found that 1 μg TBG binds either 12 ng T4 or 10 ng T3 (binding capacity).

The preparation of TBG obtained by *Tabachnik* and *Korcek* (1978) has been used to measure the binding constants for its interaction with thyroxine analogues. By means of equilibrium dialysis at pH 7.4 and 37°C the displacement of ^{125}I-labeled T4 from purified TBG by various iodothyronine compounds was measured. The changes of standard free energy, ΔG°, of the TBG-iodothyronine interaction was also measured, and from its changes the structural requirements of this interaction were approximated (Table 5). From these results it follows that a change in the configuration

Table 5. The association constants (K, × 10^{-8} M^{-1}) and changes of standard free energy (−ΔG°, kcal per mole) of the interaction of TBG with various thyroxine analogues (according to *Tabachnik* and *Korcek* 1978).

Compound	K	−ΔG°
L-thyroxine (in absence of other compounds)	60.0	13.87
D-thyroxine	10.4 ± 2.8	12.79
3,5-Diiodo-3'-isopropyl-L-thyronine	4.9 ± 0.8	12.33
3,5,3'-Triiodo-L-thyronine	3.3 ± 0.8	12.08
3,3',5'-Triiodo-DL-thyronine (reverse T3)	3.1 ± 0.2	12.05
Tetraiodothyropropionic acid	2.7 ± 0.3	11.96
Tetraiodothyroacetic acid	2.6 ± 0.3	11.94
Tetrachloro-DL-thyronine	1.0 ±	11.35
3,5-Diiodo-3'5'-diisopropyl-L-thyronine	0.89 ± 0.2	11.28
3'5'-Diiodo-DL-thyronine	0.83 ± 0.2	11.23
3,5-Diiodo-DL-thyronine	0.71 ± 0.2	11.14
3,5-Diiodo-3'5'-dimethyl-L-thyronine	0.66	11.09
3,3'-Diiodo-L-thyronine	0.59	11.02

of the alpha-amino group from L to D form reduces the free energy favoring binding by about 1 kcal per mol (this means that D forms are less firmly bound than the corresponding L forms). Both the ionized and nonionized forms of the phenolic group are involved in the interaction with the protein. The presence of an alpha-amino group on the alanine side chain contributes about 2 kcal per mole to the free energy favoring binding, that is, the presence of this alpha-amino group means more firm binding. In all, the structural requirements for optimal binding of thyronines to TBG include an intact alanine side chain, four halogens, either iodines or bro-

mines, the phenolic –OH group, and a diphenyl ether structure. Practically all these structural features agree with those found earlier (*Tabachnik* et al. 1971).

If there is a modification of the protein structure of the TBG, it would affect – at least to a certain degree – the binding of T4 to this protein. From this point of view *Siegel* et al. (1979) studied the reaction of p-iodophenylsulphonyl (pipsyl) chloride, which reacts with some amino acids bound in the molecule of a protein, e.g., TBG.

The molecule of TBG was pipsylilated to a certain degree (aproximately 40 n*M* pipsyl chloride in acetone, 8 nmol TBG, borate buffer, pH 9.0, 0°C, 2 h) and the derivatized protein was separated from the residual free pipsyl chloride by means of Sephadex G 25 gel filtration. The the T4-binding ability of such altered TBG was compared with that of native TBG, i.e., subjected to the above procedure without pipsyl chloride. The authors found that the decrease in percent binding activity for T4 is directly related to the degree of pipsylilation (the more pipsylilated TBG is, the less it binds T4).

The hydrolysis of pipsylilated TBG revealed that about 70% of pipsyl was bound to the epsilon-amino group of lysine in TBG. When the pipsylilation was carried out with TBG where its binding site was blocked with either T4 or tetraiodothyroacetic acid, a decrease in the degree of pipsylilation was observed (33% vs 29%, respectively).

The authors conclude that the reaction of epsilon-amino group of lysine in TBG with pipsyl diminished the binding of T4, because it modified the immediate environment of the T4-binding active site on the TBG molecule.

5.2 Theoretical Analyses

In two theoretical articles *Wosilait* and *Nagy* (1976) and *Wosilait* (1977) consider the interplay between T4 and three different T4-binding proteins. *Wosilait* and *Nagy* (1976) described a computer program for the estimation of free T4 in plasma and its distribution among different sets of binding sites on different proteins. As a basis for these calculations the Scatchard model for binding of a ligand to a protein carrier is employed. The input data consist of the number and concentration of binding proteins (TBG, TBPA, albumin), the number of binding sites on each protein, the association constant and binding capacity of each set of binding sites for T4, and the total concentration of T4. However, it should be noted that the Scatchard equation was devised strictly for the interaction of one protein containing one single set of binding sites for one ligand, which is hardly the case in complex natural systems. So every calculation applying

Scatchard's equation to multiple binding sites on one protein or to such complex systems as blood plasma is necessarily only approximate. Moreover, a possible mutual interdependence of binding sites should be taken into consideration.

Wosilait (1977) considers one single binding site of T4 on TBG, two sets of sites of TBPA, and two sets of sites on serum albumin. The author's analytic method makes possible the calculation of free T4 in blood under the conditions of various concentrations of its binding proteins.

6 Metabolism

6.1 Biosynthesis

Glinoer et al. (1976) gave experimental evidence that TBG is synthesized in hepatocytes. Isolated hepatocytes of normal adult rhesus monkeys were incubated with radioactive ^{14}C-leucine for 4–6 h. The incubated hepatocytes were then subjected to the following procedures to yield three fractions: the incubated cells were washed three times and then centrifuged (100 x *g*, 3 min), and the medium was recentrifuged again (105 000 x *g*, 90 min) to separate the cell debris. This solution was called "medium." The cellular pellet which remained after the first centrifugation was washed, homogenized, and subjected to centrifugation (105 000 x *g*, 90 min) which yielded the second fraction, "cytosol." The remaining particulate fraction was then extracted with 0.4% digitonin solution for 1 h and recentrifuged again (105 000 x *g*, 90 min), yielding the third solute fraction, called "particulate." TBG was isolated from all three fractions (medium, cytosol, particulate) by dialyzing the samples extensively against 0.1 *M* bicarbonate and by subsequent affinity (bioselective adsorption) chromatography on T4-Sepharose. The adsorbed TBG was desorbed from the column by 0.002 *M* KOH.

Thyroxine-binding α-globulin used for immunization was purified from monkey serum according to the methods of *Marshall* et al. (1973) employing bioselective adsorption (affinity) chromatography and anion exchange and gel chromatography. Antiserum against TBG was obtained by immunization of rabbits with TBG obtained as described above. The purified anti-TBG serum was then allowed to react (immunoprecipitation, double diffusion, and electrophoresis) with ^{14}C-labeled, newly synthesized TBG from the hepatocytes. ^{14}C-TBG was found in all three fractions. After 6 h of incubation, 59% of the TBG was found in the particulate fraction, 20% in the cytosol, and 21% in the medium. Newly synthesized TBG was present after 4 h incubation. After 6 h, the total synthesized TBG had increased to 150% of the 4-h value, while the amount present in the medium

had increased to 300%, indicating probable TBG secretion into the medium.

An implantation of beta-estradiol-containing capsules in female rhesus monkeys (*Glinoer* et al. 1977a) resulted in an increase of the TBG concentration (initially 20.6 ± 6 μg per ml serum) 24 h after the implantation; this reached a steady level (48.6 ± 5.0 μg per ml serum) in 7–10 days. The decay rate of TBG was slightly lower after the estradiol treatment. The major effect of beta-estradiol was the stimulation of TBG production rate (2.9-fold, from 1.83 ± 0.34 mg per day to 5.31 ± 0.82 mg per day after 3–4 weeks). The concentration of beta-estradiol in serum increased tenfold (from 20 ± 7 pg per ml to 212 ± 41 pg per ml) 3–4 weeks after the implantation of capsules.

The total distribution or serum equivalent volume of TBG after 3–4 weeks of implanted beta-estradiol increased 1.4-fold, from 338 ± 37 ml to 458 ± 22 ml, and the metabolic clearance rate increased 1.3-fold, from 90 ± 10 ml per day to 113 ± 12 ml per day. The preparation of TBG from rhesus monkey blood, according to *Marshall* et al. (1973), was radioiodinated and used for quantitation of TBG by means of radioimmunoassay. The kinetics of its disapperance from blood was analyzed with a five-compartment model.

Isolated liver cells of rhesus monkeys were incubated for up to 9 h with ^{3}H-leucine (*Glinoer* et al. 1977b). The washed cells were homogenized and centrifuged (105 000 × *g*), and the newly synthesized ^{3}H-TBG was determined in the cytosol. This newly synthesized TBG was first separated from the other proteins by means of affinity chromatography on agarose columns with attached T4. The determination of TBG was carried out by means of immunoprecipitation and measurement of radioactivity.

The production of nonradioactive TBG released into the medium by hepatocytes (without ^{3}H-leucine) was measured after 24 h of incubation. The cells were separated from the medium by centrifugation (100 × *g* for 3 min and 105 000 × *g* for 90 min) and dialysis against barbital buffer, pH 8.6. The TBG in the sample was detected by radioimmunoassay.

The hepatocytes of monkeys receiving beta-estradiol in a capsule (subcutaneously for 4–5 weeks) contained approximately three times as much ^{3}H-TBG in the liver cells and produced about 2.4 times as much TBG released into the medium as controls not receiving beta-estradiol (3.48 ng TBG per hour per 10^7 cells in monkeys receiving estradiol as compared with 1.46 ng TBG per hour per 10^7 cells in controls). Assuming 10.2 × 10^9 cells in the liver, the authors (*Glinoer* et al. 1977b) estimated the production rate of TBG as being about 250 μg per liver per day in monkeys with beta-estradiol and 104 μg TBG per liver per day in control monkeys.

Gershengorn et al. (1976a) investigated the biosynthesis of TBG in the human liver. TBG, purified by affinity, anion exchange, and gel chromato-

graphies, was used to immunize rabbits. The same preparation of TBG was labeled with ^{125}I and purified further on Sephadex G 25 and by affinity chromatography on T4-Sepharose. The tested plasma was diluted (1:100 or 1:200); 100 μl of this diluted sample, labeled with ^{125}I-TBG and anti-TBG serum (final dilution 1:25 000), were incubated at first at 4°C for 48 h, then with anti-rabbit IgG for 20 h at 4°C. After washing and centrifugation, the radioactivity of the precipitate was measured and the TBG content of serum was determined by means of a calibration curve representing percentage of radioactivity bound versus the amount of nonradioactive TBG added to the sample.

The authors found 1.48 ± 0.46 mg TBG in 100 ml serum of euthyroid normals. The level for females, 1.66 ± 0.56, was significantly higher than that for males, 1.37 ± 0.37. Comparison of the concentration of TBG in serum with the binding capacity of serum for T4 yielded a molar ratio of 1:1 for T4 and TBG.

Gershengorn et al. (1976b) demonstrated the synthesis and secretion of TBG by normal hepatocytes isolated from rhesus monkeys. Because of the brief survival of these hepatocytes (less than 24 h) the results were more pronounced when they used a continuous culture line of hepatocarcinoma cells of rhesus monkeys. TBG was identified by immunochemical and T4-binding techniques. De novo synthesis of TBG was shown by incorporation of ^{14}C-leucine and autoradiography. In serum-free media the cells survived for 5 days and produced TBG. The quantitation of TBG was achieved by radioimmunoassay. TBG accumulation in the medium rose linearly for 48 h. Also, albumin and other T4-binding proteins were secreted into the medium. At the end of the 48 h the authors found in the medium 4.9 ± 0.2 ng TBG per mg cell protein. At the beginning of the incubation there was no measurable TBG in the medium.

Addition of T4 to the medium affected the synthesis of TBG in a biphasic way. There was a significant progressive increase of the TBG accumulation in the range of concentrations of T4 10^{-14} M to 10^{-11} M. For comparison, the concentration of free T4 in rhesus monkeys is 6.10^{-12} M. At concentrations of T4 ranging from 10^{-11} to 10^{-10} M, the synthesis of TBG was maximal (about 6.5–7.0 ng of TBG per mg cell protein during 48 h). Any further increase of the concentration of T4 in the medium caused a progressive decrease of the amount of synthesized TBG; when the concentration of T4 was 10^{-7} M, the amount of TBG synthesized fell even below the control level to 4.0 ng TBG per mg of cell protein for 48h.

Therefore, the authors (*Gershengorn* et al. 1976b) concluded that T4 regulated the synthesis and secretion of TBG by hepatocarcinoma cells. At lower concentrations, T4 stimulates the accumulation of TBG, while at concentrations of T4 above 10^{-10} M there is an inhibition of the accumula-

tion of TBG. However, the effect of T4 upon the hepatic protein synthesis may be a general one.

Marshall et al. (1974) showed that desialylated TBG becomes bound to the hepatic cell membranes. Since there were alterations observed in glycoproteins of sera from cirrhotic patients (e.g., they contain more partially desialylated TBG than normals), this finding may have its significance in the overall metabolism of TBG.

It should be noted that *Cavalieri* et al. (1975) found the distribution space of their preparation of TBG to be about 7 liters, which corresponds to that of albumin; the half life of TBG in plasma was found to be approximately 5 days.

Glinoer et al. (1979) carried out a comprehensive study of the metabolism of TBG in rhesus monkeys. The monkey TBG was purified as described earlier (*Glinoer* et al. 1976, 1977a) and radioiodinated by means of ^{125}I. This ^{125}I-TBG has been injected into experimental animals (2-year-old *Macaca mulatta*) and studied for 9 days in order to obtain normal values. Then hyperthyroidism was induced by means of injecting the monkeys with T3 (twice daily, 10 μg T3 for 45 days) and the kinetics of ^{125}I-TBG were determined again. At the end of this period, the treatment of the animals was stopped for 4 weeks and they were subjected to total thyroidectomy. Over a period of 6 weeks the monkeys became hypothyroid and the third part of the kinetic study was performed after injecting the animals with ^{125}I-TBG for a further 8–10 days. For each study (basal, hyper-, and hypothyroid) the animals were injected intravenously with 10–20 μCi ^{125}I-TBG.

The initial concentration of TBG was 24.0 ± 1.1 μg per ml serum in normal animals. During the first 2 weeks of T3 administration this concentration dropped by about 33%, then rose again and stayd close to normal (23.8 ± 1.3). After thyroidectomy it rose and stayed high (28.8 ± 0.6). The authors measured nearly all the parameters of thyroid function (T4, T3, TSH, etc.) as well as the kinetic parameters of TBG. The total distribution volume of TBG (initial, 323 ± 23 ml) was reduced by about 33% in hyperthyroidism (217 ± 21 ml), but increased again to 17% above normal in hypothyroidism (379 ± 11 ml).

The decay rate (k) of TBG was 0.28 ± 0.01 day^{-1} (basic value); it rose in hyperthyroidism (0.36 ± 0.01) and decreased below normal in hypothyroidism (0.14 ± 0.01).

The metabolic clearance rate (MCR) was 92 ± 10 ml per day; it decreased in hyperthyroidism (81 ± 9 ml) but decreased even more in hypothyroidism (51 ± 4 ml). The production rate of TBG was 2.23 ± 0.14 mg per day in normals; it decreased to 1.93 ± 0.11 in hyperthyroid and decreased further to 1.62 ± 0.09 in hypothyroid animals.

The values presented here are similar to those observed for TBG in four female rhesus monkeys. The corresponding values for males and females, respectively, are: TBG half-lives: 2.5 ± 0.1 vs 2.7 ± 0.1 days; MCRs: 92 ± 10 vs 77 ± 5 days; production rate: 2.23 ± 0.14 vs 1.57 ± 0.17 mg per day; serum TBG levels: 24.0 ± 1.1 vs 20.2 ± 0.8 μg per ml; and total distribution volume: 323 ± 23 vs 290 ± 18 ml. All values presented here were obtained in animals with normal thyroid status.

All values presented by *Glinoer* et al. (1979) are means ± standard errors (SEM) and are expressed per 3 kg body wt. of the monkeys. Six monkeys were studied. The results obtained during hyper- and hypothyroidism were adjusted for changes in body weight for better comparison of the data. Results obtained without the adjustment of body weight were quite similar (*Glinoer* et al. 1979).

6.2 Degradation

The above papers presented conclusive evidence for the synthesis of TBG in human and monkey hepatic cells, either normal or carcinomatous. On the other hand, biodegradation of TBG as well as that of other proteins of human body is supposed to follow mainly the normal pathways through proteases. However, at least a certain amount of TBG is excreted via the kidneys into the urine. This way of biodegradation of TBG is possible because of its low molecular weight, as shown by *Hocman* et al. (1976). These authors found a protein fraction in human urine, the T4-binding ability of which was ascertained by equilibrium dialysis. Although it was not proven that this protein moiety is TBG, it is highly probable that it represents either native or modified (denaturated) human TBG excreted from the blood stream via the kidneys.

Burke and *Shakespear* (1976) measured the clearance of T3 and T4 by urine. In euthyroid persons with proteinuria the authors found higher amounts of excreted T4 than in persons with normal, low excretion of proteins. They consider this a consequence of the appearance of protein-bound T4 in urine.

Urinary protein prepared from the urine of four normal subjects by ammonium sulfate precipitation and exhaustive dialysis contained significant amounts of T3 and T4, as established by radioimmunoassay. About 0.5 μg T3 was found in 1 g urinary protein and about 0.7 μg T4 in 1 g protein. It is interesting that these authors found a "low molecular weight" binder (LMW binder) for T3 and T4 in urine. In urine, they found free T3, protein-bound T3, and LMW-bound T3 (39,4%, 8.9%, and 51.7%, respectively). For T4, the corresponding values are free T4, 20.1%, protein-bound T4, 11.9%; and LMW-binder-associated T4, 68.0%. This low-molec-

ular weight binder represents a substance which binds T3 and T4 and mirates a little beyond the salt peak on Sephadex G 50, but is able to pass a Visking membrane during dialysis. The authors suggest that the approximate molecular weight of this substance is around 500 to 2000 daltons.

Gavin et al. (1979) studied the urine of ten healthy, euthyroid males. The samples were concentrated by ultrafiltration (at least 100 times on a molecular filter with a cutoff at molecular weight of about 25 000 daltons) and dialyzed against 40 m*M* sodium barbital-HCl buffer containing EDTA, pH 7.4. This pooled concentrate from the urine contained a protein entity reacting with rabbit antiserum to human TBG which is called "urinary TBG" (TBG_u) by these authors (*Gavin* et al. 1979). The quantitation of this entity was carried out by means of radioimmunoassay and its concentration was found to be 1.74 ± 0.87 μg TBG_u per 100 ml urine. The concentration of TBG in sera of the same individuals exhibited a concentration of 1.8 ± 0.2 mg TBG per 100 ml serum. This means that the actual concentration of TBG_u in unconcentrated urine is approximately 1/1000 of that of serum TBG. The mean daily excretion of TBG was 13.2 ± 6.5 μg per gram creatinine per day. If the absolute turnover rate of TBG is 26.2 ± 10.9 μg per day, the renal excretion accounts for only about 0.15% of the total daily disposal of TBG.

During polyacrylamide gel filtration and electrophoresis, and during reverse-flow electrophoresis TBG_u showed patterns similar to those obtained with serum TBG; both showed close immunologic similarity. The association constant of TBG_u, determined by equilibrium dialysis and analyzed by Scatchard's plot, showed two kinds of binding centers: one, showing a low capacity–high affinity site ($K_a = 0.46 \pm 0.20 \cdot 10^{10}\ M^{-1}$, $n = 1$), and another, a set of high capacity–low-affinity sites (K_a less than $10^7\ M^{-1}$). Under identical conditions the K_a of serum TBG was slightly higher ($K_a = 1.43 \pm 0.23 \cdot 10^{10}\ M^{-1}$). The binding capacity of TBG_u was 1.12 ± 0.15 mol T4 per mole TBG_u (for serum TBG, the corresponding value is 1.15 ± 0.23). TBG_u bound 0.76 mol T4 per mole TBG_u, whereas serum TBG bound 0.82 mol T4.

Gavin et al. (1979) state that even if the data for both TBGs are similar, these proteins may not be identical. The electrophoretic mobility indicates that TBG_u may not be desialylated, but probably underwent conformational and/or charge alterations during renal excretion. The lower value of association constant for TBG_u could be a consequence of binding of inhibitors present in the concentrated urine. The leakage of macromolecules through normal glomerular membranes may be related to charge as well as to size and shape of the protein molecule.

Since the extent of excretion of TBG through the kidneys into the urine is rather insignificant, other means of degradation of TBG – probably by proteases – and the degradation patterns obvious for other body proteins play a decisive role in the elimination of TBG from the organism.

7 Determination

The methods for determination of TBG can be roughly divided into two groups: the methods utilizing some kind of immunoassay and methods employing the measurement of T4-binding capacity of TBG.

7.1 Immunoassay

Bradwell et al. (1976) described a method for isolation of TBG which is subsequently used as an antigen for immunologic determination of TBG. Human serum labeled with ^{125}I-T4 was chromatographed on DEAE cellulose. The adsorbed TBG was eluted by means of a Tris-HCl gradient (0.05 *M* to 0.5 *M*). The radioactive peak, concentrated by ultrafiltration, was subjected to a second ion exchange chromatography under essentially the same conditions as above. The fraction containing TBG was separated by gel chromatography on Sephadex G 150 and finally purified by polyacrylamide gel electrophoresis. The main radioactive peak was retained in both last steps. The partially purified TBG, labeled with ^{125}I-T4, was subjected to two-dimensional immunoelectrophoresis against antiserum prepared in sheep against the alpha-1 electrophoretic fraction of serum. In the agarose gel the TBG-antibody complex was identified by autoradiography, separated, and injected repeatedly into sheep. After 20 days, two circulating antibodies were identified by two-dimensional immunoelectrophoresis, the antibody against TBG was identified and isolated as above. The antibody against TBG was utilized by means of Laurell's rocket immunoelectrophoresis technique for determination of TBG in serum samples. The values of TBG in sera obtained with this technique were (in mg per liter) normal males, 11.0 ± 2.8; normal females, 12.1 ± 2.3; females on estrogen contraceptives, 16.1 ± 2.2; pregnant females, 25.0 ± 3.4.

Pure TBG makes possible the preparation of monovalent antisera and thus a simple and precise direct determination of the amount of TBG in blood plasma by means of a radioimmunoassay (*Hesch* et al. 1976a). TBG was iodinated by the chloramine T method with ^{125}I, purified on Sephadex G 25, and stored at –20°C. Before the assay it was further purified by affinity chromatography on agarose-bound T4. The authors claim that the purification of ^{125}I-labeled TBG is critical for the assay. As a standard, TBG-enriched plasma (5.1 mg per 100 ml) was used. In the assay, 0.1 ml standard (or unknown) plasma (diluted 1:300), 0.05 ml ^{125}I-labeled TBG, 0.05 ml antiserum to TBG (rabbit, diluted to 1:20 000), 0.3 ml barbital buffer (0.07 *M*, pH 8.6, contains 0.25% of bovine serum albumin) were mixed and incubated for 2 days at 4°C. The 0.5 ml DASP (Organon, Munich, Federal Republic of Germany) was added to each

sample for the separation of free and bound TBG, and the tubes were rotated for 5 h at room temperature. The samples were centrifuged (2000 rpm, 3 min), and the precipitate was washed with 0.5 ml of the buffer and recentrifuged. Bound ^{125}I-TBG in the precipitate was measured in a gamma scintillation counter.

Immunologic cross reactivity with albumin, prealbumin, and a number of other proteins was excluded or minimized. The concentration of TBG was determined by means of a standard curve (1.5–2.5 ng TBG per 100 ml).

The normal values of TBG in young adults were 0.97 mg per 100 ml of plasma. In childhood the concentration of TBG was elevated (1.34 mg per 100 ml) as well as in old age (1.28 mg per 100 ml). The authors conclude that there is no correlation between the concentration of TBG in blood and age. In mild thyrotoxicosis the concentration of TBG was slightly increased (1.20 mg per 100 ml), while in more severe hyperthyroidism its concentration did not differ markedly from normal values. In hypothyroidism the concentration of TBG was elevated (1.26 mg per 100 ml).

The same authors used the determination of TBG by means of radioimmunoassay in the course of human development (*Hesch* et al. 1976b). Purified TBG labeled with ^{125}I by the chloramine T method is first purified on a Sephadex G 25 column (0.1 *M* bicarbonate buffer, pH 7.6) and the protein is then further purified by affinity (bioselective adsorption) chromatography on T4-agarose (the ^{125}I-labeled protein is pipetted onto the column, incubated for 30 min in 0.1 *M* bicarbonate buffer, and then ^{125}I-TBG is eluted with 0.002 *M* KOH solution, pH 11.5). One hundred μg of the purified ^{125}I-TBG (5000 to 10 000 cpm) is incubated with 50 μl TBG antibody (final dilution 1:5000). One hundred μl standard TBG (ranging from 1.56–25 ng per sample, dissolved in 0.07 *M* barbital buffer, pH 8.6, containing 25% bovine serum albumin) or 100 μl of unknown plasma (diluted 1:300) is added. The volume is adjusted with buffer to 500 μl. After the reaction, the separation is performed with 500 μl DASP diluted with the same buffer to 30 ml and rotated for 5 h. The TBG content is determined by measurement of the radioactivity of the precipitate from a calibration curve of standards of known TBG concentrations.

The authors suggest that low concentrations of thyroid hormones in the aged are not due to a decreased TBG concentration, because the actual concentration of TBG is increased. The concentration of TBG in the elderly was increased significantly (to 13.0 ± 1.8 mg per liter; $n = 22$) as compared to the middle-aged group.

The concentration of T4, T3, and TBG (measured with the above method) was determined in blood plasma of healthy individuals ranging in age from newborns to 95 years (*Hesch* et al. 1977). The results are summarized in Table 6. The authors introduced the T4/TBG and T3/TBG concentra-

tion ratios, which could explain some of the changes in the metabolism of thyroid hormones during aging.

Table 6. Changes of the concentration of TBG with age (mean ± S.D.) in the blood plasma (according to *Hesch* et al. 1977). [a]

Age groups (years)	Cord blood	1–6 months	7 months – 2 years	5–12 years	19–29 years	30–45 years	60–95 years
Number of subjects	5	13	13	15	28	6	21
TBG mg/100 ml	1.14 ± 0.18	1.30 ± 0.14	1.44 ± 0.14	1.30 ± 0.16	0.95 ± 0.14	1.08 ± 0.10	1.28 ± 0.15

[a] TBG, thyroxine binding α-globulin

The preparation of TBG isolated by *Horn* et al. (1979) was used to obtain antiserum against TBG. Two rabbits were immunized (three times in 3 weeks) with 75 μg TBG each in Freund's adjuvant, and 10 days after the last injection, antiserum against human TBG was obtained. This served for immunologic assay of TBG. The isolated protein, marked with ^{125}I by the chloramine T method, was used as a standard. One hundred μl diluted serum or standard ^{125}I-TBG was incubated overnight with 100 μl antiserum. After addition of 100 μl of a 10-g-per-liter bovine gamma globulin solution and 500 μl of a 250-g-per-liter polyethylene glycol in 0.05 *M* Tris (pH 7.4), the TBG-antibody complex precipitated and its TBG content was determined according to a calibration curve.

7.2 Other Methods

Bastomsky et al. (1977) used the measurement of T4-binding ability of TBG for its determination. From a sample of human serum endogenous thyroidal hormones are extracted by means of REXYN 202 ion exchange resin, which binds them quantitatively. Thus, the serum contains the binding proteins only and is devoid of T4 or T3. Such serum is chromatographed on a Sephadex G 25 column to which 1.3 to 2.0 pmol ^{125}I-T4 was added (representing 0.01 μCi). Its concentration should be high enough to saturate the TBG present in the serum as much as possible, but low enough to avoid any significant interference by binding to TBPA or albumin. This labeled T4 is bound to Sephadex owing to the high affinity of iodothyronine to polydextrane gels. When the sample serum flows through the Sephadex column, TBG absorbs the labeled T4 from the gel. The radioactivity of the effluent is then determined, where activity is in direct relation to the amount of T4 adsorbed onto the TBG.

The concentration of TBG in unknown serum is determined by means of a calibration curve made from serial dilutions of high-TBG serum (in the range of 12 to 60 mg TBG per liter). The concentrations of TBG obtained by the above method are (in mg per liter): normals, 31.6 ± 5.4; hypothyroid, 40.6 ± 7.5; pregnant females, 50.3 ± 5.4; hyperthyroids, 28.3 ± 4.8; women using oral contraceptives, 40.1 ± 6.8; and cirrhotics, 20.7 ± 4.3.

8 Physiologic Role

Since people with raised or diminished concentration of TBG in the blood and even people with no TBG at all (see below) may be euthyroid and suffer from no known disorder attributable to changes of TBG concentration in the blood, this protein does not seem to be vitally important (*Robbins* et al. 1976, 1978).

Sterling (1979a, b) considers the physiologic role of TBG in binding and solubilizing of the poorly soluble iodinated thyroid hormones. The indefinitely soluble peptide and protein hormones need no such "carrier," whereas steroid and thyroid hormones increase their "solubility" in blood by binding to transporting protein molecules which are themselves soluble in blood plasma. This may constitute one of the important features of TBG.

However, it may be interesting to measure the changes of TBG in blood during various physiologic conditions. In an interesting experiment *Scriba* et al. (1979) measured the effects of total fasting for some 30 days upon various physiologic parameters. The initial mean concentration of TBG in obese persons (2.6 ± 0.6 mg per 100 ml) was significantly higher than the mean of age-matched controls (2.0 ± 0.4 mg per 100 ml). The level of TBG decreased significantly and linearly during fasting, but rose again during realimentation. The T4/TBG ratio increased, but T3/TBG decreased during fasting.

Bratusch-Marrain et al. (1979) investigated a kindred (three generations) with reduced TBG and found that while in males the TBG was altogether absent, in females its concentration was only reduced. However, this abnormality caused no clinical symptoms and had no discernible ill effects. The total serum concentrations of T3 and T4 were decreased. The authors confirmed the X-chromosome-linked inheritance of the absence of TBG.

Another important feature of TBG which connects it, at least in a way, with both the thyroid function and the utilization of thyroid hormones at the target tissue, is the diagnostic value of the determination of either TBG itself or the T4/TBG or T3/TBG ratio. These indicators seem to be useful for the evaluation of the thyroid status of some patients.

Using a commercially available kit for TBG assay (TBGK – CIS, EUROTOPE SERVICES, Ltd., London) *McDowell* (1979) measured the concentration of TBG in sera of several groups of patients categorized according to clinical findings. The author compared five different tests for the evaluation of thyroid function [T4, moles per liter; T3, moles per liter; T3-uptake; thyroid stimulating hormone (TSH), mIU per ml; TBG, mg per liter] from the blood serum, in several groups of persons (acutely ill, contraceptives, pregnant, borderline hypothyroid, hypothyroid, hyperthyroid, and euthyroid). In each group the TBG levels were well correlated with T3-uptake values. However, only the euthyroid and borderline hypothyroid groups showed a significant correlation between serum TBG and T4 levels. After evaluation of all the possible combinations of different tests with the various clinical groups, the author reaches the conclusion that "the assay of serum TBG clearly does not aid the diagnosis of hyperthyroidism."

Horn et al. (1977), with the help of their preparation of TBG, investigated a number of physiologic functions of this protein. They found the normal range of TBG in blood serum to be 23.0 ± 4.0 mg per liter (n = 233 patients with normal thyroid glands). No significant difference between males and females was found. The concentration of TBG is particularly high in newborns (34.3 mg per liter) and up to 1 year of age (29.4); it decreases after puberty and in middle age to its normal value and rises again in older ages. However, the T4/TBG ratio remains constant (3.2 ± 0.7) throughout the human lifespan.

In the primary thyroid disorders, the values of TBG are essentially in the normal range: hyperthyroidism, 20.0 ± 3.5; endemic goiter, 21.1 ± 4.6; and hypothyroidism, 21.6 ± 7.0. The changes of the T4/TBG ratio, which was elevated in hyperthyroidism and decreased in hypothyroidism, was caused by the changes in T4 levels in the blood.

The necessity of the determination of TBG content of blood serum for the diagnosis of thyroid disorders was stressed by *Horn* et al. (1979). Since more than 99% of thyroid hormones in blood are bound to proteins, mainly to TBG, the concentration of TBG may grossly influence the level of total serum T3 and T4. The measurement of the T4/TBG ratio could give a better assessment of the thyroid status than does the determination of total T3 or total T4 alone. The T4/TBG ratio correlates well with the concentration of free T4 in blood, and, hence, with the physiologic status of the thyroid gland. For the diagnosis of thyroid disorders the determination of the T4/TBG ratio may be better than the estimation of free thyroxine index (*Horn* et al. 1979). The determination of TBG itself, e.g., by means of radioimmunoassay, may be an additional characteristic for the determination of the status of the thyroid gland. *Pickardt* et al. (1977) correlated the concentration of TBG in blood with age as well as with thy-

roid status in the following way (TBG, mg per liter): gravidity, 40.4 ± 12.0; contraception, 31.4 ± 5.2; hypothyroidism, 21.6 ± 7.0; goiter, 21.1 ± 4.6; hyperthyroidism, 20.0 ± 3.5; physiologically diminished level of TBG, 9.0 ± 4.3; and physiologically elevated TBG level, 42.2 ± 1.7.

The preparation of TBG obtained by *Horn* and *Gärtner* (1979) has been used also for radioimmunoassay for TBG. If the concentrations of TBG and total T4 in blood are known, the value of free T4 in blood can be calculated. The normal range of TBG established by these authors was 1.0–2.8 mg TBG per 100 ml serum, and consequently, a range of values between 2.1 and 5.6 was obtained for the T4/TBG ratio. However, changes in TBG concentration do not influence the thyroid function because the levels of T4 normally change in parallel to those of TBG in order to maintain the normal T4/TBG ratio. The changes in the T4/TBG ratio may help to diagnose thyroid disorders, since elevated T4/TBG ratios were found only in hyperthyroidism and diminished T4/TBG ratios, only in hypothyroidism.

In two excellent articles *Robbins* (1976) and *Robbins* and *Johnson* (1979) presented a mathematical model for the calculation of free T4 and T3 from the values of total hormones in blood and the concentrations of their transporting proteins. They found, in good agreement with the results of other authors, that the amount of free T4 is only 0.036% of its total value in blood; its concentration is 39 p*M*. Since the binding of both T4 and T3 to TBG changes with temperature, they calculated in the range of 35°C to 41°C an increase in the amount of free forms of both hormones (3.3% per degree of T4 and 4.2% per degree for T3). These small changes in free hormone concentration may play a role in hypothermia or febrile states.

However, it should be noted (*Robbins* and *Johnson* 1979) that the physiologic role of TBG is far from important. There are genetic disorders in which TBG may be totally absent or may be increased up to four times its normal value, but neither of these disorders is accompanied by any detectable alteration in the thyroid status. The absence of hypothyroidism in TBG deficiency proves that TBG is not required even for the intracellular entry and subsequent action of the hormones. What is important for human health, however, is the concentration of free hormones (T4 and T3) in blood. "There is direct evidence that the free hormone concentration tends to remain in the normal range when the transport proteins are altered by a variety of physiologic or pathologic conditions" (*Robbins* and *Johnson* 1979).

9 Future

It is not customary in reviews of research data to speculate on the directions, trends, and further development in a given field, and even less to anticipate some of the results of future research. However, the author's opinion is that such views should be included in every review. They may help the newcomers to the field to orient themselves not only in the "state of the art" picture, but also to perceive the dynamic trends of future research in the particular field.

In the author's view, the interest of researchers is already shifting from thyroid-hormone-binding proteins in blood to those in receptor tissues. The work of *Sterling* et al. (1977) indicated that the thyroid hormones act via mitochondria and that their binding to receptor proteins at the inner surface of mitochondrial membrane of rat liver cells showed a rather high association constant to thyroid hormones (more than 10^{11} M^{-1}) and are probably in direct relationship to the enhancement of oxidative phosphorylation caused by thyroid hormones, whereas the much less firm binding to cytosol proteins may serve as a kind of intracellular storage of thyroid hormones.

It was shown that subjects who do not have any TBG in blood, probably because of a certain genetic disorder, may be completely euthyroid and healthy, indicating that the presence of TBG in blood may be of only secondary importance from the point of view of production of thyroid hormones and their utilization in target tissues. Considerable effort is, and probably will further be devoted to the study of receptor proteins to which thyroid hormones are bound in the target tissues and their cytoplasma, membranes, and cellular particles.

The determination of the exact amount of TBG in blood as a diagnostic tool is probably also of secondary importance. Since neither the production of thyroid hormones nor their utilization and action seems to depend directly upon the concentration of TBG, the determination of its amount may serve as an auxiliary diagnostic parameter.

On the other hand, it is reasonable to expect that the primary structure of human TBG (and probably of that of other species as well) will be definitely established in the near future, with the eventual subunits and complete structure of the molecule.

The mode of interaction of thyroid hormones with TBG will be further elucidated from thermodynamic and physiochemical as well as from structural points of view. Techniques like fluorescence quenching and various spectroscopic methods may be helpful in this respect. By a lucky chance, the maximal absorption of T4 in solution is approximately in the same region of the spectrum as the maximal fluoresence of proteins

(320–330 nm); this makes possible an exact fluorimetric measurement of this interaction (*Hocman* 1971).

Moreover, the interaction of TBG with thyroid hormones is very strong, and since both components are characterized, or will be in the near future, this interaction may serve as an appropriate and exact model for other interactions between protein and small molecular weight ligand.

As indicated in this review, the general principles of the biosynthesis and degradation (or excretion) of TBG are already known. In this respect we may expect a further analysis and detailed characterization of the "life" of TBG in the organism.

Since thyroxine itself contains four heavy iodide atoms and since TBG may be obtained in crystalline form, it is not excluded that the thyroxine-TBG complex may draw the attention of X-ray crystallographers. In this case, we may expect a more exact study of the structure of crystallized TBG and further elucidation of its interaction with thyroxine.

The chemical homogeneity and identity of the isolated glycoprotein, TBG, can be considered as confirmed. The next question is whether the physiologic significance of this protein lies only in its binding of thyroxine or, perhaps, in facilitating some other actions.

The statement of *Robbins* et al. (1978) that "the genetic absence of TBG in otherwise healthy persons is a strong evidence that TBG is not essential for thyroid hormone action" seems to stress that there are, in fact, some other physiologic mechanisms in which TBG may play another, more important role.

Today, in a somewhat exaggerated way, we may consider TBG to be one of the least important but most studied and best known human protein.

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Structure and Function of Phospholiphase A_2

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Contents

Abbreviations 92

1 Introduction 93

2 Different Assays 96

3 Isolation 98

4 Structural and Molecular Properties 102

5 Kinetic Analysis 111
5.1 Introduction 111
5.2 Monomeric Substrates 112
5.3 Micellar Substrates 115
5.3.1 Micelles of Short-Chain Lecithins 115
5.3.2 Mixed Micelles of Phospholipids with Detergents 119
5.4 Monomolecular Surface Films of Medium-Chain Phospholipids . . . 123
5.5 Phospholipids Present in Bilayer Structures 129
5.6 Reversible Inhibition of Phospholipase A_2 137
5.7 Monomeric or Dimeric Enzymes? 139

6 Chemical Modification Studies of Phospholipase A_2 140
6.1 Sulfhydryl Groups 140
6.2 Serine 141
6.3 Histidine 141
6.4 Tryptophan 147
6.5 Methionine 148
6.6 Lysine 150
6.7 Carboxylate Groups 150
6.8 Arginine 151
6.9 α-Amino Group 152
6.10 Tyrosine 154
6.11 Miscellaneous 157
6.11.1 Modification of Phospholipase A_2 with Ethoxyformic Acid Anhydride 157
6.11.2 Cross-Linking of Phospholipase A_2 159
6.11.3 Photoaffinity Labeling 160
6.11.4 Semisynthesis of Pancreatic Phospholipase A_2 160

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7 Direct Binding Studies 163
7.1 Binding of Ca^{2+} 163
7.1.1 Pancreatic Phospholipases A_2 163
7.1.2 Venom Phospholipases A_2 165
7.2 Binding of Monomeric Substrate Analogs 167
7.3 Binding of Aggregated Lipids 169
7.3.1 Pancreatic Phospholipase A_2 169
7.3.2 Snake Venom Phospholipase A_2 173

8 Immunology 175

9 X-Ray Analyses 176

10 Mechanism of Catalysis 181

11 Concluding Remarks 185

References 187

Abbreviations

PLA	phospholipase A_2 (EC 3.1.1.4)
pro-PLA	prophospholipase A_2
AMPA	ϵ-amidinated phospholipase A_2
des-Ala-1-AMPA	ϵ-amidinated phospholipase A_2 from which the N-terminal Ala-1 has been removed
PL	phospholipid
FA	fatty acid
PC (phosphatidylcholine, L-lecithin, di-C_n-PC, 1,2-diacyllecithin *sn*-3-lecithin)	1,2-diacyl-*sn*-glycero-3-phosphocholine
D-Lecithin (D-diC_n-PC, *sn*-1-lecithin)	2,3-diacyl-*sn*-glycero-1-phosphocholine
β-Lecithin (*sn*-2-lecithin)	1,3-diacyl-*sn*-glycero-2-phosphocholine
Lysolecithin (lyso-PC, 1-acyl-lysolecithin)	1-acyl-*sn*-glycero-3-phosphocholine
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DiC_n ether PC	1,2-dialkyl-*rac*-glycero-3-phosphocholine
PE	1,2-diacyl-*sn*-glycero-3-phosphoethanolamine
PS	1,2-diacyl-*sn*-glycero-3-phospho-L-serine
PG	1,2-diacyl-*sn*-glycero-3-phospho-1′-glycerol
AB-I	ethyl 4-azidobenzimidate. HCl
ANB-AI	ethyl *N*-azido-2-nitrobenzoylamino acetimidate. HCl
ANB-NOS	*N*-5-azido-2-nitrobenzoyloxysuccinimide
ANS	1-anilinonaphthalene-8-sufonic acid
Boc	*t*-butyloxycarbonyl
BPB	*p*-bromophenacyl bromide
CNBr	cyanogen bromide
Dansyl	5-(dimethylamino)naphthalene-1-sulfonyl
DFP	diisopropylfluorophosphate

DBE	*N*-diazoacetyl-*N'*-(2,4-dinitrophenyl)ethylene-diamine
EDC	1-ethyl-3-(*N,N*-dimethyl)aminopropyl carbo-diimide
EDTA	ethylene diamine tetracetic acid
EOFA	ethoxyformic acid anhydride
HNB	2-hydroxy-5-nitrobenzylbromide
NBS	*N*-bromosuccinimide
NPS	*o*-nitrophenylsuccinimide
TNM	tetranitromethane
RNAse S'	Ribonuclease S' (enzymatically fully active complex of equimolar amounts of S-peptide and S-protein)
CTAB	cetyl trimethylammonium bromide
SDS	sodium dodecylsulphate
Triton X-100	*p*-(1,1,3,3-tetramethylbutyl)phenoxypolyoxy-ethylene glycol
Tween	polyoxyethylenesorbitol fatty acid ester
CMC	critical micelle concentration
IRS	interface recognition site
CD	circular dichroism
NMR	nuclear magnetic resonance
Photo-CIDNP	photochemically-induced dynamic nuclear polarization
PRR	proton relaxation rate
IEP	isoelectric point

1 Introduction

Phospholipases A_2 (EC 3.1.1.4) are wide spread in Nature and are found both within and without the cell (see for reviews *Shen* and *Law* 1979; *van den Bosch* 1980). In this review we will confine ourselves to the extra-cellular phospholipases which are abundant in pancreatic tissue and in the venom of snakes and arthropods [1]. Exceptions may, however, occur as is exemplified by guinea pig pancreas which does not contain PLA. *In lieu* two lipases which are unusually active on phosphoglycerides (*White* et al. 1971; *Durand* et al. 1978; *Fauvel* et al. 1981) have been reported to be present. Irrespective of the source, the enzyme is a small (mol. wt. 14 000 for the monomeric form) water-soluble protein. Like all lipolytic enzymes it is able to hydrolyze monomeric substrate molecules, but its full activity only becomes evident in the presence of certain lipid-water interfaces. A break is observed in plots of velocity vs. substrate concentra-

1 In 1981 a more detailed review on pancreatic PLA by *J.J. Volwerk* and *G.H. de Haas* entitled "Pancreatic Phospholipase A_2. A model for lipid-protein interactions?" will appear in *Molecular Biology of Lipid-Protein Interactions* (Eds. O.H. Griffith and P. Jost), J. Wiley & Sons, Inc., New York (1981)

tion at the moment aggregation of substrate starts (Fig. 1, curve a). The precursor of the pancreatic enzyme (Fig. 1, curve b) behaves as a "normal" esterase and is not activated by the lipid-water interface.

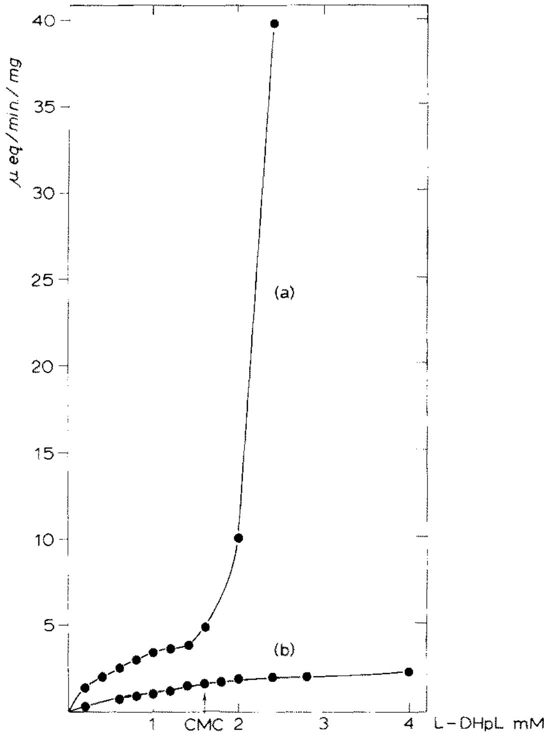

Fig. 1. Hydrolysis of di-heptanoyl lecithin by porcine pancreatic phospholipase *(curve a)* and by its precursor *(curve b)* (*Pieterson* et al. 1974b)

The enzymatic activity of PLA is calcium dependent. The naturally occurring 3-*sn*-phosphoglycerides are hydrolyzed exclusively at the 2 position, giving rise to the formation of 1-acyl-3-*sn*-lysophospholipids. However, when synthetic substrates[2] containing short (⩽ six carbon atoms) fatty acids are incubated in the presence of large amounts of the pancreatic enzymes, they loose part of their stereo- and positional specificity (this laboratory, unpublished results). From the results of the hydrolysis of dibutyryl lecithin as presented by *Wells* (1972) one is inclined to conclude that the same is true for *Crotalus adamanteus* phospholipase. It is known that hydrophobic binding forces are important in the Michaelis-Menten complex formation (vide infra). Therefore, it is conceivable that short fatty

2 For the synthesis of (short-chain) substrates and the synthesis of product analogs like *n*-alkylphosphocholine the reader is referred to recent review articles (*Slotboom* et al. 1973; *Eibl* 1980)

acids contribute less to the orientation of the substrate than do longer acyl chains. This obviously could result in aspecific binding and hydrolysis.

In the pancreas the production of (pro)PLA undoubtedly serves a digestive function. For the venom phospholipases the role is less clear, although the enzyme functions in digesting the prey in concert with the various hydrolytic enzymes found in snake venoms (*Tu* 1977). Snake venom phospholipases may serve another goal since the presynaptic toxins found in venoms are basic phospholipases or are a complex containing phospholipase. In addition to the neurotoxic action several venom phospholipases exhibit other pharmacological effects such as direct hemolytic action, anticoagulant properties, and myonecrotic and postsynaptic neurotoxic effects. For details the reader is referred to recent reviews (*Karlsson* 1978; *Howard* and *Gundersen* 1980).

In the pancreas phospholipase is produced in the form of an inactive precursor which is stored in the secretory granules. Only in the intestine does activation occur by limited tryptic proteolysis giving rise to the formation of the active phospholipase and a small polar activation peptide. For snake venoms no evidence for such a precursor has been obtained and no data about the occurrence of (pro)phospholipase in snake pancreatic tissue has been obtained so far.

Both precursor and phospholipase are about equally active on monomeric solutions of short-chain substrates (Fig. 1, curves a and b). However, only phospholipase displays full enzymatic activity on mixed micelles of bile salts and (natural) long-chain phosphoglycerides. Under these conditions the precursor is at least 10^4 times less active and for practical purposes can be considered as inactive in vivo. The inertness of the precursor toward organized substrates is due to its inability to bind to lipid-water interfaces as demonstrated by a variety of techniques (vide infra). Similarly phospholipase in which the α-amino group is blocked by a number of different reagents (*Abita* et al. 1972; *Slotboom* and *de Haas* 1975) does not bind to aggregated substrates nor degrade them. The early suggestion by *Abita* and co-workers that the α-amino group of phospholipase is locked in a fixed position, thereby stabilizing the active site geometry, has been confirmed by recent X-ray studies.

Based on the kinetic properties of phospholipase and its precursor and on specific chemical modifications *Pieterson* et al. (1974b) proposed that both PLA and precursor possess a fully functional active site in which monomeric substrates are bound and hydrolyzed. In addition to the active site the enzyme (but not the precursor) is supposed to contain an independent and topographically different site (IRS) responsible for the interaction with lipid-water interfaces. According to our present knowledge the basic concept of two different and independent sites is still attractive, although several examples of mutual effects have been found (vide infra).

Phospholipase A_2 has been used for the preparation of a number of (lyso)phospholipids (*Slotboom* et al. 1973; *Eibl* 1980), and, in conjunction with other lipolytic enzymes, for studies of the distribution of phospholipids in biomembranes and the lipid requirement of membrane-bound enzymes (see for recent reviews *Op den Kamp* 1979; *Roelofsen* and *Zwaal* 1976).

2 Different Assays

Phospholipase A_2 catalyzes the reaction: diacyl phospholipid → monoacylphospholipid + fatty acid. The enzymatic activity can be measured by the disappearance of substrate or the appearance of products. Analysis of the remaining substrate is generally less accurate since under kinetic conditions only a small portion of the substrate will be used. Hence, the determination of the remaining substrate by chromatography or the estimation of the disappearance of ester bonds (*Augustyn* and *Elliott* 1969) is only rarely employed.

Among the methods to determine reaction products many applications, advantages and drawbacks have been discussed by *Van den Bosch* and *Aarsman* (1979). The appearance of lysophospholipids is in general followed by measurement of their effect on the turbidity of lecithin emulsions (*Habermann* and *Hardt* 1972), coagulability of egg yolk (*Habermann* and *Neumann* 1954), the turbidity of egg yolk (*Marinetti* 1965; *Mebs* 1970), and hemolysis of red blood cells (*Braganca* et al. 1969). A rapid assay to determine the number of electrophoretic PLA variants has been described (*Shier* and *Trotter* 1978). This method employs disc gel electrophoresis in the absence of Ca^{2+} ions in gels containing lecithin. After electrophoresis the gels are incubated in the presence of Ca^{2+} and rhodamine 6G to detect liberated fatty acids.

Although some of these assays are easy to carry out and may be useful to screen a large number of samples for phospholipase activity, comparison of different enzymes is difficult because no absolute activities are obtained.

The liberation of fatty acids is more easily quantitated. Methods proposed include bioluminescence of a bacterium as a response to released myristic acid (*Ulitzur* and *Heller* 1978), conductometry (*Moores* and *Lawrence* 1972), thin-layer and/or gas-liquid chromatography of labeled compounds (see review *Van den Bosch* and *Aarsman* 1979), and polarography in a coupled assay with lipoxygenase (*Gale* and *Egan* 1980). The most widely used method is the titration of liberated fatty acids in a pH stat. Both purified lecithin and whole egg yolk have been used either without detergent or in the presence of detergents. After the reports of

Magee et al. (1962) and *Ute* and *Magee* (1971) deoxycholate has been widely used, although the optimal conditions with respect to Ca^{2+} and deoxycholate concentrations for enzymes from different sources can be very different (*Nieuwenhuizen* et al. 1974; *Figarella* et al. 1971; *Evenberg* et al. 1977a). The nonionic detergent Triton X-100 as introduced by *Salach* et al. (1968, 1971) and also used for kinetic studies by *Dennis* (see Sect. 5) has been applied in routine assays in many studies.

However, as with deoxycholate, often little attention has been paid to the optimal conditions. In our hands it appeared that every enzyme has its characteristic optimum for Ca^{2+} and Triton X-100 concentration. These concentrations not only affect the rate of hydrolysis but also greatly affect the linearity of the reaction with time. Outside the optimal conditions the reaction rapidly slows down with time, an effect that also has negative influence on proportionality between enzyme concentration and velocity (unpublished results). In conclusion the egg yolk assay is rapid, cheap with respect to substrate, and reproducible with a good sensitivity: specific activities are between 100–5000 μmol · min^{-1} · mg^{-1} which allows detection and determination of about 0.2 μmol/min (corresponding to about 2 μg down to 40 ng of protein).

Since long-chain phospholipids are insoluble in water, their activity can only be accurately measured in the presence of detergents. Synthetic short-chain phospholipids dissolve in water and form true (monomeric) solutions or, at higher concentrations, micelles (*Roholt* and *Schlamowitz* 1961). Assays based on the use of monomeric substrates and on the use of micellar medium-chain substrates have been used. However, these methods are quite expensive with regard to substrate and only for special purposes do these assays deserve support, i.e., for kinetic analysis in the monomeric or micellar substrate region (see also Sect. 5). The best synthetic substrate known (dioctanoyl lecithin) can be used, however, to increase the sensitivity of the test. First, the baselines are stable and allow the use of very dilute hydroxide solutions. Second, all phospholipases tested in our laboratory showed a higher activity on this substrate than on any other system, including egg yolk. For example, β-bungarotoxin, a presynaptic toxin with low PLA activity (SA $\approx$ 100 on egg yolk), is quite active when tested with dioctanoyl lecithin as a substrate (SA $\approx$ 1000) and is in fact about equally active as porcine pancreatic PLA (unpublished results).

Finally a number of specific assays deserve attention. *Aarsman* et al. (1976) introduced the use of thioester substrates. During hydrolysis thiol groups are released which can be detected spectrophotometrically after reaction with Ellmann's reagent. The introduction of the thiol ester function has been used to study the hydrolysis of monomeric lecithins by porcine pancreatic phospholipase (*Volwerk* et al. 1979) and was found to be about 100-fold more sensitive than titration of liberated fatty acids.

The use of ^{31}P NMR to study hydrolysis was introduced by *Henderson* et al. (1975) and *Brasure* et al. (1978). This method is based on the difference in chemical shifts of phosphatidylcholine and lysophosphatidylcholine. In an elegant study by *Roberts* et al. (1979) this method was applied to simultaneously analyze the hydrolysis of individual phospholipid species in phospholipid mixtures.

3 Isolation

Venom as well as pancreatic tissue contains high amounts (1%–10% of all proteins present) of (pro)PLA. As these proteins are very stable with respect to heat, variations in pH, and denaturing conditions, their isolation is relatively simple. For the pancreatic (pro)phospholipases the purification includes homogenization of the tissue, a heat treatment at low pH, a salt precipitation, and chromatography on both DEAE and CM cellulose. The purest preparations of active phospholipase are prepared by tryptic activation of the precursor followed by chromatography on CM cellulose.

In this respect it must be mentioned that the pancreatic tissue should preferably be fresh. Even freezing and thawing can modify the activation peptide of the precursor (*Nieuwenhuizen* et al. 1973a,b; *Evenberg* et al. 1977a) without changing the final phospholipases. Under more drastic conditions, however, activation to phospholipase or even proteolytic breakdown of the enzyme may occur (*de Haas* et al. 1968). The relative sensitivity of the pancreatic phospholipase to proteolysis might explain the multiple forms of the enzyme we observed using commercial pancreatic powder rather than fresh pancreas (unpublished results). Multiple forms of pancreatic phospholipase have also been described by *Tsoa* et al. (1973). These authors also used commercial pancreatin, and their disputable results once more argue against the use of this powder as enzyme source. Pure preparations of (iso) precursors and activation of these to the corresponding enzymes have been described for pancreatic tissue and juice from pig (*Nieuwenhuizen* et al. 1974 and references therein; *van Wezel* and *de Haas* 1975), ox and sheep (*Dutilh* et al. 1975), horse (*Evenberg* et al. 1977a), and man (*Figarella* et al. 1971; *Wittich* and *Schmidt* 1969; *Gratoli* et al. 1981).

As venoms from a great variety of animals can be bought and since there is no need for extensive extraction and homogenization procedures, these venoms have proven to be popular sources of PLA. Yet the elution patterns contain in general more PLA peaks than those observed with the pancreatic enzymes. Complex elution patterns can be explained because of (1) the presence of isoenzymes with different charge properties, (2) self-aggregation of the protein leading to molecular weight values between

9000 and 40 000, (3) combination of the enzyme with nonenzymatic components (these complexes may or may not be stable under the conditions employed in the isolation), and (4) changes in the charge of the phospholipase by desamidation and/or proteolytic breakdown. Possibilities (1) and (2) occur in most venoms and only a few exceptions are known (e.g., *Crotalus atrox*). Complexes [possibility (3)] have been found in several venoms, including those of *Oxyuranus scutellatus* (*Fohlman* et al. 1976), *Parademansia microlepidotus* (*Fohlman* 1979), *Vipera palestinae* (*Simon* and *Bdohlah* 1980), *Vipera ammodytes* (*Aleksiev* and *Shipolini* 1971), *Bothrops asper* (*Vidal* and *Stoppani* 1971a), *Crotalus durissus terrificus* (see minireview *Haberman* and *Breithaupt* 1978), and *Crotalus scutulatus scutulatus* (*Cate* and *Bieber* 1978). Changes in the charge properties of the PLA [possibility (4)] yield proteins with different electrophoretic mobilities but identical amino acid composition. These charges might arise from scissions in the chain while the peptides are held together by the disulfide bridges. No evidence for such splits (with retention of enzymatic activity) has been reported. The α and β forms of *C. adamanteus* PLA differ by a single Gln → Glu substitution only (*Heinrikson* et al. 1977), and although no evidence is available about the cause of their appearance, it might very well be a result of desamidation by venom proteolytic enzymes. Evidence for such activities has been presented for the venom of *Vipera palestinae* (*Shiloah* et al. 1973) where the native phospholipase was converted into a more acidic one by incubation with whole venom.

Most purification methods employ a combination of gel filtration and the use of one or more ion exchangers. The more rational order of their application undoubtedly includes first a group separation on a molecular sieve which in general improves the specific activity two- to threefold and removes small toxines (like direct lytic factor) and most other enzymatic activities from the phospholipase fraction. Subsequent chromatography on an ion exchange column gives then the separation into the isoenzymes. Because of the greater capacity of the ion exchange columns, the order is frequently reversed. In that case precautions have to be taken to avoid aggregation of the low molecular weight toxins due to lyophilization of solutions containing high salt concentrations (*Karlsson* 1978). Instead of or prior to these aspecific purification methods the use of a more specific purification should be considered. A number of potentially interesting methods have been described:

1. Precipitation of PLA from aqueous isopropanol with $NdCl_3$ (*Wells* 1975)
2. Affinity chromatography with an immobilized substrate analog (*Rock* and *Snyder* 1975) which makes use of the fact that only the enzyme-

calcium complex of *Crotalus adamanteus* phospholipase binds to the columns. [Elution was done with EDTA, but in our hands a more satisfactory elution takes place by eluting with about 30% organic solvent (acetonitril, dimethylformamide) or 6-*M* urea (unpublished results).]

3. Hydrophobic chromatography on phenyl sepharose CL-4B as described for the removal of traces of PLA from cardiotoxin preparations (*Louw* and *Carlsson* 1979)
4. Affinity chromatography using immobilized antibodies against PLA (*Apsalon* et al. 1977; *Gubenšek* and *Žunič* 1978; *Delori* and *Tessier* 1980).
5. The use of concanavalin-Sepharose 4B (*Gritsuk* et al. 1979) as an elegant way to isolate bee venom phospholipase (which contains carbohydrates)

Phospholipases or phospholipase-containing complexes have been isolated in a pure state and have been characterized from venom from the following snakes: *Agkistrodon halys blomhoffi* (*Kawauchi* et al. 1970a and b; *Hanahan* et al. 1980), *Agkistrodon piscivoris* (*Augustyn* and *Elliot* 1970), bees (*Shipolini* et al. 1971; *Gritsuk* et al. 1979), *Bitis arietans* (*Howard* 1975), *Bitis gabonica* (*Botes* and *Viljoen* 1974a), *Bothrops asper* (*Alagón* et al. 1980; *Ferlan* and *Gubensek* 1978), *Bothrops atrox, Bothrops jararaca, Bothrops jararacussu,* and *Bothrops neuwiedii* (*Vidal* and *Stoppani* 1971b), and *Bungarus caerulus* (*Abe* et al. 1977; *Moody* and *Raftery* 1978).

From *Bungarus multicinctus* venom several components with weak phospholipase activity and presynaptic activity have been isolated. The β-type toxin apparently contains two chains (mol. wt. 22 000 for the covalent complex) based on molecular weight determinations and amino acid composition of the unreduced toxin (*Abe* et al. 1977) and on the sequence analysis (*Kondo* et al. 1978a and b and references therein). However, there are also studies showing that in addition to the double-chain toxin, β toxins composed of a single chain (mol. wt. 11 000) are present in this venom (*Tobias* et al. 1978; *Hanly* et al. 1977). In addition a nontoxic phospholipase is present as well (*Wernicke* et al. 1974). A possible explanation for this confusing data might be that *B. multicinctus* contains, in addition to the α toxin (postsynaptic toxin), double chain β toxins (mol. wt. 22 000) with microheterogeneity. It is conceivable that like in other venoms, phospholipases (mol. wt. 11 000–14 000) with different IEPs are present. The acidic phospholipase is nontoxic (*Wernicke* et al. 1974) and the basic phospholipases could very well show presynaptic activity (β toxin activity), but they should be compared to other single chain presynaptic toxins (e.g., notexin) rather than to β-bungarotoxin.

The venoms of *Crotalus adamanteus* (*Wells* and *Hanahan* 1969; *Wells* 1975) and of *C. atrox* yield acidic phospholipases (*Wu* and *Tinker* 1969; *Hachimori* et al. 1971), whereas the venom of *C. durissus terrificus* contains the first venom toxin (crotoxin) ever isolated (*Slotta* and *Fraenkel-Conrat* 1938; for a review see *Habermann* and *Breithaupt* 1978). The crotoxin complex contains one or two basic isophospholipases (depending on the source of the venom; *Breithaupt* et al. 1974); an acidic nontoxic phospholipid is also present in this venom (*Breithaupt* et al. 1975). *C. scutulatus scutulatus* venom contains a toxic complex very similar in properties to crotoxin (*Cate* and *Bieber* 1978; *Gopalakrishnakone* et al. 1979). From the venom of *C. scutulatus salvinii* a phospholipase (mol. wt. 30 000) was isolated with two different amino terminal residues. The authors concluded that this enzyme is an asymmetrical dimer in analogy with the symmetrical dimer found in *C. adamanteus* and *C. atrox* venoms (*Nair* et al. 1979). However, as *Cate* and *Bieber* showed that the acidic component was easily missed in the complex isolated from *C. scutulatus scutulatus*, it could very well be that *Nair* and co-workers have isolated a similar complex from *C. scutulatus salvinii* venom. Pure phospholipases have also been isolated from the following venoms: *Enhydrina schistosa* (*Fohlman* and *Eaker* 1977), *Hemachatus haemachatus* (*Joubert* 1975a; *Yang* and *King* 1980b), *Laticauda semifasciata* (*Yoshida* et al. 1979 and references therein), *Micrurus fulvius microgalbineus* (*Possani* et al. 1979).

The Asiatic *Naja* genus (cobras) is represented by a large variety of subspecies of *Naja naja*. It has even been proposed that *N.n. oxiana* should be considered to be a different species and should be called *N. oxiana*. This uncertainty and the possibility that the venoms of the snakes from different geographical origin are mixed may explain the large variability of the phospholipase patterns in these venoms. At least nine and possibly 11 isoenzymes have been reported in the venom of *Naja naja* (*Salach* et al. 1971). However, in the venom of a single (sub)species the situation can also be complex: from the venom of *N.n. oxiana* seven isoenzymes have been reported (*Apsalon* 1977). The fact that the three main fractions (IEP 5.1–5.5) represent > 98% of the enzymatic activity but only 62% of the protein on a weight basis may be representative of the complexity of cobra venoms and stresses the need for good purification protocols. A similar situation in the venom of *N.n. naja* might explain why *Barden* et al. (1980) could remove about 20% of a protein of low specific activity from their phospholipase preparations. Purifications have been reported for:

1. *N.n. atra* (*Chang* et al. 1976)
2. *N.n. naja* (*Salach* et al. 1971; *Deems* and *Dennis* 1981 and references therein)

3. *N.n. kaouthia* (= *siamensis*) (*Andreasen* et al. 1979; *Karlsson* and *Pongsawasdi* 1980; *Joubert* and *Taljaard* 1980)
4. *N.n. oxiana* (*Apsalon* et al. 1977)
5. *N. melanoleuca* (*Joubert* and *van der Walt* 1975)
6. *N. mossambica mossambica* (*Joubert* 1977; *Martin-Moutot* and *Rochat* 1979)
7. *N. nigricollis* (*Yang* and *King* 1980a and references therein; *Evans* et al. 1980).

For *Notechis scutatus scutatus* venom the isolation of three isoenzymes, including one without phospholipase activity, has been described by *Halpert* and *Eaker* (1975, 1976a,b). Further purifications have been described for: *Oxyuranus scutellatus* (*Fohlman* et al. 1976), *Parademansia microlepidotus* (*Fohlman* 1979), *Pseudechis australis* (*Leonardi* et al. 1979; *Mebs* and *Samejima* 1980b), *P. colletti* (*Mebs* and *Samejima* 1980a,b), *P. porphyriacus* (*Mebs* and *Samejima* 1980b) and *Trimeresurus flavoviridis* (*Ishimaru* et al. 1980).

The venom of *Vipera ammodytes* contains a neurotoxic complex constituted by a basic phospholipase and an acidic subunit (*Aleksiev* and *Shipolini* 1971; *Aleksiev* and *Tchorbanov* 1976; *Tchorbanov* et al. 1977 and references therein) and several other toxic as well as nontoxic phospholipases (*Sket* et al. 1973).

Phospholipases have also been isolated from the venoms of *Vipera aspis* (*Boffa* et al. 1971) and *Vipera berus* (*Delori* 1973; *Boffa* et al. 1976). *Vipera palestinae* venom contains one phospholipase. During isolation this protein is partly converted into a species with different electrophoretic mobility but identical amino acid composition (*Shiloah* et al. 1973). The venom also contains a neurotoxin which appears to be a 1:1 complex of the acidic phospholipase and a basic polypeptide. The basic component was able to enhance the toxicity of a number of phospholipases isolated from other snake venoms but did not render porcine pancreatic PLA toxic (*Simon* and *Bdohlah* 1980). Finally two phospholipases A_2 have been isolated from the venom of *Waterinnesia aegyptica* (*Simon* and *Bdohlah* 1980).

4 Structural and Molecular Properties

The phospholipases isolated from mammalian pancreas, bee venom, and snake venom are heat stable, are resistant to denaturing agents, and are Ca^{2+} dependent. Therefore, one may expect that several structural aspects of these enzymes are similar. Because of their low molecular weight the determination of the amino acid sequence of phospholipase has become relatively easy, and the amino acid sequences of more than 20 "true" phospholipases have been determined. In addition, the sequence of a number of

homologous proteins like the γ-chain of taipoxin and the B-chain of β-bungarotoxin have been determined. The structures of these proteins are compared in Fig. 2.

It is obvious that all phospholipases shown in Fig. 2 are homologous proteins which probably have developed from a common ancestor. Bee venom PLA (*Shipolini* et al. 1974 a, b) is not included in Fig. 2, because its sequence is too different from all other phospholipases to allow a homology comparison. Only the peptide around the active center histidine (Ala-Cys-Cys-Arg-Thr-His-Asp-Met-Cys) is recognizable. Bee venom phospholipase might be an example of converging evolution. However, the observation that for example cytochrome C from insects and mammals shows a high degree of sequence homology makes the determination of a PLA from the venom of another arthropod highly desirable.

With the exception of the proteins from *Bitis gabonica*, β-bungarotoxin B-chain, and taipoxin γ-chain, all phospholipases contain seven disulfide bridges. The disulfide connections of 12 half-cysteine residues were determined for the porcine phospholipase (*de Haas* et al. 1970a, b), but since a reinvestigation of the sequence showed that this enzyme also contains 14 half cysteines (*Puijk* et al. 1977), the disulfide bridge assignment was partly incorrect. A second attempt to assign the bridges was made using a low resolution X-ray structure of porcine precursor, but unfortunately two bridges were interchanged, leaving a lot of confusion (*Drenth* et al. 1976). The three-dimensional structure of bovine pancreatic PLA at 1.7 Å resolution revealed the correct pairing beyond any doubt (*Dijkstra* et al. 1978; *Dijkstra* 1980). The disulfide bridges are indicated in Fig. 3. As no attempts have been made to determine the disulfide bridges in snake venom phospholipases we can only assume that they are present at homologous places as in bovine pancreatic phospholipases. From Fig. 2 it is obvious that in all *elapidae* and *hydrophidae* phospholipases (with the exception of β-bungarotoxin B-chain) the half-cysteine residues are completely conserved[3]. Hence one must assume that in these enzymes the disulfide bridges are connected as in the bovine pancreatic PLA (Fig. 3). As already pointed out by *Heinrikson* et al. (1977) in *viperidae* and *crotalidae* phospholipases the half-cysteine residues 11 and 77 (Figs. 2 and 3) are absent. In these enzymes two half-cysteines are found at position 50 and at the C terminus which are not present in the phospholipases from pancreas or from *elapidae* or *hydrophidae* venoms. Again in the absence of chemical evidence one must assume that these half-cysteines form a disulfide bridge. In their article *Heinrikson* et al. (1977) divided phospholipases into two groups. Group I contains the enzymes of pancreas and *elapidae*, while group II contains *viperidae* and *crotalidae* phos-

3 It should be noticed, however, that the alignment of the sequences as shown in Fig. 2 is also based on the positions of the half-cystein residues. Because of their highly conserved character they contribute much to this alignment

```
    1       5         10        15        20        25        30        35        40        45        50        55-         60        65        69
1   A L W Q F R S M I K C A I P G S H P L M D F N N Y G C Y C G L G G S G T P V D E L D R C C E T H D N C Y R D A - K N L D S C K F L V D N P Y
2   * V * * * * * * * Q * T * * N * K * Y L E * * D * * * * * * * * * * * * * * * * * * A * * Q V * * * * * T Q * - * E * S * * R * * * * * * *
3   * * * * * N G * * * * K * * S * E * * L * * * * * * * * * * * * * * * * * * * D * * * * * Q * * * * * * K Q * - * K * * * * * V * * * * * *
4   * * * * * * * * * * * T * * * * D * * L * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * - * * * * * * * * * * * * * *
5   N * V * * S N L * Q * N V K * * R A S Y H Y A D * * * * * * A * * * * * * * * * * * * * * K I * * * * * G E * E * - M - G * - Y - - - - * K
6   N * V * * T Y L * Q * * N S * K R A S Y H Y A D * * * * * * A * * * * * * * * * * * * * * K I * * * * * G E * E * - M - G * - Y - - - - * K
7   N * V * * S Y L * Q * * N T * K R A S Y H Y A D * * * * * * A * * * * * * * * * * * * * * K I * * * * * G E * E * - M - G * - Y - - - - * K
8   N * V * * S Y V * T * * N H N R R S S L * Y A D * * * * * * A * * * * * * * * * * * * * * K I * * D * * G E * E * - Q - G * - Y - - - - * K
9
10  N * V * * S Y L * Q * * N H * K R * T W H Y M D * * * * * * A * * * * * * * * * * * * * * K I * * D * * D E * G * - K - G * - * - - - - * K
11  N * V * * S Y L * Q * * N H * R R * T R H Y M D * * * * * * W * * * * * * * * * * * * * * K I * * D * * S * * E * - K - G * - S - - - - * K
12  N * V * * S N * * Q * * N H * * R * S L A Y A D * * * * * S A * * * * * * * * * * * * * * K * * * D * * A R * T * - S Y S * - T - - - - * *
13  N * Y * * K N * * * * T V * S - R S W W H * A * * * * * * * R * * * * * * * * D * * * * * Q * * * * * * S * * E * - I S G * - R - - - - * *
14  N * Y * * K N * * H * T V * N - R * W W H * A * * * * * * * R * * K * * * * * D * * * * * Q I * * K * * D E * E * - I S G * - W - - - - * *
15  N * Y * * K N * * Q * T V * N - R S W W H * A * * * * * * * R * * * * * * * * D * * * * * Q I * * * * * G E * E * - I S G * - W - - - - * *
16  N * Y * * K N * * H * T V * N - R S W W H * A * * * * * * * R * * * * * * * * D * * * * * Q I * * * * * G E * E * - I S G * - W - - - - * *
17  N * Y * * K N * * H * T V * S - R * W W H * A D * * * * * * R * * K * * A * * D * * * * * Q V * * * * * G E * E * - * - G * - W - - - - * *
18  N * Y * * K N * * H * T V * S - R * W W H * A D * * * * * * R * * K * * A * * D * * * * * Q V * * * * * G E * E * - * - G * - W - - - - * *
19  N * Y * * K N * * H * T V * S - R * W W H * A D * * * * * * R * * K * * * * * D * * * * * Q V * * * * * E K * G * - M - G * - W - - - - * *
20  N * Y * * K N * * H * T V * S - R * W W H * A D * * * * * * R * * K * * * * * D * * * * * Q V * * * * * E K * G * - M - G * - W - - - - * *
21  N * Y * * K N * * * * T V * S - R S W L * * A * * * * * * * R * * * * * * * * D * * * * * Q I * * * * * N E * G * - I S G * - W - - - - * *
22  N * Y * * K N * * Q * T V * N - R S W W * * A D * * * * * * R * * * * * * * * D * * * * * Q V * * * * * D E * E * - I S R * - W - - - - * *
23  N * Y * * K N * * Q * T V * S - R S W W N * A D * * * * * * R * * * * * * * * D * * * * * Q V * * * * * D E * E * - I S G * - W - - - - * *
24  N * Y * * K N * * Q * T V * S - R S W W * * A D * * * * * * R * * * * * * * * D * * * * * Q V * * * * * N E * E * - I S G * - W - - - - * *
25  D F E * * S N * * Q * T * * C(G/S)E C L A Y M D * * * * * * F * * * * * * I * D * * * * * K * * * E * * A E * G * - * S A * * S V L S E * N
26  N * I N * M E * * R Y T * * C E K T W G E Y A D * * * * * * A * * * * R * I * A * * * * * Y V * * * * * G * * E * - K H K * - - - - - * * K
27
28  N * I * * G N * * S - * M T * - K S S L A Y A S * * * * * * W * * K * Q * K * D T * * * * F V * * C * * G K * D * - - - - * - - - - - S * K
29  D * T * * G N * * N - K M - * - Q S V F * Y I Y * * * * * * W * * K * K * I * A T * * * * F V * * C * * G K M G T - - - - Y - - - - - D T K
30  S * V * * E T L * M - K V A K R S G * L W Y S A * * * * * * W * * H * R * Q * A T * * * * F V * * C * * G K * T N - - - - * - - - - - * * K
31  S * V * * E T L * M - K * A * R S G * L W Y S A * * * * * * W * * H * L * Q * A T * * * * F V * * C * * G K * T D - - - - * - - - - - * * K
32  H * L * * N K * * * - F Z T R K B A I P F Y A F * * * * * * W * * Z * R * K B A T B * * * F V * B C * * G K L A * - - - - * - - - - B T K *
33  S                                   V                 L
34  S * M * * E T L * M - K * A * R S G I W Y S G S * * * * * * A * * Q * R * S * A S * * * * F V * * C * * G K V T G - - - - * - - - - * B * K
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    70        75  -         80      -    85           - 90          95          100         105       - -110          115         120        125         130
1   T E S Y S Y S - C S N T E I T - C N S K N N - A C E A F I C N C D R N A A I C F S K A - - F Y N K E H K N L D T K - K Y C
2   * * * * K F * - * * G * * V * - * S D * * * - * * * * * * * * * * * * * * * * * * * * - - * * * F * N * * * * S * R * A *
3   * N N * * * * - * * * * * * * - * S * E * * - * * * * * * * * * * * * * * * * * * * V - - * * * * * * * * * * - * - * N *
4   * N * * * * * - * * * * * * * - * * * * * * - * * * * * * * * * * * * * * * * * * * * - - * * * * * * * * * * * * - * * *
5   W T L * T * E S * T D * S P - - * D E * T - - G * Q G * V * A * * L E * * K * * A R S - - * * * N K N Y * I * * S - * R * K
6   L T M * N * Y - * G T Q S P * - * D D * T - - G * Q R Y V * A * * L E * * K * * A R S - - * * * N K N Y * I * * S - * R * K
7   L T M * N * Y - * G T Q S P * - * * D * T - - G * Q R Y V * A * * L E * * K * * A R S - - * * * N K N Y * I * * S - * R * K
8   M L M * D * Y - * G S N G P Y - * R N V K K - K * N R K V * D * * V A * * E * * A R N - - A * * N A N Y * I * * * - * R * K
9   L                                                                    K
```

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10 M S A * D * Y - * G E N G P Y - * R N I K K - K * L R * V * D * * V E * * F * * A * * - - * * * N A N W * I * * * - * R * Q
11 M S A * D * Y - * G E N G P Y - * R N I K K - K * L R * V * D * * V E * * F * * A * * - - * * * N A N W * I * * * - * R * Q
12 W T L * * W Q - * I E K T P * - * D * * T - - G * Q R * V * D * * A T * * K * * A * * - - * * * * * N Y * I * P * - * R * Q
13 F K T * * * D - * T K G K L * - * K E G * * - E * A * * V * K * * * L * * * * * A G * - - H * * D N N N Y I * L A - R H * Q
14 I K T * T * E S * - Q G T L * - * K D G G - - K * A * S V * D * * * V * * N * * A R * - - T * * D K N Y * I * F N - A R * Q
15 I K T * T * E S * - Q G T L * S * G A N * - - K * A * S V * D * * * V * * N * * A R * - - T * * D K N Y * I * F N - A R * Q
16 I K T * T * D S * - Q G T L * S * G A A * - - N * A * S V * D * * * V * * N * * A R * - - * * I D K N Y * I * F N - A R * Q
17 L T L * K * E - * * Q G K L * - * S G G * * - K * * * A V * * * * L V * * N * * A G * - - * * I D A N Y * V N L * - E R * Q
18 L T L * K * E - * * Q G K L * - * S G G * * - K * A * A V * * * * L V * * N * * A G * - - R * I D A N Y * I N L * - E R * Q
19 F T L * K * K - * * Q G K L * - * S G G * S - K * G * A V * * * * L V * * N * * A G * - - R * I D A N Y * I N F * - * R * Q
20 L T L * K * K - * * Q G K L * - * S G G * S - K * G * A V * * * * L V * * N * * A G * - - R * I D A N Y * I N F * - * R * Q
21 F K T * * * E - * * Q G T L * - * K G D * * - S * A * S V * D * * * L * * * * * A G * - - * * * N D N Y * I N L * - A R * Q
22 F K T * * * E - * * Q G T L * - * K N G * * - * * A * A V * D * * * L * * * * * A G * - - * * * N N N Y * I * L * - A R * Q
23 F K T * * * E - * * Q G T L * - * K G G * * - * * A * A V * D * * * L * * * * * A G * - - * * * N N N Y * I * L * - A R * Q
24 F K T * * * E C S * Q G T L * - * K G G * * - - * A * A V * D * * * L * * * * * G G * - - * * * D N N N Y I * L * - A R * Q
25 N D T * * * E - * N E G Q L * - * * D D N D - E * K * * * * * * * * T * V T * * A G * - - * * * D D L Y * I G M I - E - * H K
26 * S Q * * * K - L T K R T * I - * Y G A A G G T * - R I V * D * * * T * * L * * G Q S - - D * I E * * * * I * * A - R F * Q
27                                                      V
28 M I L * * * K - F H * G N * V - * G D * * - - * * K K K V * E * * * V * * * * * A A S K H S * * - K N L - W R Y P S S K * T G T A E K C
29 W T * * N * E - I Q * G G * D - * D E D P - - Q - K K E L * E * * * V * * * * * A N N R N T * * - S N Y - F G H S S S K * T G T - E Q C
30 * V * * T * S - E E * G * * V - * G G D D - - P * G T Q * * E * * K A * * * * * R D N I P S * D - N K Y - W L F P P * D * R Q E P E F C
31 * V * * T * S - E E * G * * I - * G G D D - - P * G T Q * * E * * K A * * * * * R D N I P S * D - N K Y - W L F P P * D * R E E P E P C
32 W B I * R * S - L K S G Y * * - * G K G T - - W * Z Z Z * * Z * B * V * * Z * L R R B L S T * K - B G Y - M F Y P D S R * R G P S E T C
33                                                                                                      Z
34 L D V * T * T - E E B G A * V - * G G B B - - X * X X X * * E * * K D * * * * * R D N I D T * D - N E Y - W W F P F * N * Q E S P E E C
```

Fig. 2. Comparison of amino acid sequences of phospholipases from various sources. Sequences compared are: *1,* pig (*Puijk* et al. 1977); *2,* horse (*Evenberg* et al. 1977b); *3,* ox (*Fleer* et al. 1978); *4,* iso-pig (*Puijk* et al. 1979); *5–7, Laticauda semifasciata,* fractions I, III and IV (*Nishida* et al., unpublished results); *8, Enhydrina schistosa* (*Lind* and *Eaker* 1980b); *9,* ibid, microheterogeneity; *10, Notechis scutatus,* notexin (*Halpert* and *Eaker* 1975); *11, N. scutatus,* fraction II-5 (*Halpert* and *Eaker* 1981); *12, N. scutatus,* fraction II-1 (*Lind* and *Eaker* 1980); *13, Hemachatus haemachatus* (*Joubert* 1975a); *14–16, Naja melanoleuca,* fractions DEI, DEII and DEIII (*Joubert* 1975b, c); *17–19, N. mossambica mossambica,* fractions CMI, CMII, and CMIII (*Joubert* 1977); *20, N. nigricollis,* basic (*Obidairo* et al., unpublished results); *21, N.n. oxiana* (*Ovchinnikov* et al. 1979); *22–23, N.n. kaouthia* (*Joubert* and *Taljaard* 1981); *24, N.n. atra* (*Tsai* et al. 1981); *25, Oxyuranus scutellatus,* taipoxin γ-chain (*Fohlman* et al. 1977); *26, Bungarus multicinctus, β-bungarotoxin,* B-chain (*Kondo* et al. 1978b); *27,* ibid, microheterogeneity; *28, Bitis caudalis* (*Viljoen,* unpublished results); *29, B. gabonica* (*Botes* and *Viljoen* 1974b); *30, Crotalus adamanteus,* fraction α (*Heinrikson* et al. 1977); *31, C. atrox* (*Randolph* et al. 1980); *32, C. durissus terrificus* (*Fraenkel-Conrat* et al. 1980); *33,* ibid microheterogeneity (sequences 32 and 33 probably represent the isoenzymes described by *Breithaupt* et al. 1975) and *34, Agkistrodon halys blomhoffii,* parts of the published sequence (*Samejima* et al. 1974) have been rearranged (see also *Randolph* et al. 1980). Gaps (-) have been introduced in order to get maximal alignments of half-cysteine and maximal homology. Residues identical to the corresponding residue in porcine pancreatic PLA are indicated with an *asterix.* The numbering has been based on horse pancreas PLA. Note that gaps introduced in the pancreatic model do not affect the numbering. Note also that the numbers used here do not necessarily correspond to the numbers used in the original publications. The IUPAC one-letter notation for amino acids (Eur J Biochem 5, 1968) 151–153 has been used

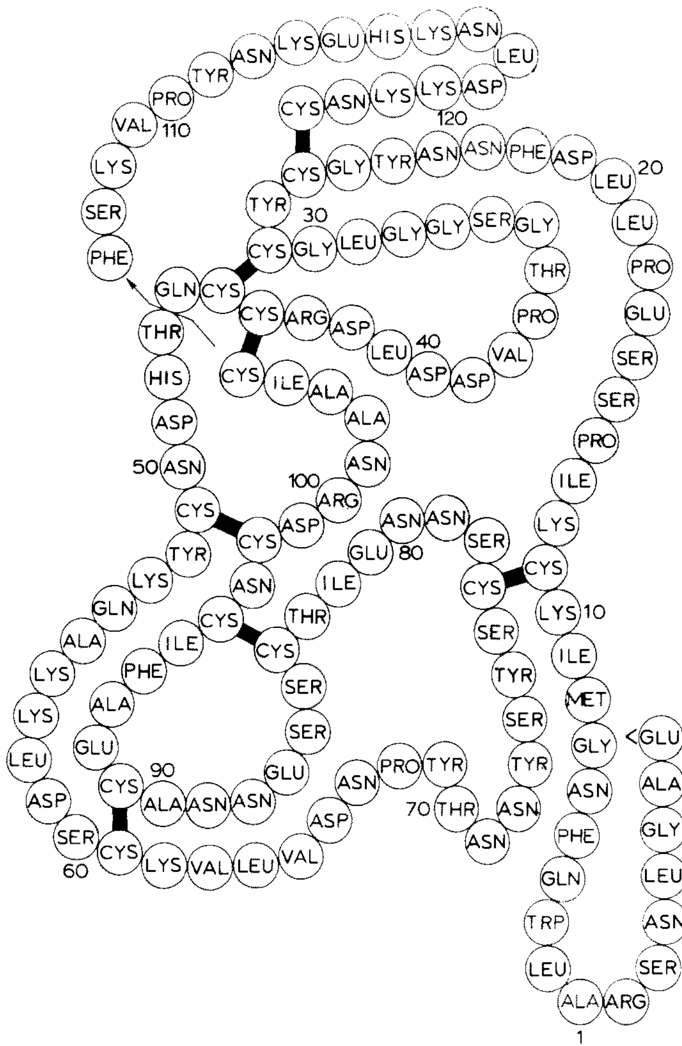

Fig. 3. Amino acid sequence of bovine pro-PLA and the connection of the disulfide bridges

pholipases. However, since then more sequences (or partial) sequences have appeared indicating that there are exceptions to this division: in the B-chain of β-bungarotoxin (*Kondo* et al. 1978b) and the phospholipase from *Micrurus microgalbineus*, both elapids, the bridge Cys^{11}-Cys^{77} is missing.

The high number of disulfide bridges contributes to the stability of the enzyme and their correct pairing must be a prerequisite for enzymatic activity. When the disulfide bridges are broken by reduction the activity is lost and, without special precautions, the activity is only partly or not at all recovered following reoxidation (*van Scharrenburg* et al. 1980).

Using porcine pancreatic PLA *van Scharrenburg* et al. (1980) showed that reduction led to a complete loss of activity. When the reoxidation was carried out in the absence of thiols only about 35% of the enzymatic activity was recovered. The authors assumed that the relatively low recovery was due to the formation of mismatched disulfide bridges. When the reoxidation was carried out in the presence of cysteine and in

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	PIG		0	28	19	4	68	68	67	71	65	65	58	60	69	68	68	64	66	66	66	59	59	60	63	65	70	83	91	85	83
2	HORSE		28	0	32	27	71	71	71	74	69	70	63	65	68	67	68	61	63	63	63	62	57	58	62	66	74	86	96	89	88
3	OX		19	32	0	17	72	73	73	73	71	71	62	59	68	67	67	61	63	63	63	56	58	58	61	65	72	84	92	84	83
4	Iso-pig		4	27	17	0	68	69	68	71	66	66	58	59	68	67	67	63	65	65	65	57	58	59	62	63	69	83	91	84	82
5	L. semif.	I	68	71	72	68	0	18	18	41	42	43	39	57	51	48	50	53	53	54	54	55	53	52	57	69	66	73	79	81	81
6	ibid.	III	68	71	73	69	18	0	3	34	36	38	40	60	54	52	53	54	54	55	54	56	54	53	59	70	65	72	81	82	81
7	ibid.	IV	67	71	73	68	18	3	0	33	35	37	40	60	54	52	53	54	54	55	54	56	54	53	59	68	65	71	82	82	81
8	E. schist.		71	74	73	71	41	34	33	0	26	26	46	62	56	54	56	58	57	58	58	54	52	54	59	72	66	74	83	80	88
9	Notexin		65	69	71	66	42	36	35	26	0	7	41	60	55	56	58	56	56	54	54	55	52	52	58	67	66	79	86	85	84
10	N. scut.	II-5	65	70	71	66	43	38	37	26	7	0	42	58	57	57	59	57	57	56	56	57	54	54	59	68	65	78	86	83	82
11	ibid.	II-1	58	63	62	58	39	40	40	46	41	42	0	58	56	56	57	57	57	55	55	53	51	51	56	62	66	74	85	86	85
12	H. haem.		60	65	59	59	57	60	60	62	60	58	58	0	33	32	32	37	35	37	38	25	24	22	22	60	62	74	84	87	88
13	N. mel.	I	69	68	68	68	51	54	54	56	55	57	56	33	0	10	13	32	30	32	32	27	25	25	29	63	68	74	82	86	87
14	ibid.	II	68	67	67	67	48	52	52	54	56	57	56	32	10	0	6	34	32	35	35	25	24	24	27	62	66	73	82	85	86
15	ibid.	III	68	68	67	67	50	53	53	56	58	59	57	32	13	6	0	33	32	34	34	26	26	26	29	64	64	74	85	85	86
16	N.m mos.	I	64	61	61	63	53	54	54	58	56	57	57	37	32	34	33	0	3	13	12	31	29	26	30	62	67	74	82	85	86
17	ibid.	II	66	63	63	65	53	54	54	57	56	57	57	35	30	32	32	3	0	11	10	30	28	25	29	62	66	74	82	85	86
18	ibid.	III	66	63	63	65	54	55	55	58	54	56	55	37	32	35	34	13	11	0	1	32	32	29	33	64	66	72	81	81	82
19	N. nigri.		66	63	63	65	54	54	54	58	54	56	55	38	32	35	34	12	10	1	0	33	33	30	34	64	66	72	81	81	82
20	N.n. oxian.		59	62	56	57	55	56	56	54	55	57	53	25	27	25	26	31	30	32	33	0	14	12	16	55	65	73	80	84	85
21	N.n.kaouth.	I	59	57	58	58	53	54	54	52	52	54	51	24	25	24	26	29	28	32	33	14	0	4	11	57	62	72	81	85	86
22	ibid.	III	60	58	58	59	52	53	53	54	52	54	51	22	25	24	26	26	25	29	30	12	4	0	9	57	61	72	82	84	85
23	N.n. atra		63	62	61	62	57	59	59	59	58	59	56	22	29	27	29	30	29	33	34	16	11	9	0	61	62	75	84	86	87
24	Taip.		65	66	65	63	69	70	68	72	67	68	62	60	63	62	64	62	62	64	64	55	57	57	61	0	75	82	86	90	89
25	β-bung.		70	74	72	69	66	65	65	66	66	65	66	62	68	66	64	67	66	66	66	65	62	61	62	75	0	76	87	82	81
26	B. Caud.		83	86	84	83	73	72	71	74	79	78	74	74	74	73	74	74	74	72	72	73	72	72	75	82	76	0	50	61	61
27	B. Gabon		91	96	92	91	79	81	82	83	86	86	85	84	82	82	85	82	82	81	81	80	81	82	84	86	87	50	0	65	64
28	C. Adam.		85	89	84	84	81	82	82	80	85	83	86	87	86	85	85	85	85	81	81	84	85	84	86	90	82	61	65	0	6
29	C. Atrox		83	88	83	82	81	81	81	80	84	82	85	88	87	86	86	86	86	82	82	85	86	85	87	89	81	61	64	6	0

Fig. 4. Sequence difference matrix for phospholipases from various sources. Sequences were aligned as shown in Fig. 2 and the comparison is based on a total number of residues (including deletions) of 138. The values shown are the number of positions (including deletions) where a change has occurred; a value of 69 in the figure therefore indicates a 50% homology. For the full names of the phospholipase sources see Fig. 2

the presence of 0.9-*M* guanidine chloride to increase the solubility of the reduced protein, 90%–95% of the enzymatic activity could be recovered. After purification this enzyme was indistinguishable from the native enzyme.

Figure 4 shows a sequence difference matrix constructed from the sequence alignment as shown in Fig. 2 for 29 PLAs. Included are four pancreatic, four hydrophid, 17 elapid (four Australian, five Asian and eight African), and four viper sequences. The average sequence difference of about 50% shows the strong homology among all PLAs. The homology between hydrophidae and the Australian *Notechis* enzymes is much greater than that between hydrophid and the other elapids. *Hemachatus haemachatus* is phylogenically distinct from the African cobras (*Naja* species). This is confirmed by the sequence of its PLA. In fact this structure is closer to that of the Asian than to that of the African cobra. Both rattlesnake PLAs (*C. adamanteus* and *C. atrox*) show a strong homology as could be expected for such related snakes. In contrast, both *viperidae* sequences are very different from each other, a fact that casts serious doubt on the correct classification of both snakes in the genus *Bitis.*

From Fig. 4 one might conclude that cobras are more closely related to mammals than to vipers, even if one does not take into regard the C terminal appendage which is distinctive for viper PLAs. Apparently the elapid venom and pancreatic PLA have undergone only a limited parallel divergent evolution from the ancestral enzyme within the same framework of disulfide bridges and number of peptide loops between the bridges. The sequence changes in viper venom PLA resulting in the introduction of the C terminal appendage and the loss of disulfide bridge Cys^{11}-Cys^{77} apparently has opened the way to other more pronounced sequence changes.

When all sequences are compared it appears that 32 amino acids are absolutely conserved. In addition 29 residues are usually substituted by residues with similar properties with respect to size, charge, or hydrophobicity. When only pancreatic and elapid phospholipases are compared these numbers are as high as 36 and 45, respectively. The residues which are absolutely conserved are so because of two major reasons: either they are catalytic residues (His-48, Asp-99), residues involved in binding of the cofactor Ca^{2+} (Asp-49), or they have an important structural function (e.g., all half-cysteines, five glycine residues).

Since it is known that upon binding of substrate (either monomers or aggregated substrate) hydrophobic interactions are involved, it is of interest to analyze the residues which surround the active site of bovine pancreatic phospholipase. Inspection of the X-ray model shows the astonishing fact that several hydrophobic side chains surrounding the active site are not buried but point toward the surrounding water. This creates

a large surface area with hydrophobic properties suitable for interactions with lipids. These surface residues are: Leu-2, Trp-3, Leu-19, Leu-20, Leu-31, Lys-56, Leu-58 (Val-Leu-Val-65), Tyr-69, and Thr-70. Figure 2 shows that in all phospholipases these side chains are highly variable (as could be expected for exposed residues), but mainly hydrophobic residues are present. Among the side chains carrying a charge only a single negatively charged side chain is found, although several arginine and lysine residues are present. This might suggest that interactions with lipid-water interfaces not only require a large hydrophobic surface area but also that a positive charge on the protein may add favorably to this interaction. Two regions rich in lysine may be important for binding. In bovine pancreatic PLA the lysine residues 53, 56, 57, and 62 form a cluster that might be important for binding (*Dijkstra* et al., 1981a). Also the C terminal part of the sequence (residues 116–121) may be important. Especially in venom PLAs this part contains a cluster of hydrophobic side chains (see Fig. 2). Since more than ten residues contribute to the hydrophobicity of the protein surface one might expect that substitution (or chemical modification) of only one of these side chains will not drastically alter the interaction with lipid-water interfaces per se.

Only a few insertions and deletions are needed to achieve maximal homology except for residues 55–68. This part of the sequence is present as two long external loops around disulfide bridge Cys^{61}-Cys^{91} in bovine PLA. Deletion would shorten these loops but would not affect the gross shape of the whole molecule. For this reason a tryptophan found in most elapid phospholipases is placed in the middle of two deletions; it is supposed to replace the bovine sequence Val-63-Leu-Val-65.

The B chain of β-bungarotoxin occurs as a covalent complex with the A chain. The complex does not contain free sulfhydryl groups. Since 12 half-cysteines are present at conserved sites, the A chain might very well be attached via the unique cysteine at position 15.

Two proteins are reported to be devoid of phospholipase activity: *Notechis* II-1 and taipoxin γ-chain. The former, which binds Ca^{2+} and does react with active site irreversible inhibitors, has a normal elapid phospholipase structure except for the substitution of Ser for the otherwise invariant Gly-30 (*Lind* and *Eaker* 1980). Since this part of the main chain participates in Ca^{2+} binding one might suppose that although the enzyme binds Ca^{2+} ions the Ca is not bound at the proper position. This situation might then resemble the enzyme-barium complex which is very similar to the enzyme-calcium complex but lacks enzymatic activity (*Verheij* et al. 1980a).

The taipoxin γ-chain has several salient structural features different from other phospholipases:

1. At the N terminus it contains eight additional residues as do the zymogens of the pancreatic phospholipases.
2. If the cysteines present at positions 15 and 19 form a disulfide bridge, a short extra loop is present near the entrance of the active site.
3. It is the only sequence with Pro-31 in a part of the sequence important for Ca binding.
4. There is no deletion between residues 55 and 68.
5. A polysaccharide is attached to Asn-70 which is located at the entrance of the active site.

The precursors of the pancreatic enzymes, which are devoid of activity on micellar substrates but efficiently hydrolyze monomeric substrates, differ from the active enzymes only by the presence of a polar activation peptide at the N terminus. Activation peptides containing three, five, or seven residues have been reported (*Nieuwenhuizen* et al. 1973a; *Dutilh* et al. 1975; *Evenberg* et al. 1977a), all containing an invariant arginine residue at the C terminal end.

In 1972 it had already been suggested that the α-amino group of PLA forms an internal salt bridge, thereby stabilizing the active site geometry (*Abita* et al. 1972). This hypothesis has been supported by the high (8.3–8.9) pK values of this group (*Jansen* 1979; *Janssen* et al. 1972). Also the finding that replacement of Ala-1 by other amino acids can have drastic effects (see Sect. 6) stresses the importance of this bridge. Finally, the refined X-ray structure of bovine PLA shows that Ala-1 is indeed buried in the interior of the enzyme. The α-amino group is linked via a water molecule to the side chain of Asp-99; moreover, the α-ammonium group is hydrogen bonded to the side chain of Gln-4 and to the main chain carbonyl carbon of Asn-71 (see also Sect. 9).

Despite a remarkable sequence homology of the enzymes isolated from pancreatic tissue and from the venoms of all classes of venomous snakes their behavior in solution is quite different. Whereas the enzymes from *C. adamanteus* and *C. atrox* only occur as dimers even at concentrations as low as 50 μg/ml (*Wells* and *Hanahan* 1969; *Hachimori* et al. 1971), the enzyme from porcine pancreas exists as monomer even at concentrations of 5 mg/ml (*de Araujo* et al. 1979). Several other phospholipases show a concentration-dependent association, generally in the concentration range between 0.05 and 0.5 mg/ml. This equilibrium is shifted to the monomeric form at low pH, whereas calcium ions display a more complex behavior, showing either no influence on the monomer-dimer equilibrium or shifting it toward the monomeric or to the dimeric form (*Shiloah* et al. 1973; *Roberts* et al. 1977a; *Joubert* and *van der Walt* 1975; *Yang* and *King* 1980b). *Mal'tsev* et al. (1979) showed that Ca^{2+} ions alter the association-dissociation rate constants of the monomer-dimer

equilibrium of *Naja n. oxiana* PLA, but the equilibrium constant was hardly affected.

Based on the concentration-dependent dimerization one might expect molecular weights between about 14 000 and about 28 000. However, a much broader range is reported in the literature with values ranging from 10 000 to 38 000. The higher molecular weight may reflect association beyond a dimer. The lower values may be due to systematic errors caused by hydrophobic interactions with the gel matrices or to abnormal SDS binding properties caused by the relatively large proportion of hydrophobic residues (*de Jong* et al. 1978). In any case, since no sequenced "true" phospholipase has a molecular weight outside the range of 13 000–14 000, characterization of phospholipases by only molecular weight determination must be considered of limited value.

Since all extracellular PLAs are calcium dependent, it is not surprising that those phospholipases that were tested are able to bind calcium ions. In general the observed dissociation constants fall in the range of 0.1–1 m*M* at pH 7–8. For a limited number of enzymes detailed studies pertaining to spectral and conformational changes as well as to amino acid side chains involved in the binding have been published (see Sect. 7).

5 Kinetic Analyses

5.1 Introduction

The kinetic behavior of a large number of water-soluble enzymes acting on molecularly dispersed substrates (including esterases) has been analyzed in detail. Usually these enzymes display classical Michaelis-Menten kinetics and important information has been obtained on the mechanism of action of these proteins.

Phospholipase A_2 (EC 3.1.1.4.) belongs to a special group of esterases, the lipolytic enzymes, the specific activity of which strongly depends on the state of aggregation of the substrate. The rate of hydrolysis of phospholipids increases by several orders of magnitude on passing from monomolecularly dispersed to micellar solutions.

The analysis of the kinetic properties of this enzyme acting on monomolecularly dispersed substrates has provided a theory about the mechanism of catalysis (cf. Sect. 10). Attempts to reveal kinetic pathways for these enzymes acting upon their biologically relevant aggregated substrates have not met with success so far, notwithstanding extensive efforts. Up till now no general agreement even exists on the model of lipolysis from which the kinetic equations have to be derived.

As has been discussed in recent review papers (*Brockerhoff* and *Jensen* 1974; *Verger* and *de Haas* 1976; *Sémériva* and *Desnuelle* 1978; *Verger* 1980), the main difficulty in understanding lipolysis is our lack of information concerning the mechanisms leading to the observed enhanced rates as induced by certain organized lipid-water interfaces. Although it is evident that the physicochemical properties of the aggregated phospholipid systems play a predominant role in lipolysis, the effects of important factors such as steric environment and hydration of polar headgroups, chain packing density and surface defects, surface charge, and -pH still are poorly understood. This results in the use of rather vague terms as "quality of interface", "supersubstrate", etc.

Three speculative hypotheses have been suggested to explain the burst in enzyme activity upon substrate aggregation.

1. "Enzyme theory", which assumes a conformational change in the adsorbed enzyme controlled by the microenvironment of the lipid-water interface and resulting in an optimization of the active site.
2. "Substrate theory", which assumes a much higher susceptibility of substrate molecules toward the enzyme in the lipid-water interface.
3. "Product theory", which assumes that the rate limiting step of product release, being very slow in water, markedly increases in the hydrophobic lipid-water interface.

The in vivo function of PLAs is a controlled degradation of aggregated long-chain phospholipids, and our final aim should be the elucidation of the mechanism of action under these conditions. Based on the above mentioned difficulties, we will try, however, to evaluate kinetic data obtained with other systems as well in the following order: (1) Monomeric substrates, (2) micellar substrates (micelles of short-chain lecithins and mixed micelles of phospholipids with detergents), (3) monomolecular surface films of medium-chain phospholipids, and (4) phospholipids present in bilayer structures.

5.2 Monomeric Substrates

As early as 1961, *Roholt* and *Schlamowitz* in a remarkable study investigated the kinetics of crude PLA from *Crotalus durissus terrificus* on molecularly dispersed dihexanoyl lecithin. The enzyme was found to act optimally at pH 8, and Ba^{2+} ions were shown to inhibit the hydrolysis by competition with the essential cofactor Ca^{2+} for binding to the protein. The highly water-soluble reaction products, hexanoic acid and 1-hexanoyl-lysolecithin [4], did

4 Most probably this lysolecithin would have inhibited the enzyme at higher concentrations. Cf. *Wells* (1972)

not appear to influence the reaction rate. On the other hand a number of monoalkyl long-chain surfactants such as egg lysolecithin, sodiumdodecylsulphate or Tween, strongly influenced the hydrolysis rate, and it is now evident that these effects have to be attributed to the incorporation of the substrate in the detergent micelle (see Sect. 5.3).

The first very detailed kinetic analysis of a highly purified PLA from *Crotalus adamanteus*, using as substrate monomeric 1,2 dibutyryl lecithin, was reported in 1972 by *Wells*. The pH activity profile of this enzyme (optimum pH 8–8.5) is in agreement with the results of *Roholt* and *Schlamowitz* (1961), and under no circumstances was it possible to find any cation which could replace Ca^{2+} in the enzymatic reaction. The pH dependence of the reaction suggests that a group with pK 7.6 is involved in the catalytic step as well as in Ca^{2+} binding (see *Wells* 1974b). Besides the important consequences of these studies for our understanding of the mechanism of catalysis of PLA, the author clearly demonstrated that his results are consistent with an ordered addition of ligands to the venom enzyme. Ca^{2+} adds first, followed by monomeric substrate. In addition the kinetic results point to an ordered release of products where fatty acid is released first from the enzyme, followed by the lysolecithin. It has to be remarked that the *Crotalus adamanteus* PLA has an exceptionally strong tendency to form dimeric enzyme complexes in aqueous solution. Very recently *Smith* and *Wells* (1981) demonstrated by "active enzyme ultracentrifugation" that it is the dimeric form of the enzyme which catalyzes the hydrolysis of monomeric substrate.

Using a series of homologous short-chain diacyl lecithins varying in chain length between C_2 and C_5, *Zhelkovskii* et al. (1978a) also showed that a homogeneous preparation of PLA from the cobra *Naja naja oxiana* is able to hydrolyze these short-chain lecithins at concentrations far below their CMC. Although the individual kinetic constants k_{cat} and K_m could not be derived because the Michaelis constants are considerably higher than the CMC values, it is evident that the efficiency of the catalytic transformation of the substrate strongly depends on chain length of the hydrocarbon moiety of the substrate. From the results obtained it follows that the PLA molecule must possess an apolar region and most probably both acyl chains participate in the hydrophobic interaction between substrate and enzyme.

Viljoen and *Botes* (1979) investigated the kinetic properties of pure PLA from *Bitis gabonica* on monomeric dihexanoyl lecithin as a function of pH. The authors confirmed the results of *Wells* (1972) that these enzymes follow a kinetic mechanism of the ordered bi-ter type and found a k_{cat}/pH dependence controlled by a group active in catalysis with a pK of 6.8 which probably is a histidine residue. It is not clear why the authors used 0.5 m*M* lipid as highest substrate concentration, taking

into account the CMC of dihexanoyl-lecithin which is about 10 m*M*. Although the value of k_{cat}/K_m can be determined in this way, the absolute values of k_{cat} and K_m could have been estimated with more accuracy by using higher substrate concentrations. The enzyme-Ca^{2+} dissociation constant was found to be pH dependent and controlled by a group with a pK of 6.0–6.4 which was assigned a carboxylate function. This assignment is based, however, on disputable experimental evidence. In addition they reported that the Michaelis constant K_b is pH independent in the range 5.5–9.0 which could be in agreement with a predominantly hydrophobic interaction between enzyme and substrate. The comparison made by the authors between their present results (obtained with molecularly dispersed dihexanoyl lecithin) and those reported previously by them (obtained with dihexadecanoyl lecithin) should be re-evaluated (see Sect. 5.5).

Although the highly purified pancreatic (pro)PLAs are also known to be able to hydrolyze molecularly dispersed short-chain lecithins (*de Haas* et al. 1971; *Pieterson* et al. 1974b), technical difficulties connected with the use of the titrimetric assay (see also *Wells* 1972) have prevented so far more extensive kinetic analyses. Using specific chromogenic short-chain lecithins containing thioester bonds, *Volwerk* et al. (1979) reported kinetic data of porcine pancreatic PLA in the monomeric substrate region. In contrast to the venom enzymes, the initial velocity patterns of the pancreatic phospholipase are consistent with random addition of substrate and Ca^{2+} to the protein. Enzyme-monomer substrate binding in the absence of Ca^{2+} was confirmed by direct binding studies. This binding is most probably not aspecific as shown by *Volwerk* et al. (1974): an increasing protective effect against irreversible active-site His-48 modification was observed for a series of monomeric substrates and lysolecithins. This technique allowed the quantitative determination of dissociation constants of monomeric phospholipids. The increase in ΔG (~ 600 cal per extra methylene group) upon binding to the active center of the enzyme is in agreement with predominant hydrophobic binding. Although both studies were hampered by unfavorable K_m-CMC ratios, hydrophobic interaction again seems to be the main driving force for binding of monomeric substrates to the enzyme. The V_{max}-pH profiles show that the activity of the pancreatic enzyme is controlled by a group of approximately pK 5.5, tentatively assigned to His-48.

In summary, although most of our present knowledge of the mechanisms of catalysis of PLA has been obtained by analyses of the kinetics of hydrolysis of monomeric substrates, it must be emphasized that these studies are often seriously hampered because of comparable values of K_m and CMC. In such cases the monomeric substrate region is so limited that Michaelis-Menten plots of velocity as function of substrate concentration

do not allow the separate determination of k_{cat} and K_m, and only their ratio can be determined. Of course in these situations potential inhibitors which increase the apparent K_m value cannot be investigated. Despite extensive synthetic efforts in the authors' laboratory involving chemical changes both in the polar head group and in the alkyl chain, hardly any success was obtained in attempts to raise the CMC of the substrate while at the same time keeping the K_m low. Most probably the hydrophobic binding forces which control both micelle stability and enzyme-monomer attraction are very similar and the K_m-CMC ratio hardly changes.

5.3 Micellar Substrates

5.3.1 Micelles of Short-Chain Lecithins

The above-mentioned difficulties in obtaining detailed kinetic data on PLA with monomeric substrates combined with the fact that lipolytic enzymes in vivo act on aggregated phospholipids led various investigators to examine the kinetics of PLA acting on micellar short-chain lecithins. *De Haas* et al. (1971) studied the action of porcine pancreatic PLA on a series of short-chain diacyl lecithins varying in acyl chain length from C_6 to C_9. Large increases in reaction rates were observed upon passing the CMC, and in the micellar region seemingly normal Michaelis curves were obtained describing the progressive adsorption of the enzyme at the surface of the micelles. Notwithstanding their slight differences in chemical structure, the various lecithins are degraded with very different rates, indicating the importance of the "quality" of the lipid-water interface for hydrolysis.

Initial rate measurements were interpreted to be consistent with a random addition of Ca^{2+} and substrate to the enzyme which is in agreement with the results obtained for this enzyme in the monomeric substrate region (*Volwerk* et al. 1979). These results would support the existence of separate and independent binding sites for substrate and metal activator on the enzyme, although *Pieterson* et al. (1974a) in direct binding studies reported a synergistic effect for Ca^{2+} and substrate binding between pH 5 and 8. The porcine pancreatic enzyme works optimally at a pH of about 6, but such values obtained with aggregated substrates have to be considered as apparent and are essentially uninterpretable [cf. also *Wells* (1974a) and *Kensil* and *Dennis* (1979)].

A dramatic activation of the enzyme was found at high salt concentrations. No clear-cut explanation was provided, but the concomitant decrease of the apparent K_m supports the idea that micellar binding to this enzyme also involves mainly hydrophobic forces.

Detailed kinetic analyses of PLA from *Crotalus adamanteus* acting on dibutyryl, dihexanoyl, and dioctanoyl lecithin both below and above the CMC were reported by *Wells* (1974a). Also for the venom enzyme a dramatic increase in catalytic efficiency was observed when the substrate concentration exceeded the CMC. In contrast to the pancreatic enzyme, this venom PLA requires an ordered addition of Ca^{2+} and substrate both in micellar and monomeric form. No activation of the venom enzyme was observed in the presence of high salt concentrations. Although the V_{max} of the phospholipase acting on *monomeric* dibutyryl lecithin is some 3000 times lower than the V_{max} measured on dioctanoyl lecithin *micelles,* dibutyryl PC concentrations near the K_m of this substrate (~ 40 m*M*) were found to competitively inhibit the enzyme action on micellar dioctanoyl PC. This result was interpreted as a support for a mechanism of PLA in which the enzyme after each single encounter with the micellar interface and a catalytic cycle returns to the aqueous phase.

This argument, however, is valid only if diC_4-PC is not present in the diC_8-PC micelle. If part of the diC_4 PC is incorporated into mixed micelles together with diC_8-PC, the quality of the lipid-water interface will change and inhibition is to be expected. The observation that no hydrolysis of diC_4-PC occurs cannot be adduced as evidence that diC_4-PC does not partition between solvent and diC_8-PC micelles. Even if present in the micelle, the diC_4-PC monomer will hardly be able to compete for the monomer binding site on the enzyme with the monomeric diC_8-PC molecule. Compare the monomer-E dissociation constants:

1. K_m diC_4-PC ~ 40 m*M*
2. K_m diC_6-PC ~ 4 m*M*
3. K_m diC_8-PC ~ 0.4 m*M*

Indeed, such a "single encounter mechanism" in which the enzyme "hops" up and down between bulk and micelle surface would not be fundamentally different from its interaction with monomeric substrate. The large rate enhancements attendent upon substrate aggregation were tentatively explained by assuming (1) marked increase in the rate of product release [5], (2) a much lower entropy of activation, or (3) conformational constraints placed on the glycerophosphoryl-choline moiety of the substrate in the aggregated state.

In an attempt to improve our understanding of the large rate enhancement observed with PLA when the substrate concentration exceeds the CMC, *Pieterson* et al. (1974b) compared the kinetic data of the "active" pancreatic enzyme with that of its natural zymogen using short-chain substrates below and above the CMC. Both proteins catalyze the hydro-

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5 This means that the interface can affect the apparent kinetic mechanism of PLA

lysis of short-chain monomeric 3-*sn*-phosphatidyl cholines with a similar albeit low efficiency, indicating the pre-existence of the catalytic site in the zymogen [see also *Volwerk* et al. (1979)]. Direct binding studies involving Ca^{2+} and monomeric substrate analogs and irreversible inactivation characteristics also point to a very similar architecture of the active center in PLA and its zymogen (*Volwerk* et al. 1974). The aggregated (micellar) form of the lecithins is hydrolyzed effectively only by PLA and not by the zymogen. Apparently only the active form of the pancreatic enzyme recognizes certain organized lipid-water interfaces and hydrolyzes such substrates in a very efficient way. These results together with a previous monolayer study (*Verger* et al. 1973; see also Sect. 5.4) led to the hypothesis that "active" PLA, in contrast to its zymogen, contains a hydrophobic surface region, the interface recognition site (IRS), through which the enzyme binds[6] to the lipid-water interface. Direct binding studies involving both active PLA and its zymogen with micellar substrates and analogs confirmed that only the "active" enzyme interacts with interfaces (*Pieterson* et al. 1974b). The fact that irreversible modification of the active center in PLA does not impede the binding of the protein to interfaces (*Volwerk* et al. 1974) suggests a functional and topographic separation of IRS and the active center. Nuclear magnetic relaxation studies by *Hershberg* et al. (1976b) are in agreement with such topologically distinct sites. A similar conclusion was reached by *Roberts* et al. (1977c) for the *Naja naja* PLA. As shown in Fig. 5, two successive equilibria are supposed to exist, first a rate-limiting, reversible

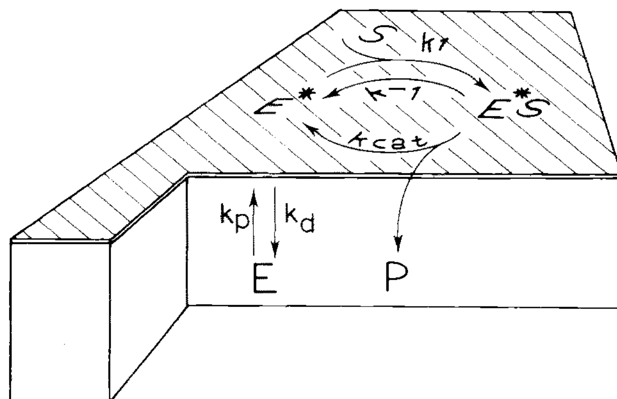

Fig. 5. Proposed model for the action of PLA *(E)* at an interface (*Verger* et al. (1973). A similar model has been proposed by *Vidal* et al. (1978) to explain the activation kinetics of liver 3-D-(−)-hydroxybutyrate apodehydrogenase by phosphocholine containing lipids. For description of the model see text

6 A comparable "hydrophobic head" or "interfacial affinity region" in lipolytic enzymes has been independently postulated by *Brockerhoff* (1973). Because the mode of interaction of the enzyme with the interface is still under discussion, "binding" is used in a rather loose sense and stands for different forms of interaction such as "adsorption", penetration", "anchoring", etc.

penetration [7] of the enzyme into the interface ($E \rightleftharpoons E^*$), followed by the formation of a "two-dimensional Michaelis complex" ($E^* + S \rightleftharpoons E^*S$). The dramatic rate enhancement observed for PLA from various sources when the substrate concentration exceeds the CMC and lipid-water interfaces are formed has been attributed to a conformational change in the bound protein (E^*) resulting in an optimal alignment of the active site amino acid residues.

This model could also explain why irreversible active-site inhibition of PLA by *p*-bromophenacyl bromide is stimulated in the presence of certain micellar interfaces (*Volwerk* et al. 1974). Although the apolar reagent is incorporated in various forms of lipid aggregates, such as micelles and lamellar structures, only those interfaces which allow binding of PLA to the interface gave rise to increased inhibition.

In a very interesting study *Allgyer* and *Wells* (1979) reanalyzed the hydrolysis kinetics of *Crotalus adamanteus* PLA acting on monomeric and micellar diC_6-, diC_7-, and diC_8-PC. The abnormal parabolic velocity dependence on substrate concentration near the CMC was tentatively explained by a thermodynamic model for micelle formation in which two species of micelles exist. In this formulation the first micelle is formed at lecithin concentrations near the CMC and the second micelle arises from the first at higher concentrations of lecithin (*Hershberg* et al. 1976a). A satisfactory fit to the kinetic data was achieved, assuming that the second micelle is the form of substrate responsible for the large rate enhancement observed above the CMC. In agreement with an early hypothesis of *Brockerhoff* (1968) and with recent ^{13}C-NMR results of *Schmidt* et al. (1977) the authors suggest that dehydration of the carbonyl groups in micelle II might be the main reason for the enhanced activity of PLA. The enzyme's extreme sensitivity for small changes in lipid hydration was noted earlier by *Wells* and colleagues (*Wells* 1974c; *Misiorowski* and *Wells* 1974; *Poon* and *Wells* 1974).

In summary, from the foregoing it is clear that PLAs from different sources display dramatic rate enhancements when their substrates pass from the monomeric into the micellar form. Both for the *Crotalus* PLA and the pancreatic enzyme it has been demonstrated that substrate molecules at concentrations below their CMC are hydrolyzed much more rapidly after incorporation into mixed micelles, even with nonsubstrates or with competitive inhibitors. No agreement, however, exists on the origin of this interfacial activation.

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7 Penetration is used because of the multiple indications that at least for the pancreatic enzyme hydrophobic interactions play a major role in the binding process (*Verger* et al. 1973; *Verheij* et al. 1981). Most probably an insertion of apolar amino acid side chains in the hydrophobic lipid core is preceded by a more loose adsorption process

Wells (1972, 1974a, 1978) prefers the "substrate" hypothesis: it is the lipid-water interface which confers a preferred conformation[8] on the substrate molecule which would allow for a higher fraction of productive single encounters with the enzyme. On the other hand the investigators working with the pancreatic enzymes favor the "enzyme" theory in which PLA reversibly "binds" to the lipid-water interface, followed by a conformational change in the protein with increased catalytic activity. Although it could be argued that PLAs from various sources might follow different pathways, the high structural resemblance of these enzymes makes such an idea unattractive. In the reviewers' opinion the "enzyme" theory does not exclude the "substrate" hypothesis: both could be acting together and result in the large rate enhancement observed. However, the assumption that the enzyme necessarily leaves the interface after each catalytic cycle is based on disputable arguments, and it is not clear why such a mechanism would lead to accelerated catalysis.

5.3.2 Mixed Micelles of Phospholipids with Detergents

Detergent solutions with a low CMC solubilize phospholipids by incorporation into mixed micelles. Such systems are attractive for kinetic investigations of lipolytic enzymes because, at least at the first glance, they combine all the advantages of isotropy of micellar solutions with the possibility of investigating long-chain natural phospholipids by classical pH stat assay techniques. In a series of papers *Dennis* (1973a,b; 1974a,b), *Deems* and *Dennis* (1975), and *Roberts* et al. (1978b) extensively analyzed the kinetic behavior of PLA from *Naja naja naja* acting on lecithins (varying in chain length from C_6 to C_{16}) solubilized in the nonionic detergent Triton X-100. Although this detergent is somewhat polydisperse, its neutral character constitutes a distinct advantage over charged amphiphiles such as bile salts, CTAB, SDS, etc. in kinetic studies of phospholipases which are dependent on metal cofactors. Biologically relevant phospholipids, such as the long-chain lecithins DMPC and DPPC, form bilayer structures in water (liposomes, vesicles) interfaces which are hardly attacked by most PLAs (compare Sect. 5.5). Addition of increasing amounts of Triton gradually transforms these lamellar structures into mixed micells, and at a molar ratio of Triton to lecithin of about 2:1, isotropic solutions are obtained which are optimally susceptible to the action of the cobra enzyme[9].

8 Support for a change in monomer PL conformation/orientation occurring as the molecules become packed in an interface was obtained in ^{1}H and ^{13}C-NMR studies of *Roberts* and colleagues (*Roberts* et al. 1978a; *Burns* and *Roberts* 1980)

9 The authors demonstrated (*Robson* and *Dennis* 1979; *Dennis* 1974b) that this formation of mixed micelles takes place only above the thermotropic phase transition temperature of the phospholipid. Formation of mixed micelles at temperatures below the transition temperature requires much higher ratios of Triton to phospholipid

Higher mol fractions of the detergent gave rise to increasing "inhibition" of the phospholipase, a kinetic effect which has been ascribed to "surface dilution" of the substrate. To explain the observed "surface dilution" kinetics, *Deems* et al. (1975) used a model of lipolysis comparable to the one shown above in Fig. 5. By changing the lecithin concentration in the interface of the mixed micelle with Triton, they calculated approximate values of K_S^A (= k_p/k_d in Fig. 5), the dissociation constant for the enzyme-mixed micelle complex, and K_M^B (= K_M^* in Fig. 5), the two-dimensional Michaelis constant for the catalytic step. Credit should be given to the authors for the originality of the idea to quantitatively separate the affinity constant of the enzyme for the interface and the binding to the substrate in the interface. Unfortunately, the numerical values reported have to be considered as rather rough estimates, taking into account the simplifying assumptions which were required to apply the kinetic equations. As has been extensively discussed before (*Verger* and *de Haas* 1976), changes in the molar ratio of Triton to phospholipid might induce differences in the quality of the lipid-water interface and thereby influence K_S^A. Such changes have been detected in fact by the authors (*Dennis* 1974a; *Roberts* et al. 1979). On the other hand reliable estimates of K_M^B are even more difficult to obtain, Under "saturating" conditions when all enzyme molecules were bound to the mixed micellar surface, the authors showed that the velocity remained linearly proportional with the amount of lecithin in the interface of the mixed micelle up to a mol fraction of 0.33 (*Dennis* 1973b; *Deems* et al. 1975). This implies that the two-dimensional lecithin concentration is far below K_M^B, and even rough estimates of its absolute value become impossible.

In a similar attempt to separate K_M^* from k_p/k_d (Fig. 5) and to obtain a numerical value for the two-dimensional Michaelis constant, *Slotboom* et al. (1976) used two enantiomeric 2-*sn*-lecithins containing fatty acids of different chain length in positions 1 and 3. By incorporating mixtures of both β-lecithins into Triton micelles, keeping *total* phospholipid concentrations and *total* amount of Triton constant, the enzyme activity could be followed as a function of the mol fraction of each of the β-lecithins. Because of the identical physicochemical properties of enantiomers, the quality of the interface remains constant. Although this technique clearly showed that the K_M^* values for stereoisomers are not identical, a quantitative relationship can be obtained only under interfacial saturation conditions (all E in form E*). Pancreatic PLA has a very low affinity for pure Triton micelles, as was found also for the Cobra enzyme (*Roberts* et al. 1977c), and therefore the distribution of enzyme over bulk interface (E ⇋ E*) will strongly depend on the total amount of β-lecithin incorporated into the mixed micelles. This implies for this detergent that interfacial saturation is difficult to reach. Using

n-alkylphosphorylcholine as a carrier micelle for which the enzyme has a high affinity, k_{cat} and K_M^* values could be obtained for both stereoisomers. It must be pointed out, however, that also in this case a simplifying assumption had to be made because the molecules of the carrier matrix are competitive inhibitors of the enzyme. In addition in this study one might also wonder whether the quality of the lipid-water interface remained rigorously constant upon incorporation of increasing amounts of β-lecithin.

Roberts et al. (1977c) proposed a new model for the interaction between *Naja naja* PLA and mixed micelles of Triton and phospholipid: two phospholipid molecules should be required, one to sequester the enzyme to the interface and the other for subsequent catalysis. Based on cross-linking experiments of the enzyme in the presence of excess substrate it was concluded that the substrate is essential for enzyme aggregation and that probably the resulting dimer unit is the active form of the enzyme. This "dual-phospholipid" model, however, was heavily based on the presumed "half-site reactivity" of this enzyme (*Roberts* et al. 1977a), which is now known to be incorrect (*Darke* et al. 1980). Of course, the withdrawal of the "half-site" reactivity does not need to invalidate the proposal that the cobra enzyme aggregates to its enzymatically active dimer form in the presence of substrate. On the other hand, the results of the cross-linking experiments, where under optimal conditions trimer formation is relatively more important than dimerization, are not fully convincing.

Maybe the strongest evidence for the "dual-phospholipid" model has to be found in the "specificity reversal" of this enzyme (vide infra). An interesting observation in this study is that the cobra enzyme, like the pancreatic PLA, has no affinity for pure Triton micelles. Only mixed micelles containing phospholipids (including sphingomyelin) in the presence of Ca^{2+} or Ba^{2+} ions bind to the enzyme. Also lysolecithin or free fatty acid incorporated in the Triton micelle enable the enzyme to bind to the mixed micelles and with these products no bivalent metal ions were required for binding. Although these findings might be interpreted as a support for a mechanism in which PLA initially interacts with a *single* lipid molecule in the interface, other explanations are possible as well. An interesting case of specificity reversal of the *Naja naja* PLA was described by *Dennis* and co-workers (*Adamich* and *Dennis* 1978; *Roberts* et al. 1979; *Adamich* et al. 1979) which might have a direct relevance to the mechanism of action of this enzyme. Comparing the action of the enzyme on mixed micelles of Triton and long-chain lecithin with that on mixed micelles of Triton and long-chain PE, the cobra PLA hydrolyzes the lecithin-containing micelles at a much higher rate. However, in Triton micelles containing both PE and PC in equimolar amounts, the enzyme

was shown to possess a clear preference for PE as substrate. The activating effect on PE hydrolysis appeared not to be limited to long chain PC, but several other phosphoryl choline containing lipids showed a similar behavior, such as lyso-PC, sphingomyelin, and even dibutyryl lecithin. These results were tentatively explained by the possible existence of two binding sites on the enzyme molecule: (1) an activator site which requires a lipid molecule containing the phosphorylcholine moiety and at least one fatty acyl chain and (2) a head group nonspecific catalytic site.

While it might be argued that activation of PLA towards PE by long-chain phosphoryl choline lipids could be caused by subtle changes in the lipid-water interface of the mixed micelle, the activating effect of the highly water soluble dibutyryl lecithin constitutes the strongest evidence for the proposed direct interaction of the PC molecule with the enzyme. Taking into account the relatively weak activating effect of dibutyryl PC (four times) as compared to the twofold activation by an aspecific, non-phosphorylcholine-containing lipid such as oleic acid, it is, however, of the utmost importance to be certain that dibutyryl lecithin is not partially incorporated into the mixed micelle. The experimental techniques used by the authors (*Roberts* et al. 1979; *Adamich* et al. 1979), namely, equilibrium gel filtration in the *absence* of PE and ^{31}P-NMR, would probably not detect a low incorporation of dibutyryl PC in the mixed micelle. The activating effects observed here of phosphorylcholine containing lipids on the venom PLA hydrolysis rate of more negatively charged phospholipids are in agreement with previous reports on similar activation by *n*-alkylphosphorylcholine of *Crotalus adamanteus* venom PLA hydrolysis of negatively charged phospholipids such as cardiolipin, phosphatidylglycerol, and phosphatidic acid (*van Deenen* and *de Haas* 1963; *de Haas* et al. 1966). The small size of a PLA molecule, however, makes it difficult to suppose the presence of two binding sites for the relatively large phospholipid molecules.
The previous suggestion of *Roberts* et al. (1977c) that the substrate might induce enzyme aggregation and that probably the resulting dimer is the active form of the enzyme would solve the "sterical" problem, but in that case the dimer structure should be asymmetrical.

In summary, the combined efforts of *Dennis* and colleagues lead to the following model for hydrolysis of mixed micelles by the *Naja naja naja* PLA. The enzyme, present as monomer under catalytic conditions, binds to a single substrate molecule in the interface. A conformational change occurs in the enzyme molecule which gives rise to dimerization of the protein. The second PLA molecule binds then a second phospholipid molecule of the interface to a functional active site and hydrolysis takes place. Binding of the enzyme to the phospholipid requires Ca^{2+} and the resulting dimeric structure is asymmetrical.

5.4 Monomolecular Surface Films of Medium-Chain Phospholipids

The principles, advantages, and drawbacks of this attractive technique to investigate the kinetics of lipolytic enzymes have been discussed in considerable detail in two recent reviews (*Verger* and *de Haas* 1976; *Verger* 1980). Therefore, we will limit ourselves here to a discussion of a few very recent papers. Although a number of them deal with lipase-substrate interactions in monolayers and therefore, strictly speaking, do not fit in this review on PLA, the general conclusions which can be drawn from these studies may have a large bearing on our understanding of the interaction of PLA with their specific substrates.

In a series of papers *Dervichian* and *Barque* (1979) and *Barque* and *Dervichian* (1979a,b) investigated the kinetics of pancreatic lipase acting on surface films of 1,3 didecanoylglycerol. The authors showed that the enzyme adsorbs in a reversible way to the lipid monolayer and that the equilibrium surface concentration is a linear function of the bulk lipase concentration. In agreement with previous studies (*Zografi* et al. 1971) in which a similar "constant surface pressure" setup was used, it was found that a rapid establishment of the adsorption equilibrium required efficient stirring of the subphase.

However, in contrast to most other monolayer studies using lipolytic enzymes (cf. *Verger* and *de Haas* 1976), *Dervichian* and *Barque* stopped stirring after the initial adsorption equilibrium was reached and followed enzymatic velocity as function of various variables such as surface pressure, enzyme concentration, pH, etc. after film transfer to an enzyme-free bulk phase. The fact that after this film transfer the lipase initially displays the same hydrolysis rate as before transfer is in good agreement with the results of *Rietsch* et al. (1977) and *Pattus* et al. (1979a) obtained with pancreatic PLA. Moreover, these experiments clearly demonstrate that some kind of fixation of the water-soluble enzyme to the lipid-water interface must have taken place. The decline of enzyme activity after transfer, the velocity of which is dependent on the surface pressure used, is also in agreement with the reports by *Pattus* et al. (1979a) and this enzyme desorption from the interface is clearly in favor of a *reversible* adsorption process.

In their second paper *Barque* and *Dervichian* (1979a) studied the enzymic velocity dependence on bulk enzyme concentration and on the surface substrate density, which is proportional to the surface pressure π. It is interesting to note that notwithstanding the proportionality between bulk enzyme concentration and velocity of hydrolysis, the extrapolated curve does not pass through the origin. This behavior has also been reported by other investigators (*Verger* and *de Haas* 1973; *Pieroni* and *Verger* 1979)

and so far no adequate explanation has been provided. To study the relationship between enzyme velocity and substrate density at the interface the authors applied two techniques: (1) the initial adsorption of the enzyme and the rate measurement were made at each individual surface pressure π and (2) all initial adsorptions of the enzyme were effected at a constant π, and after readjustment to the desired surface pressure, the hydrolysis rate was measured. The authors claim that in this way the effects of the *amount* of adsorbed enzyme and the *specific activity* of the enzyme on the hydrolysis rate can be separated. Although the observation that the amount of enzyme adsorbed to the monolayer at a certain surface pressure remained constant upon changing π contradicts the findings of *Verger* et al. (1976) and *Pattus* et al. (1979a), it should be realized that the experimental conditions of *Dervichian* and *Barque* were totally different from those of *Verger* and *Pattus.* The latter investigators applied continuous and efficient stirring during the whole assay which will rapidly readjust the distribution equilibrium of enzyme between bulk and monolayer upon surface pressure changes.

Dervichian and *Barque,* however, stopped agitation immediately after the establishment of the adsorption equilibrium. Perhaps under the latter conditions the amount of enzyme adsorbed to the film remains constant ("frozen") during changes in surface pressure or area and the mixed monolayer (lipid plus adsorbed enzyme) might be considered as a segregated phase. The main conclusion of *Dervichian* and *Barque* is that the variations of enzymatic hydrolysis rate as a function of surface pressure are caused by two effects: changes in amount of adsorbed enzyme and changes in the specific activity of the enzyme. In their third paper *Barque* and *Dervichian* (1979b) studied the enzymatic velocity at constant surface area, i.e., under conditions where the surface pressure is continuously decreasing. Because of the limited stirring procedure described earlier it was assumed again that substrate and enzyme together form a segregated and well-defined system on the surface. This implies that notwithstanding the gradually decreasing substrate density at the surface the total amount of adsorbed enzyme ($E^* + E^*S$ in Fig. 5) remained constant. The explanation given by the authors is that only E^* (cf. Fig. 5) is in equilibrium with the bulk enzyme concentration. Upon decreasing the surface pressure the equilibrium $E^* + S \rightleftharpoons E^*S$ is supposed to shift to the left and only when $[E^*]$ gets higher than the bulk equilibrium enzyme concentration would the enzyme desorb.

The model of lipolysis proposed by *Verger* et al. (1973) was recently checked by *Pattus* et al. (1979a,b,c) using two radioactively labeled preparations of porcine pancreatic PLA and a series of medium-chain lecithins containing C_8, C_9, C_{10}, and C_{12} acyl chains. The lag time observed during pre-steady-state kinetics reflects the rate limiting step of the pene-

tration of the enzyme in the monolayer. Film transfer experiments showed this penetration to be reversible, but the desorption of the enzyme from the film is slow as compared to the adsorption which is in agreement with the results of *Barque* and *Dervichian.* The kinetics of the penetration process is governed by the packing density of the substrate molecules, and it seems that the polar head group of the phospholipid molecule and its hydration state play an important role. The steady state surface concentration of the enzyme decreases with increasing film pressure. However, this surface concentration increases with fatty acyl chain length of the substrate which is in agreement with the idea that hydrophobic interaction dominates the penetration process.

The influence of bulk pH on the pre-steady-state kinetics of the porcine enzyme was investigated, and it was found that at alkaline pH the penetration capacity strongly decreases (increase of induction time). In the presence of Ca^{2+}, the equilibrium surface concentration of the enzyme was found, however, to be pH independent until the pH region where deprotonation of the α-NH_3^+ group of Ala-1 occurs. Deprotonation of this function results in a rapid desorption of the enzyme from the interface. At slightly acidic pH values ($\leqslant$ 6.0) enzyme substrate binding occurs in the absence of Ca^{2+}, but at higher pH only the E-Ca^{2+} complex is able to interact with the PC film. The rapid decomposition of the E-Ca^{2+}-PC complex at basic pH upon addition of EDTA again is a strong indication for the reversibility of the binding process.

Willman and *Stewart-Hendrickson* (1978) investigated the influence of positive charge on the kinetics of hydrolysis of diC_{10}-PC monolayers by PLA from porcine pancreas and *Crotalus adamanteus.* Different insoluble long-chain amines were incorporated in the substrate PC film and hydrolysis rates were followed in a "zero-order" trough as function of pH and amine mol fraction. Because the amines possess very different apparent pK_a values in the mixed surface films, it was possible to follow hydrolysis rates as a function of the surface charge of the monolayer. The authors conclude that the inhibition of both PLAs is caused exclusively by the positive surface charge of the film and not by changes in film packing. Unfortunately no use was made of radiolabeled enzymes, so it is not clear whether the surface penetration step or the two-dimensional Michaelis parameters K_m^* and k_{cat} are modified by the positive charge of the film. Most probably more meaningful kinetics would have been obtained by the mixed-film technique (which will be described later) which avoids a continuous change of the quality of the mixed film.

Until now all recent kinetic studies on lipolytic enzymes using the monolayer technique have been performed with surface films consisting of one type of lipid. This is inherent to the technique, as it does not allow one to follow individually in one experiment the hydrolysis of

more than one substrate species. In the most popular rectangular trough design it is also not possible to study the influence of surface inhibitors on the kinetics of lipolysis. Hydrolysis of substrate molecules would continuously modify the chemical composition of the film by enrichment of the inhibitor, resulting in a change of the quality of the interface. Application, however, of the "zero-order" trough (*Verger* and *de Haas* 1973) enabled *Verger* and colleagues to study the hydrolysis of mixed monomolecular films of triglyceride and lecithin by pancreatic lipase (*Pieroni* and *Verger* 1979) and by pancreatic PLA (*Pieroni* and *Verger,* to be published). Such studies are of particular relevance since lipolysis in vivo involves the participation of several classes of lipids. The principle of the method is shown in Fig. 6 where a mixed film of trioctanoylglycerol-didodecanoyl lecithin is hydrolyzed by lipase. A most remarkable result

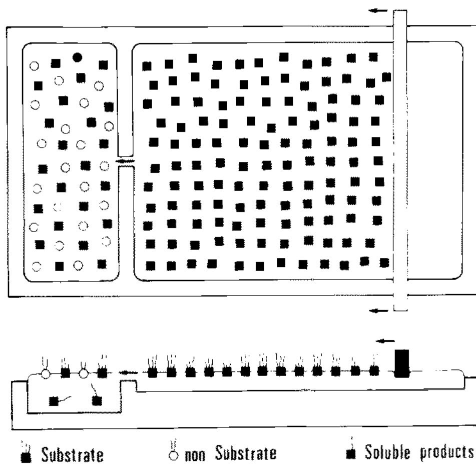

Fig. 6. Principle of the method for the study of enzymatic lipolysis of mixed monomolecular films (*Pieroni* and *Verger* 1979)

from this study is that the mixed films containing increasing mol fractions of the PC (which is not degraded) are hydrolyzed at a continuously increasing rate up to a PC mol fraction of about 0.4. This rate increase is not caused by the presence of higher amounts of enzyme in the film – on the contrary, radioactivity measurements of the ^{14}C-labeled lipase present in the surface showed that *less* enzyme is present in the interface when the mol fraction of PC increases! The observed rate increase is the more remarkable because one would expect that substitution of triglyceride molecules by PC molecules would give rise to substrate dilution and would thereby lower the velocity. In terms of the lipolysis model of Fig. 5 the accelerating effect was attributed to a better binding between enzyme and triglyceride *in* the film (lower K_m^*)and/or to an increased k_{cat}. It is our feeling that a decrease in K_m^* cannot be used as

explanation for the observed enhanced lipolysis rates. According to the model of Fig. 5 a displacement of the equilibrium $E^* + S \rightleftharpoons E^*S$ to the right would result in a concomitant shift of the equilibrium $E \rightleftharpoons E^*$, and more enzyme would be attached to the film. Therefore, to explain the above results one must assume that the surface defects introduced in the triglyceride film by the PC molecules create a microenvironment which is favorable for the decomposition of E*S, in other words these surface defects must increase k_{cat}. A drawback of this mixed-lipid system is the use of triglyceride as a substrate for lipase. Additional complications of the kinetics can be expected because the first hydrolysis product, dioctanoin, is insoluble and remains at the lipid-water interface, changing the quality of that interface. Although it will be ultimately hydrolyzed by lipase into completely soluble products, this is a slow reaction. In a subsequent study *Pieronie* and *Verger* (to be published) investigated the hydrolysis kinetics of the same mixed monolayer of trioctanoin/diC_{10} PC by pancreatic PLA. In this case only PC is degraded and the influence of increasing mol fraction of triglyceride on the hydrolysis rate was studied as function of film pressure.

At low surface pressure (10 dynes/cm) which is the optimum for hydrolysis of pure diC_{10}PC, an increase in mol fraction of triglyceride results in a proportional decrease of hydrolysis rate. The amount of radioactive enzyme in the film remains roughly constant and independent of the chemical composition of the mixed film. This behavior was explained by substrate dilution, assuming that the two-dimensional substrate concentration is smaller than K_M^*. At higher film pressures, however, where the pure PC film is not hydrolyzed at all because the enzyme is unable to penetrate, the substitution of 40%–50% of the substrate molecules by triglyceride results in a sudden and very sharp increase of penetrated enzyme. This behavior was explained by the creation of surface defects ("cracks"[10]) at certain molar ratios of triglyceride and PC. Such cracks might be caused by isothermal phase separation in the surface film. A consequence of this surface heterogeneity is the fact that the surface pressure optimum of the enzyme shifts to much higher values than measured with the pure PC substrate.

Mixed monolayer films of diC_{12}PC and bovine brain sphingomyelin were used by *Barenholz* et al. (to be published). They investigated two radiolabeled PLAs from porcine pancreas and from the venom of *Vipera berus* and studied the kinetics at different surface pressures and molar ratios of the phospholipids. Taking into account the complex thermotropic behavior of natural sphingomyelins which are composed of various

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10 Following a proposal of *M.K. Jain* such still ill-defined surface defects will occasionally be indicated by "cracks"

acyl chains (broad phase transition between 22°–45°C), it can be expected that mixtures of this phospholipid with $diC_{12}PC$ will show nonideal mixing in surface films (compare *Untracht* and *Shipley* 1977). *V. berus* PLA, an enzyme characterized by a high penetrating power (*Boffa* et al. 1980; *Verheij* et al. 1980b), is relatively insensitive for the cracks introduced in the surface film by increasing mol fractions of sphingomyelin. Its surface pressure-activity profile does not shift, and the lower hydrolysis rates observed with increasing sphingomyelin content could be explained just by substrate dilution. However, these experiments demonstrate again the high sensitivity of the weakly penetrating pancreatic PLA for surface defects. At low film pressures (10 dynes/cm) where the enzyme experiences no penetration problems, addition of sphingomyelin decreases enzymatic activity by substrate dilution. At high surface pressures, however, where the enzyme is unable to penetrate pure PC films, the insertion of sphingomyelin molecules in the film gives rise to phase separation and the resulting cracks are immediately recognized by the pancreatic enzyme[11] which enters the film and high hydrolysis rates are found. This results in a dramatic shift in the activity-surface pressure profile. It would be very interesting to repeat these experiments with a better defined synthetic sphingomyelin.

In summary, notwithstanding its inherent drawback – a small interface to volume ratio which makes interfacial enzyme saturation impossible – the monolayer technique continues to yield valuable information on the interaction between lipolytic enzymes and interfaces. Notably film transfer experiments, use of radiolabeled enzymes, and mixed film kinetics have considerably extended our insight into lipolysis. Moreover the possibility to study pre-steady-state kinetics in a relatively simple way should not be underestimated. Although the technique demands highly purified materials, the amounts of enzyme and substrate are extremely low.

The extension of the technique to long-chain substrate films by incorporation of albumin or bile salt micelles in the subphase as recently described by *Scow* et al. (1980) and *Lairon* et al. (1980), respectively, can be expected to yield important results on the interaction of lipolytic enzymes with biologically relevant lipid-water interfaces. Careful controls should be made, however, to make sure that albumin does not introduce surface defects in the film.

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11 The observation that the even weaker penetrating zymogen of pancreatic PLA (cf. *Pattus* et al. 1979a) is able to adsorb to sphingomyelin monolayers up to 21 dynes/cm indicates that surface films of this natural material contain many surface defects

5.5 Phospholipids Present in Bilayer Structures

One of the earliest kinetic analyses of a pure PLA *(Bitis gabonica)* acting on DPPC was reported by *Viljoen* et al. (1974). Although the authors were under the impression that they studied *monomer* catalysis, the substrate concentrations applied in their assays were so far above the CMC reported by *Tanford* (1973) for DPPC ($\pm\ 10^{-10}\ M$) that we must assume that they worked with lipid *aggregates*, presumably bilayers. Using a somewhat obsolete enzyme assay technique in which proton release is followed by pH drop they were able to measure initial hydrolysis rates at substrate concentrations ranging from 5–80 μM. The very low maximal velocity of the enzyme under these conditions (calculated from the figures to be about 0.5 μmol min^{-1} mg^{-1} protein) is not in agreement with the V_{max} value given in Table 1 of their paper which is more than 200 times higher.

Initial rate measurements in which substrate and Ca^{2+} concentrations were varied confirm the mechanism proposed by *Wells* (1972) for the *Crotalus adamanteus* PLA in which Ca^{2+} adds first to the enzyme before the substrate molecule. Product inhibition experiments suggest that also in the *Bitis gabonica* enzyme the products are released in an obligatory order: fatty acid first and lysolecithin second. In summary, the results of *Viljoen* et al. (1974) might be interpreted by stating that the mechanism of action of both venom PLAs are very similar and are independent of the aggregation state of the substrate. On the other hand the ill-defined physicochemical state of the substrate under the conditions used, together with the uncertainty about the maximal velocity, make such conclusions premature. Similar remarks have to be made on the kinetic experiments with PLA from *Naja mossambica mossambica* reported by *Martin-Moutot* and *Rochat* (1979).

Long-chain diacylphospholipids such as PC which form aggregated bilayer structures in water have been known for a long time to be very poor substrates for pancreatic PLAs (*van Deenen* et al. 1963; *de Haas* et al. 1968) and accurate kinetic analyses seemed to be impossible. However, after the initial reports of *Op den Kamp* et al. (1974, 1975) that several fully saturated long-chain lecithins become very susceptible to hydrolysis by porcine pancreatic PLA at the thermotropic phase transition, a renewed interest has come up. At the transition temperature domains of frozen molecules are separated from surface areas where the lipids are in the liquid crystalline state, and most probably at the borders surface defects (cracks) exist which allow the penetration of the enzyme. Both below and above the phase transition the more regular and tighter packing of the phospholipid molecules prevent the anchoring of the enzyme into the interface, and no hydrolysis is observed. It has to be

remarked that this sharp differentiation is found only with PLAs characterized by a weak penetrating power such as the pancreatic enzymes, β-bungarotoxin (*Strong* and *Kelly* 1977), or platelet phospholipase (*Kainagi* and *Koizumi* 1979), in combination with multilayered liposomes of fully saturated lecithins. With increasing unsaturation of the lecithin acyl chains resulting in looser packing of the phospholipid molecules in the interface, the more powerful penetrating PLAs in particular are also able to enter the bilayer to a certain extent at temperatures above the thermotropic phase transition and hydrolysis occurs.

Wilschut et al. (1976, 1978) extended these studies and showed that sonicates of PC dispersions, especially those containing small unilamellar vesicles, are more susceptible to PLA hydrolysis than the multilamellar liposomes. They also observed that if sonication is done below the phase transition temperature, the resulting vesicles are hydrolyzed over a much wider temperature range. Most likely the high curvature of the vesicles results in surface defects which facilitate penetration on the enzyme. These systems, however, are still hardly of any use in kinetic studies because of difficulties in determining initial rates and the variable effects of reaction products on the enzymatic velocity.

In order to overcome these difficulties, *Jain* and *Cordes* (1973a,b) proposed the incorporation of medium chain *n*-alkanols (C_6, C_8) in the aqueous dispersions of long-chain lecithins. By a number of different techniques, including trapping experiments, they showed that the bilayers remained closed. They concluded that at optimal concentration of activating alcohols egg PC liposomes and vesicles behave as excellent substrates for various PLAs and that normal Michaelis kinetics can be obtained. Most probably the alcohol chains inserted in the bilayer cause an increased spacing of the substrate molecules and thus allow a facilitated penetration of the PLA molecule[12]. However, effects of the alcohol molecule on the catalytic factors K_m^* and k_{cat} could not be excluded. In a subsequent study *Upreti* and *Jain* (1978) improved their assay system by an osmotic shock of the multilamellar vesicles before addition of the enzyme. A major disadvantage of the original substrate, phospholipid liposomes plus alkanol, was the rather high apparent K_m of the lipolytic enzymes used. Because only the outer layer of the multilamellar vesicles is exposed to the enzyme, large amounts of substrate were required to obtain interfacial saturation. Moreover initial rate measurements were complicated because the rate of hydrolysis was increasing with time as

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12 An exceptional case seems to be the highly unsaturated cabbage lecithin (> 4 double bonds/mol). Without any addition of alcohols, normal Michaelis kinetics were obtained with Russels viper venom PLA. Moreover an unusually high apparent K_m (13 m*M*) appeared to be accompanied by a very high k_{cat} (25 and 40 times higher than for egg PC and DPPC, respectively)

successive bilayers were "opened" and more substrate became exposed. By a sudden decrease of the ionic strength of the assay solution the liposomes transiently "open" and such osmotically shocked bilayers offer a nearly complete access of the enzyme to the substrate molecules. Because resealing of the liposomes is a rather slow process ($t_{1/2} \approx 10$ min), initial rate measurements were possible and the apparent K_m values were much lower.

It has to be remarked that even with these osmotically shocked liposomes, the pancreatic PLA, in contrast to all venom enzymes tested, shows a lag phase at the beginning of hydrolysis and only after a certain induction time (τ) is a steady-state rate obtained (*Jain* and *Apitz-Castro* 1978). This lag phase is strongly reminiscent of the behavior of the pancreatic enzyme towards densely-packed medium-chain PC monolayers (*Verger* et al. 1973). *Jain* and *Apitz-Castro* showed that the lag period preceding the steady state phase is not caused by increasing amounts of hydrolysis products. Moreover, the induction time appeared to be independent of concentrations of enzyme, substrate, alkanol, and Ca^{2+}. These facts led the authors to a hypothetical kinetic mechanism for this enzyme which is very similar to the model of *Verger* et al. (1973) (cf, Fig. 5) in which the latency period is due to a slow, rate-limiting penetration of the enzyme into the lipid-water interface (*Pattus* et al. 1979a). It is difficult to understand, however, how in this model τ could be independent of the concentration of the bilayer perturbing alcohol. Moreover the observation that calcium is not required for the slow penetration step is not in agreement with the monolayer results.

Recently, *Upreti* et al. (1980) in a very detailed study investigated the bilayer perturbing capacity of an impressive series of different alkanols and the effect of the alcohol-modified bilayer on the kinetics of PLA. Whereas insertion of all alkanols into egg PC liposomes resulted in an increase of free space in the substrate bilayer (surface defects) as evidenced by a higher accessibility to the enzyme and increasing velocities, estimation of the individual kinetic constants (cf. Fig. 5) remained impossible. The fact that the increasing chain length of straight-chain *n*-alcohols results in a higher apparent K_m, whereas insertion of branched alcohols seems to have no influence on this parameter, suggests that the former alcohols might compete with substrate molecules for the hydrophobic binding site in the active center (cf. *Slotboom* et al. 1976). In this study the authors confirmed the original observation made by *Bonsen* et al. (1972a) that in mixtures of *sn*-3 and *sn*-1 lecithins having the same chain length the D-isomer behaves as a pure competitive inhibitor characterized by the same binding constant to the enzyme. This makes the stereoisomeric *sn*-1-phospholipid the most ideal phospholipid for determination of dissociation constants by direct binding experiments. Using

sn-1 DPPC bilayers and radioactive PLA preparations from bee venom and porcine pancreas *Upreti* et al. clearly showed that addition of increasing amounts of alkanol to the PC bilayer increases the amount of PLA bound to the lipid-water interface. Usually higher enzyme concentrations in the bilayer result in higher hydrolysis rates. The observed decrease in enzymatic activity at very high alcohol concentration, where even more enzyme was shown to be bound to the bilayer, is similar to the findings of *Dennis* (1973a,b) working with Triton-PC mixed micelles. Most probably this effect is caused by competitive inhibition and substrate dilution and/or unfavorable effects of the microenvironment on k_{cat}. It goes without saying that at least for the venom PLAs a most relevant approach to study the kinetics of the enzymes would be the use of an aqueous system containing only long-chain substrate, enzyme, and Ca^{2+} ions.

Several groups investigated such systems using PLAs of different origin (*Tinker* et al. 1978; *Tinker* and *Wei* 1979; *Kensil* and *Dennis* 1979; *Upreti* and *Jain* 1980). *Tinker* et al. (1978), working with dispersions [13] of DPPC and of DMPC, analyzed the kinetics of hydrolysis by *Crotalus atrox* PLA at different temperatures, both below and above the phase transition temperature. They observed that the hydrolysis of gel-phase lecithins showed hyperbolic dependence of initial steady state rates on bulk lipid concentration, which is in agreeement with the results of *Viljoen* et al. (1974) and of *Martin-Moutot* and *Rochat* (1979). However, hydrolysis of liquid crystalline preparations showed a short initial burst of proton release and then a long lag period of very slow reaction, which was followed by a dramatic increase in the reaction rate. The accelerated proton release during the last stage is probably caused by the presence of considerable amounts of hydrolysis products in the interface. The lag period could indeed be abolished by preaddition of the reaction products to the substrate bilayer before the reaction was started, an observation which was reported also by *Roholt* and *Schlamowitz* (1961).

Based on this results the authors proposed a kinetic model of lipolysis which is quite different from that of Fig. 5, which had been proposed by *Verger* et al. (1973), *Brockerhoff* (1973), *Deems* et al. (1975), and *Jain* and *Apitz-Castro* (1978). As shown in Fig. 7, the key feature of this new model implies that the enzyme can only bind to the lipid-water interface by forming a 1:1 complex of enzyme and a single *substrate* molecule. This complex formation is supposed to involve a conformational change in the enzyme resulting in exposure of hydrophobic sites which subsequently

13 Unfortunately the authors prepared their vesicles by sonication below the phase transition temperature and no annealing was attempted. This procedure is known (*Szoka* and *Paphadjopoulos* 1980) to give unstable, very heterogeneous particles. The relatively low apparent K_m values reported by the authors (100–200 μM) suggest that most of the bilayers contained structural defects (cracks)

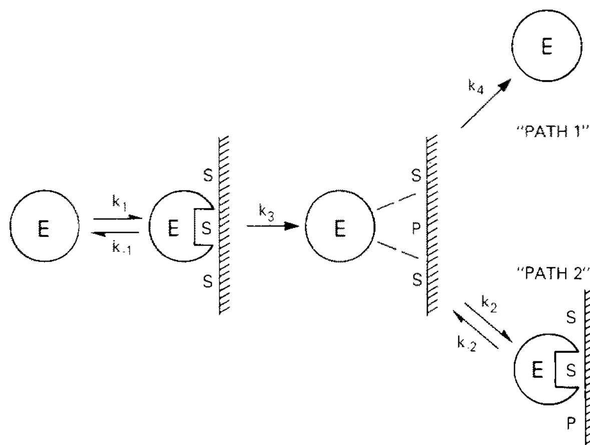

Fig. 7. Kinetic model for hydrolysis of phosphatidyl choline aggregates by *C. atrox* PLA (*Tinker* et al. 1978). For description of model, see text

penetrate the lipid surface. After the performance of *one* catalytic cycle, the enzyme molecule can either desorb from the surface and return to the aqueous phase ("hopping"[14]) or diffuse along the surface to an adjacent substrate molecule ("scooting"[14]). The authors proposed that the hopping model describes the rapid hydrolysis of the gel-phase phospholipids, whereas the slower hydrolysis of the liquid crystalline phase would proceed by the scooting pathway. In a second paper (*Tinker* and *Wei* 1979) the authors worked out a mathematical treatment of the observed kinetics in the liquid crystalline state and concluded "that the proposed model is consistent with current ideas on the mechanism of catalysis by this enzyme".

Very recently, the same group (*Tinker* et al. 1980) analyzed the hydrolysis of the gel phase and studied the effects of reaction products on hydrolysis rates. Gel filtration experiments demonstrated that the enzyme binds to egg PC bilayers even in the absence of Ca^{2+} and that incorporation of hydrolysis products in the bilayer *weakened* the enzyme binding. These observations together with the observed increase in hydrolysis rate at later stages of the reaction where substantial amounts of lyso-PC and free fatty acids are present were ascribed to a product-facilitated desorption of the enzyme from the surface. In this latter study both annealed and unannealed sonicated DPPC vesicles were used, but no attempt was made to separate the larger multilamellar structures from small unilamellar vesicles.

Kensil and *Dennis* (1979) examined the action of *Naja naja naja* PLA on single-walled, sonicated vesicles of DPPC, DMPC, and egg PC as a function of temperature. They confirmed the observation of *Tinker* et al.

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14 "Hopping" and scooting" are expressions used by *Upreti* and *Jain* (1980) to differentiate between these pathways

(1978) that the venom PLA hydrolyzes the gel phase phospholipids at a higher rate than the same substrate in the liquid crystalline state. In addition they also found an apparent stimulation of activity as the reaction proceeded above the phase transition temperature. This observation was tentatively attributed to an increase in phase transition temperature caused by increasing amounts of reaction products by which the enzyme could actually be hydrolyzing *gel* state phospholipid, the preferred physical form. As possible explanation for the enhanced hydrolysis of gel state phospholipids, the authors consider decreased hydration of head groups and better accessibility of the 2-ester function to the enzyme by a tilt of the acyl chains. In this study well-characterized, annealed, small unilammellar vesicles were used, and consequently the apparent K_m values are about 30 times higher than reported by *Tinker* et al. (1978).

Finally *Upreti* and *Jain* (1980) reported on the kinetics of bee venom PLA acting on unmodified PC bilayers. Packing alterations in the substrate aggregate were made by sonication, temperature change, and osmotic shock. Again biphasic progress curves were found: after an initial rapid proton release in which less than 7% total available substrate is hydrolyzed, the reaction slows down and only after production of a certain amount of lyso-PC plus fatty acid does fast hydrolysis start again. As a very attractive hypothesis to explain the observed kinetics the authors propose that any treatment of the bilayer which introduces defect structures (cracks), and therefore free space, will enhance PLA activity. In terms of the model in Fig. 5 they do not preclude effects of the cracks on the catalytic parameters K_m^* and k_{cat}, but a highly important function of the surface defects is thought to be the shift of the equilibrium $E \rightleftharpoons E^*$ to the right side. The specific influence on phosphatidyl-choline bilayer packing exerted by the simultaneous presence of the hydrolysis products, lysolecithin and free fatty acid, has been demonstrated by *Jain* et al. (1980) and *Jain* and *de Haas* (1981). While the pancreatic PLA is unable to penetrate into the closely packed bilayers of pure lecithin, the presence of *both* lysolecithin and fatty acid results in surface defects (phase separation), and the enzyme displays a high affinity and catalytic power to such "cracked" interfaces (*Jain* et al., to be published).

The hypothesis that cracks or irregularities in the lipid bilayer enhance PLA activity is furthermore illustrated by studies on a natural membrane using pancreatic PLA (*Bevers* et al. 1977, 1978; *Bouvier* et al. 1981). The *Acholeplasma laidlawii* membrane contains glycolipids (70%) and PG (30%) as the only substrate for PLAs. The physicochemical condition of the membrane can be manipulated by growth of the organisms on different fatty acids: e.g. palmitate addition yields membranes in which 80% of the esterified fatty acids present consists of palmitate, and the lipids undergo a phase transition between 15 and 40°C. At tem-

peratures above the lipid phase transition PG is accessible for hydrolysis, below the lipid phase transition no PG is hydrolyzed. In the latter condition proteins are aggregated, eliminating to a large extent the presence of irregularities in the gel state bilayer (*Bevers* et al. 1977). That membrane proteins may be responsible for irregularities in the membrane is illustrated by experiments on membranes which are enriched with branched chain fatty acids. In this case protein aggregation does not occur upon a decrease in temperature and PG remains accessible also below the onset of the transition (*Bouvier* et al. 1981). Another type of cracks can be induced by binding the membranes at temperatures between the onset and termination of the lipid-phase transition. Now phase separation occurs between domains of gel like lipids surrounded by liquid crystalline lipid molecules. Pancreatic PLA has access only to those PG molecules which are present in the fluid, protein-containing areas of the lipid bilayer (*Bevers* et al. 1978).

In a very recent study *Menashe* et al. (1981) reported on the action of porcine pancreatic PLA in annealed DPPC unilammelar vesicles. At or above the phase transition temperature long lag times were observed. Preincubation of the enzyme with substrate for a short period of time below the transition temperature followed by enzymatic assay at high temperature abolished the lag time. These results were explained by a slow substrate-enzyme organizational step above the phase transition, whereas this process is much more rapid with gel state phospholipids. The intrinsic activity of the enzyme is maximal when the substrate is in the liquid crystalline state.

Summary. What is the additional information obtained from kinetic studies of PLA acting on intact PC bilayers? One remarkable result seems to be the observation of *Tinker* et al. (1978) and *Kensil* and *Dennis* (1979) that gel phase PC bilayers are hydrolyzed with a higher rate than the corresponding liquid crystalline phase. These reports are in agreement with an early observation of *Smith* et al. (1972). He found at 30°C a faster hydrolysis rate of DPPC as compared to dioleoyl PC or dilinoleoyl PC. It is clear, however, that independent of the physical structure of the PC bilayers used (multilamellar liposomes, single walled vesicles, annealed and unannealed), the kinetics are all characterized by similar, very complex progress curves. The reviewers feel that initial rate measurements with an acceptable accuracy are hardly possible and that therefore mathematical analyses of these systems using rate equations such as developed by *Gatt* and *Bartzai* (1977a,b) are premature. On the other hand, the experimental results obtained by the various investigators appear to be in good agreement and therefore one should try, if only in a rather qualitative and intuitive way for the present, to explain the reported observations

and to fit them in a common and generalized model of lipolysis. At this moment two hypothetical models are under discussion: (1) the model of *Verger* et al. (1973) (cf. Fig. 5) and (2) the model of *Tinker* et al. (1978) (cf, Fig. 7). It seems that in general investigators working with snake venom PLAs are more inclined to model (2), whereas most people investigating the pancreatic enzyme prefer model (1).

Yet these two models are fundamentally different: while in the *Verger* model the enzyme is supposed to interact hydrophobically with the interface (by penetration or anchoring) before Michaelis-Menten type ES formation and hydrolysis occurs, the prevailing pathway in the *Tinker* model (hopping) implies initial formation by collision of an ES complex at the interface and a return of the enzyme into the aqueous bulk phase after each catalytic cycle. The generally observed accelerated hydrolysis of substrates in aggregated form is tentatively explained in the *Verger* model by a conformational change in the penetrated[15] enzyme with a concomitant optimization of the active site. On the other hand, in the *Tinker* model the high interface activity is attributed to a hopping of the enzyme from interface to bulk solution and vice versa, and a prolonged stay of the enzyme at the surface of the aggregate (scooting) is supposed to yield low hydrolysis rates. While the effective hydrolysis of gel-phase phospholipids and the observed rate increases upon product formation in the *Tinker* model are explained by product-facilitated *desorption* of enzyme from the interface, in the *Verger* model these phenomena are ascribed to a product-facilitated *adsorption* of enzyme to an interface containing more surface defects!

An often reported objection against the *Verger* model is that with several venom enzymes no indications could be found for initial adsorption to or penetration in the lipid-water interface using optical techniques such as ultraviolet difference spectroscopy or fluorescence spectroscopy. Most probably, however, these negative results are caused by the particular lipid-water aggregates used. In titration experiments with single-chain substrate or product analogs such as lysolecithin, glycol lecithins and *n*-alkylphosphorylcholines, ultraviolet and fluorescence signals were obtained for a number of venom PLAs (*Verheij* et al. 1980b; *Prigent-Dachery* et al. 1980) and usually saturation was observed. A second argument against this model could be the observation that the enzyme hydrolyzes gel-phase phsopholipids more rapidly than the liquid crystalline phase. A priori, one would expect in the *Verger* model that adsorption of the enzyme and surface diffusion *in* the interface would be favored by

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15 Although the penetration process by various techniques has been shown to be reversible, the enzyme is thought to remain bound to the interface during a number of catalytic cycles

the more loosely packed liquid crystalline phase and would result in increased hydrolysis rates. It has to be remarked, however, that besides the difficulties mentioned above to determine initial velocities with bilayer systems, comparison of the steady state hydrolysis rates is hampered because of the unknown amounts of enzyme present in the interface. In addition, all investigators agree upon the fact that in phase-separated mixtures of lecithins the most liquid component is hydrolyzed more extensively. As regards the *Tinker* model the following points seem to be relevant:

1. PLAs, independent of their origin, are known to possess an unusual affinity for all kinds of interfaces, and adsorption occurs not only to lipid-water aggregates but also to glass, teflon, and many other surfaces, including the air-water interface. Therefore, an ordered mechanism in which a Michaelis type ES complex would be required before hydrophobic interaction of the enzyme with the interface can occur seems to be superfluous.

2. A product (lyso-PC and/or fatty acid)-stimulated desorption of PLA from the lipid aggregate, assumed to explain the observed higher hydrolysis rates, seems to be in contrast with the results of many direct binding studies. Several PLAs adsorb very well to micelles of single-chain detergents such as lyso-PC, fatty acid, *n*-alkylphosphocholines, etc. Moreover, the pancreatic PLAs which have no affinity to pure lecithin aggregates in bilayer form (liposomes or vesicles) strongly adsorb to these structures if low percentages of hydrolysis products are incorporated (*Jain* et al., to be published).

3. The hopping mechanism implies that desorption of PLA from the surface is a faster process than the formation of a new ES complex. This argument is based on a supposed slow surface diffusion of the enzyme in the lipid bilayer, a medium of higher viscosity than water, but does not take into account the well-known high mobility of free substrate molecules in the plane of the bilayer.

5.6 Reversible Inhibition of Phospholipase A_2

Studies of inhibition kinetics have contributed to a large extent to our present knowledge of the mechanism of many enzymes. Unfortunately this approach has yielded only limited information on the mechanism of action of lipolytic enzymes. With the exception of the earlier work of *Wells* (1972) in which product inhibition was successfully studied with *Crotalus adamanteus* PLA acting on monomeric substrate, similar studies on several other PLAs were seriously impeded by unfavorable CMC-K_m ratios. An important problem is that inhibition studies of PLA acting on

aggregated substrates are plagued by even greater difficulties. Any incorporation of a possible inhibitor in an organized lipid-water interface will change the quality of the interface and influence not only the Michaelis parameters K_m^* and k_{cat} (cf. Fig. 5) but also the amount of enzyme present *in* the interface (k_p/k_d in Fig. 5). In this way several potential inhibitors of PLA act in fact as potent activators (*Roholt* and *Schlamovitz* 1961; *Bonsen* et al. 1972a; *Drainas* and *Lawrence* 1978; *Jain* et al., to be published; *Rosenthal* and *Ching-Hsien Han* 1970). This subject has been discussed previously by *Verger* and *de Haas* (1976) and up till now it has not been possible to separate the effects of inhibition in the classical chemical sense from pure physical effects.

Recent reports showed that several dyes are able to bind with a high affinity to the enzyme. Hydrophobic binding forces seem to be predominant and the competitive behavior with monomeric substrates suggests that the catalytic site is involved. Indirect evidence that flavin analogs bind to a particular surface region of pancreatic PLA was obtained by photo-CIDNP experiments (*Jansen* et al. 1978). *Zhelkovskii* et al. (1978a) demonstrated that the acridine dye proflavine interacts with PLA from *Naja naja oxiana* and inhibits the enzymatic hydrolysis of diC_4-PC in a competitive way. *Barden* et al. (1980) showed that *Naja naja naja* PLA is effectively inhibited by the dye Cibracron blue F_3GA. Again the displacement of the dye from the enzyme by the monomeric substrate diC_6-PC suggests that binding occurred at the hydrophobic active center region. Inhibitory effects of a number of local anesthetics on PLAs of different origin have been reported by several investigators (*Scherphof* et al. 1972; *Waite* and *Sisson* 1972; *Kunze* et al. 1974, 1976). Interference with Ca^{2+} binding, perturbation of the lipid aggregate, and direct binding to the enzyme have been evoked to explain the inhibition of the enzyme. Often, however, crude enzyme preparations and nonhomogeneous PL systems have been used, and it is difficult to draw definite conclusions. *Stewart-Hendrickson* and *van Dam-Mieras* (1976) investigated the action of local anesthetics on the porcine pancreatic PLA using the monomolecular surface film technique to avoid the influence of enzymatic breakdown products.

Naturally occurring PLA-inhibitor complexes have been shown to be present in several snake venoms (*Braganca* et al. 1970; *Vidal* and *Stoppani* 1971a; *Breithaupt* 1976; *Simon* and *Bdohlah* 1980). These inhibitors are relatively small peptides with an opposite charge at neutral pH as compared to the PLA molecule. Notwithstanding tight binding between both polypeptides, in detergent-activated assay systems for PLA the complex often slowly dissociates and accelerating kinetics (lag phase) are observed.

5.7 Monomeric or Dimeric Enzymes?

The question whether PLAs are catalytically active as monomeric or dimeric proteins becomes particularly important after the reports of *Wells* (1973b) and *Roberts* et al. (1977a) that *Crotalus adamanteus* and *Naja naja naja* PLAs demonstrate "half of the sites" reactivity. Although the original evidence for a dimeric form of the *Crotalus adamanteus* enzyme as presented by *Wells* (1971b) and confirmed by *Shen* et al. (1975) has been criticized (*Volwerk* et al. 1979), there is now little doubt that this PLA under catalytically meaningful concentrations is present as a dimer in aqueous solution. Moreover, very recently *Wells* showed by "active enzyme ultracentrifugation" that this PLA hydrolyzes diC_6-PC *monomers* as a dimeric protein (*Smith* and *Wells* 1981).

Although the half-site reactivity for the *Naja naja naja* PLA has been withdrawn (*Darke* et al. 1980), this enzyme demonstrates a concentration-dependent aggregation in aqueous solution (*Deems* and *Dennis* 1975): at concentrations below 50 $\mu g \cdot ml^{-1}$ enzyme exists predominantly in the monomeric form, however additional evidence has been reported that aggregated lipids shift this equilibrium to the dimeric state and that in fact the (asymmetric) dimer of this PLA is the catalytically active form of the enzyme.

A similar substrate-induced shift of monomeric into dimeric protein has been proposed for PLA from *Naja naja oxiana* (*Zhelkovskii* et al. 1978a; *Mal'tsev* et al. 1979). Again the enzyme dimer is assumed to be organized asymmetrically, but it is not clear why the enzyme should dimerize into asymmetric units in order to be able to hydrolyze monomeric diC_4-PC molecules. As regards the porcine pancreatic PLA, in aqueous solutions without lipids this enzyme exists as monomeric protein up to concentrations of several $mg \cdot ml^{-1}$.

Addition of monoacyl zwitter ionic substrate analogs in concentrations up to the CMC does not induce aggregation of the enzyme (*Volwerk* et al. 1979), suggesting that this enzyme is catalytically active as monomer. On the other hand direct binding studies of porcine pancreatic PLA with micellar substrate analogs and analysis of the resulting lipoprotein complexes (*Araujo* et al. 1979; *Hille* et al. 1981) showed the presence of particles containing two or three enzyme molecules per 80–100 lipid monomers.

In summary, with the exceptions of the *Crotalus adamanteus* and *Agkistrodon halys blomhoffii* PLAs which act as dimers in the hydrolysis of monomeric substrate molecules, the functional role of enzyme aggregation in the hydrolysis of organized lipid-water interfaces seems to be uncertain for all phospholipases investigated.

6 Chemical Modification Studies of Phospholipases A_2

In the past decade a wide variety of more or less specific reagents have been used to modify almost all functional groups present in PLAs. As cited previously (cf. *Jeng* and *Fraenkel-Conrat* 1978) one has to bear in mind that there exist no specific protein reagents but only specific protein reactions. From this statement it may already be clear that it is necessary to first purify the modified protein to homogeneity before studying the effects produced by the modification. Obviously, the major goal of these studies is to pinpoint active site residues in order to gain more insight into the mechanism of action of PLA. For some of these modifications it has been concluded – based almost exclusively on the observed loss of enzymatic activity toward substrate present as a lipid-water interface – that the residue modified is an active site residue. Although this form of the substrate enables the enzyme to display its full enzymatic activity, PLA has also a distinct, though considerably lower, activity toward the same substrate present as monomers. The enzymatic activity of PLAs on aggregated substrates can be completely lost by modification of a particular residue, while its active site remains intact. As a matter of fact such modifications lead to zymogen-like proteins. The loss of enzymatic activity toward aggregated substrates can be ascribed to the inability of the modified PLA to bind to lipid-water interfaces or, alternatively, to bind nonspecifically, preventing the formation of products. In these cases the residue modified is quite often termed "essential" without further proving its function. In order to avoid equivocal explanations it is therefore preferable for PLAs to reserve the term "active site residues" to those residues directly involved in binding of the monomeric substrate and the essential Ca^{2+} ion and to the residues performing the actual splitting of the ester bond. Modification of such residues will lead to loss of enzymatic activity of PLA toward substrate present as organized lipid-water interfaces *and* toward monomeric substrate. Residues which upon modification give rise to loss of PLA activity toward aggregated substrate but which do not significantly affect enzymatic activity toward monomeric substrates are most likely involved in the binding to aggregated substrates.

6.1 Sulfhydryl Groups

Based on studies with inhibitors and the absence of any free sulfhydryl groups in all known PLAs it is generally agreed that no sulfhydryl group is essential for activity or binding of PLA (*Long* and *Penny* 1957; *Saito* and *Hanahan* 1962; *Kurup* 1965; *de Haas* et al. 1968; *Wells* and *Hanahan*

1969; *Salach* et al. 1971; *Shipolini* et al. 1971). Reports describing inhibition of PLA activity by sulfhydryl reagents therefore must be ascribed to reaction with residues other than cysteine (*Brown* and *Bowles* 1966; *Wu* and *Tinker* 1969; *Munjall* and *Elliott* 1971).

6.2 Serine

It is now well established that various organic phosphorous compounds do not cause inhibition of PLAs from different sources (*Saito* and *Hanahan* 1962; *de Haas* et al. 1968; *Salach* et al. 1971; *Shipolini* et al. 1971; *Vidal* et al. 1972; *Howard* and *Truog* 1977). The fact that PLA activity is not destroyed by DFP and similar compounds suggests that no Ser is present in the active site of this enzyme. However, since the active site of PLA contains a hydrophobic region, the possibility could not be excluded that more apolar organic phosphorous inhibitors would destroy the enzymatic activity. Both di (1-methylheptyl)phosphofluoridate and 1-methylheptyl-methylphosphofluoridate did not inactivate porcine pancreatic PLA, neither when present as a pure emulsion nor when present as a mixed micelle with various detergents (*Volwerk* 1979). It can thus be concluded that a Ser residue is not involved as an active site residue in PLA. In good agreement with this conclusion is the fact that no Ser residue close to the active site could be detected in the recently reported X-ray structure of bovine PLA (*Dijkstra* et al. 1981b). Most likely the observed inhibition of PLA activity of *Crotalus atrox* by DFP is not due to modification of an active site Ser residue (*Brown* and *Bowles* 1966; *Wu* and *Tinker* 1969).

6.3 Histidine

In contrast to PLA from *Crotalus adamanteus* (*Wells* 1973b), PLA from *Naja naja naja* was inactivated by photo-oxidation, most likely due to modification of histidine groups (*Kocholaty* 1966; *Salach* et al. 1971).

Soon after *Erlanger* et al. (1966, 1967) showed that *p*-bromophenacyl bromide (BPB) inactivated pepsin by reaction with one aspartyl group, this reagent turned out to inhibit also porcine pancreatic PLA (*Postema* 1968). In contrast to the inhibited pepsin, no recovery of enzymatic activity was observed upon treatment of the BPB-inhibited PLA with thiophenol, pointing to the modification of a functional group other than a carboxylate. Also the results of the pH dependence of the BPB inactivation of the porcine PLA (vide infra) suggested that instead of a carboxylate a histidine residue was modified (*Bonsen* et al. 1972b).

More systematic studies (*Volwerk* et al. 1974; *Volwerk* 1979) revealed that the inactivation of porcine PLA and its zymogen follows similar pseudo first order kinetics, suggesting that in both proteins one class of groups was modified. When the residual enzymatic activity was less than 5%, amino acid analyses showed the loss of about one residue of His per mole of PLA or its zymogen in good agreement with the incorporation of 1.1–1.2 mole of [^{14}C] BPB per mole of protein. The [^{14}C] BPB incorporated was shown to be localized mainly on His-48, while 10% of the radioactivity was associated with His-115. Similar experiments with horse pancreatic PLA (*Verheij* et al. 1980a) lacking His-115 showed His-48 to be the only residue reacted with BPB, demonstrating that His-48 is the primary site of modification and that alkylation of this residue produces a PLA inactive both toward micellar and monomeric substrate.

Kinetic studies have shown that Ca^{2+} is an absolute cofactor of PLA. In agreement with the metal ion binding properties of the enzyme and its zymogen (*de Haas* et al. 1971; *Pieterson* et al. 1974a; *Pieterson* 1973) both proteins are protected against BPB inactivation very efficiently by Ca^{2+} and Ba^{2+}, while Mg^{2+} has no effect. In addition short-chain D-lecithins, previously shown to be competitive inhibitors (*Bonsen* et al. 1972a), the products of the PLA hydrolysis (lysolecithin and fatty acid), and the nondegradable substrate analogs (*n*-alkylphosphocholines), when present below their respective CMCs, all protect the enzyme and the zymogen efficiently against the inactivation by BPB. The most effective protection was obtained when both Ca^{2+} and a monomeric D-lecithin were present. On account of the stoichiometric relationship between the loss of enzymatic activity and the incorporation of one mole of BPB per mole of protein and the effective protection by Me^{2+} and substrate analogs against the inactivation, His-48 was assigned to be an active site residue in PLA.

From the effect of pH on the BPB inactivation of porcine PLA the apparent pK of His-48 was found to be 6.2 (*Bonsen* et al. 1972b; *Volwerk* et al. 1974), while His-48 in the bovine PLA was shown to have a pK_{app} of 6.8 (*Dutilh* 1976). The BPB inactivation of this latter PLA was shown to be ten times faster than that of the porcine enzyme using similar conditions (*Dutilh* 1976).

It should be emphasized that the protection against BPB inactivation with all lipids was observed *only below* their CMCs and was thus a result of the formation of the protein-monomer complex. Anomalous behavior was observed when the rate of inactivation of PLA was studied with D-diC_6 or D-diC_7 lecithins in a concentration range above the respective CMCs. When the CMCs are exceeded there is no longer protection but rather an enhancement of the inactivation of PLA. The observed inclusion of BPB into the lipid-water interface and the interaction of PLA with this lipid-water interface would increase the BPB concentration

close to the reaction site, so that enhanced rates of inactivation are to be expected. Taking into account the increase in the rate of PLA catalyzed hydrolysis when the substrate goes from a monomeric to an aggregated form, the observed enhancement is difficult to account for by a concentration effect alone. The stimulated inactivation of PLA was not observed when aggregates of D-diC_{10} lecithin, which are of the lamellar type and to which pancreatic PLA does not bind, were used, although BPB is incorporated into these structures as well. No enhancement of the inactivation rate is observed for the zymogen in the presence of micellar substrate analogs because this protein has no affinity for these aggregated structures.

The identical rates of inactivation of PLA and the zymogen and their similar protection by divalent metal ions and monomeric substrate analogs suggest that the active site pre-exists, at least partially, in the zymogen. This idea is supported by the observation that the zymogen is capable of hydrolyzing monomeric substrates (*Pieterson* et al. 1974b; *Volwerk* et al. 1974, 1979), whereas it is inert towards micellar substrates. These results provide the strongest basis for the hypothesis that PLA contains an additional site for the interaction with lipid-water interfaces (IRS) which is absent in the zymogen.

The values of the second order rate constants for the inactivation of porcine pancreatic PLA by various bromoketones follow the expected chemical reactivity and show that the presence of a phenyl ring is not a structural requirement of the inhibitor. The most important requirement seems to be a certain degree of hydrophobicity (*Volwerk* 1979; *Verheij* et al. 1980a). Essentially similar findings were reported by *Roberts* et al. (1977a) for PLA from *Naja naja naja*. From inactivation of both porcine and equine PLAs with *N*-bromoacetylbenzylamine it was established that exclusively the N-1 position of His-48 is alkylated, pointing to a specific orientation of the imidazole ring. This was confirmed by methylation of His-48 with methyl *p*-nitrobenzenesulfonate (*Verheij* et al. 1980a).

Since efficient protection by monomeric substrate analogs is observed and since the affinity of PLA for these compounds is predominantly dependent on the length of the fatty acyl chains, it was concluded that the reactive His-48 is close to a hydrophobic site on the protein. This site might help to orient the apolar BPB in such a way that covalent bond formation is favored. However, so far no evidence for saturation kinetics with BPB has been obtained for porcine PLA. Therefore, we must conclude that if modification of His-48 proceeds via a noncovalent E-I complex, the K_D of this complex will be considerably larger than the solubility limit of the apolar haloketone (± 0.1 m*M*), so that for all practical purposes the reaction follows normal second-order kinetics. Although all data obtained from the BPB modification support the importance of

His-48, which is conserved in the primary structure of all vertebrate PLAs, it does not specify its catalytic role.

More conclusive evidence on this point was obtained recently by *Verheij* et al. (1980a) who used methyl *p*-nitrobenzenesulfonate to introduce a methyl group specifically on the N-1 position in His-48 of pancreatic PLAs. The methylated pancreatic PLAs have lost all their enzymatic activity both towards micellar and monomeric substrates but still bind monomeric substrate analogs and Ca^{2+} with comparable affinities as the native enzymes. Binding of these ligands to the BPB or 1-bromo-octan-2-one inhibited PLAs is, however, greatly impaired, most probably due to steric hindrance of these more bulky moieties (*Volwerk* 1979; *Verheij* et al. 1980a). Binding to lipid-water interfaces of PLA inhibited with BPB, 1-bromo-octan-2-one or methyl-*p*-nitrobenzenesulfonate is almost identical to that of the unmodified enzyme, thus indicating that the IRS and active site are topographically distinct (*Pieterson* et al. 1974b). Also BPB-inactivated *Naja naja naja* PLA retained its affinity for mixed micelles (*Roberts* et al. 1977a). Introduction of a [^{13}C]-methyl group on His-48 enabled the determination of the pK value of the modified His residue by ^{13}C NMR measurements. From the results obtained it was concluded that the proton on N-3 in the imidazole ring is involved in a strong interaction with a buried carboxylate group, thereby hindering rotation of the imidazole ring, and that the N-1 is involved in catalysis. Based on this result and other observations of the methylated PLA together with Y-ray data, a catalytic mechanism for PLA was proposed (see Sects. 9 and 10).

Since the publication for porcine PLA several reports have appeared describing the selective modification of one His residue per protein molecule by BPB in various PLAs and presynaptic snake venom neurotoxins (*Halpert* et al. 1976; *Halpert* and *Eaker* 1976b; *Fohlman* and *Eaker* 1977; *Kondo* et al. 1978c,d; *Jeng* and *Fraenkel-Conrat* 1978; *Martin-Moutot* and *Rochat* 1979; *Abe* et al. 1977; *Viljoen* et al. 1977; *Magazanik* et al. 1979; *Fohlman* et al. 1979; *Yang* and *King* 1980a,b; *Eaker* 1978). These neurotoxins are basic proteins which have a high degree of homology with PLA and possess PLA activity. Reaction of these neurotoxins with BPB causes complete loss of PLA activity as well as of neurotoxicity. Apparently, an intact active site is not only important for PLA activity but is also a prerequisite for neurotoxicity. It has to be mentioned that in the crotoxin and taipoxin complexes, which consist of two and three polypeptide chains, respectively, only one His residue in the former and two in the latter are modified (*Jeng* and *Fraenkel-Conrat* 1978; *Fohlman* et al. 1979). Crotoxin, consisting of an acidic (crotoxin A) and a basic (crotoxin B) subunit, does not react with BPB and retains its PLA activity. In contrast, crotoxin B alone, the subunit possessing the PLA activity,

incorporates one mole of BPB with the concomitant loss of one His and all PLA activity and neurotoxicity (*Jeng* and *Fraenkel-Conrat* 1978). Most likely the presence of the A chain protects the His-48 in the active site of the B chain. The BPB-modified crotoxin B is still able to form the noncovalent complex with the A chain, in a way similar to that of the unmodified crotoxin B.

Taipoxin consists of three subunits, two of which (α and β) are homologous with PLA, while the third subunit (γ) is homologous to the porcine pancreatic zymogen (*Fohlman* et al. 1979). Both in the α and β subunit one His residue reacts with BPB, whereas it has been suggested that the presence of a carbohydrate moiety situated at or very close to the active site in the γ subunit prevents the reaction with BPB of His in this subunit. It is remarkable that the β subunit of taipoxin, just like *notechis* II-1 from *Notechis scutatus scutatus* (*Halpert* and *Eaker* 1976b), has no PLA activity but does react with BPB. It has been suggested that alkylation of His-48 by BPB might be used generally for the production of high-titer antibodies against snake venoms in a short time and with no ill effects in the antisera-producing animals (*Ramlau* et al. 1979).

Until very recently PLA from *Naja naja naja* was shown to be the only PLA in which one His residue per dimer reacted with BPB, with the complete loss of all its enzymatic activity ("half-site reactivity") (*Roberts* et al. 1977a). However, more recent experiments revealed that the proposed concept of "half-site reactivity" has to be abandoned (*Darke* et al. 1980).

The His residue modified with BPB has been positively assigned to be His-48 in a large number of PLAs and neurotoxic PLAs (*Halpert* et al. 1976; *Halpert* and *Eaker* 1976b; *Kondo* et al. 1978c,d; *Jeng* and *Fraenkel-Conrat* 1978; *Viljoen* et al. 1977; *Magazanik* et al. 1979; *Yang* and *King* 1980a,b). Both for β-bungarotoxin (*Kondo* et al. 1978c,d) and PLA from *Naja naja naja* (*Roberts* et al. 1977a) the His residue modified was shown to have a pK of 6.9.

Ca^{2+} has been demonstrated to protect the inactivation by BPB for a number of these PLAs and neurotoxins (*Halpert* et al. 1976; *Kondo* et al. 1978c,d; *Abe* et al. 1977; *Viljoen* et al. 1977; *Roberts* et al. 1977a; *Yang* and *King* 1980a,b). Only for crotoxin B could no such protecting effect be demonstrated, even at 25 m*M* Ca^{2+} (*Jeng* and *Fraenkel-Conrat* 1978). Modified notexin (*Halpert* et al. 1976) as well as modified β-bungarotoxin (*Abe* et al. 1977) have almost completely lost its Ca^{2+} binding properties just as have modified pancreatic PLAs. In contrast, it has been reported that the BPB-inactivated PLAs from *Naja naja naja* (*Roberts* et al. 1977a), from *Naja nigricollis* (*Yang* and *King* 1980a), and from *Hemachatus haemachatus* (*Yang* and *King* 1980b) still bind Ca^{2+} with comparable affinities as the corresponding native enzymes.

For a number of PLAs and neurotoxic PLAs the protective effect by substrate analogs against BPB modification was also investigated. Although it was claimed that β-bungarotoxin is protected by 5 m*M* egg lecithin (*Kondo* et al. 1980c,d) or 50 m*M* lysolecithin (*Abe* et al. 1977) and PLA from *Bitis gabonica* by lysolecithin (*Viljoen* et al. 1977), it seems more likely that this effect is due to selective sequestering of BPB in the liposomal or micellar systems used. Under the experimental conditions used the enzyme most likely does not bind to the aggregates, causing a protective effect rather than an increased inactivation as observed for the porcine pancreatic PLA (*Volwerk* et al. 1974). This explanation was put forward also by *Roberts* et al. (1977a) to explain the observed "protecting effect" of Triton on the BPB inactivation of *Naja naja naja* PLA, which does not bind to Triton micelles alone. Using micellar phospholipids in the presence of Ca^{2+} ions (*Jeng* and *Fraenkel-Conrat* 1978; *Viljoen* et al. 1977), under conditions favoring binding, it is difficult to draw conclusions about protecting effects of the micellar phospholipid alone. As pointed out already the inactivation is protected by Ca^{2+} and monomers of the phospholipid, whereas the incorporation of BPB into the micelles also affects the inactivation process.

Recently, it has been shown for PLAs from *Naja naja naja* (*Barden* et al. 1980) and from *Naja nigricollis* and *Hemachatus haemachatus* (*Yang* and *King* 1980a,b) that Cibacron blue or ANS protects against BPB modification. Cibacron blue is supposed to bind to the active site of the enzyme, although the dye still can bind to modified enzyme. ANS is supposed to bind to the hydrophobic pocket of the active site and prevents the bulky BPB group from reaching His-48 due to steric hindrance. In contrast to the previously described binding of BPB-inactivated *Naja naja naja* PLA with Cibacron blue, the BPB-modified *Naja nigricollis* and *Hemachatus haemachatus* PLAs lost their ability to bind ANS.

Inactivation of the basic anticoagulant PLAs from *Vipera berus* and *Naja nigricollis* by reaction with 1-bromo-octan-2-one abolished all catalytic and anticoagulant activities of these enzymes, despite the retention of their lipid binding properties (*Verheij* et al. 1980b). Finally BPB has been used as a specific reagent to demonstrate the presence of PLA activity in amniotic fluid (*Gebhardt* et al. 1978) and to investigate the role of PLA in the release of prostaglandins from platelets (*Vargaftig* et al. 1980).

6.4 Tryptophan

The oxidation of two Trp residues per dimer in *Crotalus adamanteus* PLA by *N*-bromosuccinimide (NBS) renders the enzyme inactive and leads to the loss of both anomalous solvent-induced spectral perturbations and cation-related spectral changes (*Wells* 1973b). Although these properties would suggest that oxidation leads to dissociation, the oxidized protein is still a dimer. As pointed out by the auhtor it is unlikely that Trp participates directly in the catalytic process. Most likely the oxidized Trp residue is involved in the binding to lipid-water interfaces. It would therefore be of interest to show whether this modified PLA possesses enzymatic activity toward monomeric substrates.

Reaction of 2-hydroxy-5-nitrobenzylbromide (HNB) with *Crotalus adamanteus* PLA also modified two Trp residues per dimer (*Wells* 1973a). In contrast to the NBS-oxidized PLA, the HNB-modified PLA retains full catalytic activity and also exhibits spectral perturbations in the presence of divalent cations. Because of the presence of three Trp residues per subunit (*Heinrikson* et al. 1977) it seems very likely that the reagents modify different Trp residues.

Viljoen et al. (1976) carried out Trp modification for PLA from *Bitis gabonica* with NBS. They were able to show that oxidation of Trp-31 was responsible for the observed loss of enzymatic activity toward substrate present as organized lipid-water interfaces. In addition these investigators found that Ca^{2+} or $diC_{16}PC$ (30 μM) does not or only very weakly protect against the oxidation. In contrast micelles of lyso-PC and particularly in the presence of Ca^{2+} do protect against oxidation of Trp-31. Although *Viljoen* et al. (1976) claim that Trp-31 is an active site residue, their second explanation that Trp-31 is involved in the binding to lipid-water interfaces seems more likely. This explanation is consistent with the fact that Trp-31 is variable in most PLAs. Moreover, Ca^{2+} ions alone do not protect against inactivation, whereas Ca^{2+} ions plus micelles do protect. Unfortunately, the enzymatic activity of the oxidized PLA toward monomeric substrate has not been tested. Apparently NBS is not incorporated in micelles of lyso-PC, otherwise a more rapid modification would be expected. PLA from *Bitis gabonica* was reacted also with *o*-nitrophenylsulfenylchloride (NPS) (*Viljoen* et al. 1976), modifying predominantly Trp-70 with retention of full enzymatic activity. More drastic conditions lead to the modification of an additional Trp residue, which was found to be Trp-31, with a concomitant loss of enzymatic activity. Also NBS-modified Trp-31 PLA incorporates NPS.

Modification of the single Trp-3 residue in porcine pancreatic PLA with NPS did not affect the enzymatic activity when assayed on micellar L-diC_8PC (*Slotboom* and *de Haas* 1975). In the egg yolk assay the Trp-3-

modified PLA possesses only half of the activity as compared to the native enzyme.

Yoshida et al. (1979) modified the single Trp at position 70 by NBS oxidation in one of the four iso-PLAs isolated from the sea snake *Laticauda semifasciata* and found that the activity decreased considerably and becomes comparable to those of the other three isoenzymes lacking this Trp residue. Moreover the authors reported the interesting observation that the Trp modification changed the kinetic properties of this isoenzyme. NBS oxidation of the Trp-containing enzyme produced a PLA which, just as the native Trp-free isoenzymes, displays biphasic kinetics. The lag periods were shown to disappear in the presence of the reaction products lyso-PC and fatty acid. The mechanism of the product activation of the Trp-free proteins is not clear at present. It is tempting, however, to relate this behavior to a Trp-involving, lipid-binding site on this enzyme.

NBS was reported by *Howard* and *Truog* (1977) to oxidize Trp in β-bungarotoxin with loss of PLA activity and neurotoxicity. Both NBS and 2-hydroxy-5-nitrobenzylbromide modified all of the tryptophan present in *Naja naja naja* PLA with the loss of almost all activity toward substrate present in lipid-water interfaces (*Roberts* et al. 1977a). It is not certain whether all three Trp residues now known to be present in this PLA (*Darke* et al. 1980) were modified. In these latter two modifications it seems very likely that the observed losses in enzymatic activities are due to impaired binding of the modified enzymes to lipid-water interfaces.

6.5 Methionine

PLA from *Crotalus adamanteus* venom was found to slowly react with 2-bromoacetamido-4-nitrophenol by modification of the single Met-10 residue (*Wells* 1973a). When about 0.75 moles of *p*-nitrophenol groups were incorporated per subunit, all enzymatic activity was still present. No detectable spectral perturbations of the *p*-nitrophenol group were observed in the presence of divalent cations, demonstrating that these ions do not bind in the environment of Met.

Carboxymethylation of horse, bovine, and pig iso-PLAs, all possessing only one Met residue at position 8, resulted in a rather slow loss of enzymatic activity (*van Wezel* et al. 1976; *Meyer* 1979). After approximately 22 h reaction 65% of the enzymatic activity of these enzymes is still present. When, however, 8-*M* urea is present, inactivation of porcine iso-PLA is fast (*van Wezel* et al. 1976). The modified enzyme has lost its enzymatic activity toward both micellar and monomeric substrates. Direct binding studies of this carboxymethylated iso-PLA showed

that it does not bind any longer to lipid-water interfaces but that it still could bind a monomeric substrate analog and Ca^{2+}, though with a lower affinity than the native enzyme. Based on these observations it was proposed that Met-8 was part of the IRS. From the X-ray structure of bovine PLA (*Dijkstra* et al. 1978, 1981b) it turns out that Met-8 is buried in the interior of the protein. Apparently upon introduction of the zwitter ionic group under rather vigorous conditions part of the tertiary structure of the enzyme is considerably distorted. Contrary to native PLA, removal of urea does not result in proper refolding to the active conformation, resulting in the loss of enzymatic activity upon modification. Therefore the previous conclusion that Met-8 is part of the IRS is no longer tenable.

Porcine PLA, having an additional Met residue at position 20, is rapidly carboxymethylated in the absence of urea under conditions where Met-8 of the iso-PLA is hardly reactive. The modified protein retained about 50% of its activity (*Meyer* 1979).

Although no inactivation was observed upon prolonged reaction of porcine PLA with methyliodide, the reagent slowly alkylates Met-20 as was demonstrated by incorporation of [^{14}C]-methyliodide. Similarly as observed for carboxymethylation, it was found that methylation of iso-PLA was considerably slower than that of normal porcine PLA. The observed differences in rates of alkylation of Met-8 and Met-20 in porcine PLA enabled *Meyer* (1979) to selectively prepare both S-carboxymethyl Met-20 and S-methyl Met-20 porcine PLA. After purification of the alkylated PLAs he found a 1:1 stoichiometric incorporation of the alkylating agents, which were found to be located exclusively at Met-20. Both modified proteins possess similar enzymatic activities toward monomeric substrates as the native enzyme. Also, the affinities of both alkylated PLAs for monomeric and micellar substrate analogs as well as for Ca^{2+} were not affected. Furthermore, the specific activity of S-methyl Met-20 PLA when tested with the egg yolk assay was found also to be similar to that of native PLA, whereas that of the S-carboxylmethyl Met-20 PLA was only about 50%. Monolayer experiments of these two modified PLAs revealed that the penetrating power was noticeably decreased, in particular for that of the carboxymethyl analog. Most likely the more drastic effects on the properties of the enzyme upon carboxymethylation of Met-20 as compared to those upon methylation are due to the introduction of a positive and a negative charge (carboxymethylation) or a positive charge only (methylation). The finding that the introduction of a positive charge on Met-20 has little influence on the properties of the pancreatic PLA is compatible with the occurrence of a positively charged Arg residue at this position in some snake venom PLAs (see Sect. 4). These results together with the three-dimensional X-ray structure of the bovine PLA suggest that Met-20 is part of the IRS.

6.6 Lysine

Viljoen et al. (1977) concluded that Lys is a residue essential for enzymatic activity of *Bitis gabonica* PLA based on the observation that reaction of pyridoxal-5′-phosphate followed by reduction with sodium borohydride inactivated the enzyme toward the substrate present as a lipid-water interface. The enzyme is protected against inactivation by micellar lysolecithin but not by Ca^{2+}. It is therefore very likely that the modified residue(s) is involved in some way in the binding to aggregated substrate. The loss of enzymatic activity was not due to modification of *one* particular Lys residue per enzyme molecule but to four different Lys residues, each modified by about 25%. First-order plots were obtained which, according to the authors, indicate that the modification does not involve groups with different reactivities. Since the inhibition reaction velocities, measured as a function of pyridoxal-5′-phosphate concentration, showed saturation kinetics and complete loss of enzyme activity was found at the stage where one mole of pyridoxal-5′-phosphate had been incorporated per mole of enzyme, it was concluded that this modification is of the active site-directed type.

Pyridoxilation followed by reduction with ^{3}H-labeled sodium borohydride was used to radioactively label β-bungarotoxin (*MacDermot* et al. 1978). The dissociation constant for binding to several tissue subfragments of nervous tissue was found to increase tenfold upon pyridoxylation. No data were reported for loss of PLA activity.

6.7 Carboxylate Groups

Recently PLA from *Naja naja oxiana* has been modified with *N*-diazoacetyl-*N*′-(2,4-dinitrophenyl)-ethylenediamine (DBE) in the presence of Ca^{2+} (*Zhelkovskii* et al. 1977, 1978b). When one carboxylate group per dimer was modified, the authors found complete inactivation of PLA, using monomeric L-diC_4 PC as substrate. Their evidence, however, seems to be based heavily on the previously observed "half-site reactivity" by *Dennis* and co-workers (cf. *Roberts* et al. 1977a) which is no longer valid (*Darke* et al. 1980). Proflavin, a competitive inhibitor for this enzyme, and Ca^{2+} ions did not have any effect or increased the incorporation. After reduction of the modified protein with sodium borohydride, indications were obtained for selective modification of an Asp residue, which has not yet been assigned to a particular Asp residue.

In order to obtain information about the involvement of particular carboxylate groups in the active site and in Ca^{2+} binding of bovine pancreatic PLA, *Fleer* et al. (1981a) used the water-soluble 1-ethyl-3-(*N*,*N*-dimethyl)

amino propyl carbodimide (EDC) and semicarbazide as the nucleophile. Depending on the conditions they were able to block all carboxylates except one (Asp-99) or two (Asp-39 and Asp-99). Both modified proteins have lost their enzymatic activity toward micellar and monomeric substrates and have also lost their Ca^{2+} binding properties. Repeating these experiments in the presence of Ca^{2+} ions, the carboxylate of Asp-49, in addition to those of Asp-39 and Asp-99, was not modified. This protein still possesses enzymatic activity. Its Ca^{2+} binding properties were lost upon further modification in the absence of Ca^{2+} under conditions where only Asp-49 reacted. Therefore, it was concluded that Asp-49 is the Ca^{2+} binding ligand, which is in good agreement with the results from the X-ray structure of bovine pancreatic PLA (*Dijkstra* et al. 1981b). From the pH dependence of the Ca^{2+} binding to bovine PLA a group with an apparent pK of 5.25 was found which was tentatively assigned to Asp-49.

6.8 Arginine

Recently, *Vensel* and *Kantrowitz* (1980) reported the modification of an essential Arg residue in porcine pancreatic PLA by reaction with phenylglyoxal. About one Arg residue per PLA molecule was modified, based on the assumption of a stoichiometry of two phenylglyoxal molecules per Arg residue, which is not necessarily always valid.

Moreover, the correlation of the inactivation of PLA with the number of Arg residues modified, derived in a rather unsatisfactorily way, does not show very convincingly that modification of one Arg residue per PLA molecule correlates with the loss of enzymatic activity. Almost no protection by Ca^{2+} was found, whereas a good protection against the inactivation was exhibited by micellar *n*-alkylphosphocholines. Increasing the pH from 6.5 to 9.5 leads to a more rapid inactivation, whereas it decreases the efficiency of protection by the micellar substrate analogs. It is known, however, that phenylglyoxal can transaminate α-amino groups even more rapidly than it modifies Arg residues (*Takahashi* 1968). Because the presence of a free α-amino group is essential for enzymatic activity and binding of porcine pancreatic PLA to lipid-water interfaces, *Vensel* and *Kantrowitz* (1980) tried to prove by amino acid analysis and qualitative end group analysis that the inactivation was not due to transamination.

In the reviewers' opinion the methods used to show that transamination had not occurred are not sensitive enough. The effects of pH and micellar substrate analogs hold equally well for transamination of the α-amino group. Moreover, 2,3-butanedione and 1,2-cyclohexanedione, being more specific for Arg than phenylglyoxal, cause a much slower

inactivation, despite the large excess used of each of these reagents. From extensive model studies in the reviewers' laboratory it turned out that phenylglyoxal gives rise to excessive transamination of porcine pancreatic PLA with simultaneous modification of Arg residues, the number of which depends on the reagent concentration. Using phenylglyoxal concentrations lower than those of *Vensel* and *Kantrowitz,* complete inactivation of porcine PLA was observed. Then the protein was subjected to CNBr cleavage. After separation of the liberated N terminal octapeptide from the remainder of the protein, it was found by amino acid analysis that in addition to the disappearance of 80% of Arg-6, Ala-1 was almost completely absent. In order to prevent transamination, the α-amino group in porcine AMPA was protected with an *N-t*-Boc group prior to modification by phenylglyoxal using similar conditions as for PLA. As a result the modified protein after deblocking of the α-amino group still possessed considerable enzymatic activity, while approximately one Arg (presumably Arg-6) per AMPA molecule was modified (*Fleer* et al. 1981b). Therefore, *Fleer* et al. preferred the use of [^{14}C]-labeled 1,2-cyclohexanedione in the presence of borate to modify Arg residues in porcine PLA. Despite the formation of some transaminated PLA they were able to isolate a PLA modified exclusively at Arg-6. Extensive characterization revealed that the modification had almost no effect on the V_{max} values when assayed both on micellar and monomeric substrates, and on V_{max} values, assayed both on micellar and monomeric substrates, and on the Ca^{2+} binding properties as compared to unmodified PLA. The affinity of the modified PLA to micellar substrate analogs as well as its penetrating capacity into monomolecular lecithin films was improved as compared to the unmodified PLA.

6.9 α-Amino Group

Transamination of proteins by glyoxylic acid in the presence of Cu^{2+} is assumed to be specific for the α-amino group (*Dixon* and *Fields* 1972). This assumption is based on the mechanism proposed for transamination in which the peptide carbonyl group participates in the formation of an intermediate. A rather rapid inactivation was observed for both porcine and equine PLA ($t_{1/2} \approx 53$ min and 16 min, respectively, under conditions as described by *Dixon* and *Fields* 1972), whereas bovine PLA is much more stable ($t_{1/2} \approx 400$ min) (*Slotboom* et al., to be published). Ca^{2+} and monomeric substrate analogs do not appreciably protect against the inactivation, whereas micellar substrate analogs almost completely protect porcine PLA against the modification. In order to check the specificity of the reaction, porcine pro-PLA, devoid of an α-amino group, was reacted

under similar conditions as PLA. It was found that at a stage where PLA was approximately 80% inactivated about 15% of the potential activity of the zymogen was lost, indicative of some kind of side reaction.

Prolonged reaction times lead to an increased loss of the potential activity of the zymogen. Because of the structural similarity of glyoxylic acid with arginine modifying reagents (α, β-diketone structure) it could not be precluded a priori that one or more Arg residues were modified. In particular, the C terminal Arg residue of the activation peptide could possibly be modified, thereby preventing tryptic activation of the zymogen. This could indeed be the case because the lost potential activity of the zymogen could partially be restored upon storage at pH 8, conditions which favor the dissociation of Arg-phenylglyoxal complex (*Takahashi* 1968).

When the transamination reaction was performed in the presence of 6-*M* guanidine hydrochloride or 8-*M* urea, complete inactivation of bovine, porcine, and equine PLAs within 30–60 min was observed. After similar treatment of porcine pro-PLA, all potential activity was recovered, indicating no additional inactivation[16].

Transaminated porcine PLA prepared in this way was subsequently purified by ion exchange chromatography, and the only detectable modification was the conversion of the α-amino group into a keto group. The transaminated porcine PLA had lost its enzymatic activity toward micellar substrate due to its considerably decreased affinity for lipid-water interfaces but still retained its enzymatic activity toward monomeric substrate. In these respects the transaminated PLA thus resembles very much the zymogen. As a matter of fact, the results of Photo CIDNP NMR spectroscopy (*Egmond* et al. 1980) as well as the tentative 2.4-Å X-ray structure of transaminated bovine PLA (*B.W. Dijkstra,* personal communication) support this conclusion. Subsequent treatment of a transaminated protein with *o*-phenylene diamine is reported (*Dixon* and *Fields* 1972) to selectively remove the N terminal amino acid residue. This sequence of reactions was applied to the enzymatically inactive Ala^{-1}-AMPA[17], which indeed produced in about 30% overall yield enzymatically active AMPA having the same specific activity as authentic AMPA (*Slotboom* et al., to be published).

The use of glyoxylic acid to selectively modify the α-amino group is of particular interest for the snake venom PLAs because it allows one to study whether it has similar effects on enzymatic activity and lipid binding

16 Using radioactive glyoxylic acid incorporation of ^{14}C radioactivity was observed both in PLA and pro-PLA. Upon subsequent purification and dialysis almost all of the incorporated radioactivity disappears

17 AMPA in which an Ala residue has covalently been attached to the N terminal Ala^1

properties as observed for the pancreatic PLAs. PLAs from *Crotalus atrox, Vipera berus,* and *Naja melanoleuca* were rapidly inactivated by glyoxylic acid in the presence of 4-*M* tetramethylurea (*Verheij* et al. 1981). After purification, the modified proteins have no enzymatic activity when tested with micellar substrate but partially retained their activity toward substrate in monomeric form. Direct binding studies revealed that the affinity of the transaminated snake venom PLAs for lipid-water interfaces was decreased five- to tenfold, but in contrast to transaminated porcine PLA, a strong interaction was still observed. A possible explanation for this difference could be that the rather weak binding of the pancreatic PLAs is mainly induced by its N terminal region, whereas the stronger binding of the snake venom PLAs is predominantly due to interaction with other hydrophobic regions in the protein. Therefore, upon transamination of the α-amino groups relatively small effects on the affinity to lipid-water interfaces could be expected in the latter case as compared to the former. The lack of enzymatic activity of the modified pancreatic enzyme can be explained by the impaired interaction with lipid-water interfaces. However, even though the modified venom PLAs do bind to lipid-water interfaces, no enhanced activity induced by the interface was observed. This was explained (*Verheij* et al. 1981) by the assumption that PLA bound to lipid-water interfaces can occur in two conformations characterized by low and high turnover numbers, respectively, when acting on these aggregated substrates.

6.10 Tyrosine

From direct binding studies of pancreatic PLAs with Ca^{2+}, monomeric, and micellar substrates analogs using spectroscopic methods it was found that binding of these ligands perturbed one or more Tyr residues (*Pieterson* et al. 1974a; *van Dam-Mieras* et al. 1975; *Donné-Op den Kelder* et al. 1981). *Meyer* et al. (1979a,b) nitrated Tyr residues in horse, porcine, and bovine (pro)-PLAs with tetranitromethane (TNM), giving rise to a rapid, partial loss of enzymatic activity, which is even more rapid in the presence of lysolecithin micelles and Ca^{2+}. This latter effect was attributed to the incorporation of the reagent into the lysolecithin micelles, thus enhancing the rate of nitration of those Tyr residues involved in the micellar binding site of PLA. The presence of lysolecithin also protects against polymerization which was a side reaction in its absence. After purification of the mono- and di-NO_2 monomeric proteins it was found that in all three pancreatic PLAs Tyr-69 was always nitrated. In addition Tyr-124 in porcine and Tyr-19 in horse PLA were also nitrated. All these mononitrated PLAs still posses 15%–50% of the

enzymatic activities of the respective unmodified enzymes when assayed on micellar substrates, indicating that the modified Tyr residues are not active site residues. The NO_2-Tyr residues could be reduced by sodium dithionite into NH_2-Tyr residues. The various NH_2-Tyr PLAs are still enzymatically active, and due to the low pK values of these NH_2 groups, they could easily be transformed into the corresponding dansyl-NH_2-Tyr PLAs also possessing enzymatic activity.

From direct binding studies using ultraviolet difference spectroscopy it was found that NO_2-Tyr-69 as well as the dansyl-NH_2-Tyr-69 porcine and equine PLAs and in particular NO_2-Tyr-19 and dansyl-NH_2-Tyr-19 equine PLA possess a higher affinity for lipid-water interfaces than the native enzymes. Upon interaction of the latter dansyl-NH_2-Tyr PLAs with micellar substrate analogs a considerable increase in fluorescence and a concomitant blue shift of the emission maximum of the dansyl group was observed. No such effects occurred for the corresponding dansyl-NH_2-Tyr pro-PLAs nor for dansyl-NH_2-Tyr-124 porcine PLA. It has therefore been concluded that Tyr-19 and Tyr-69 are part of the IRS in pancreatic PLA.

Monomer phospholipid binding at pH 6 as monitored by ultraviolet difference spectroscopy induces a strong hydrophobic perturbation of NO_2-Tyr-69 and -19, and again the microenvironment of NO_2-Tyr-124 is not changed. When measured at pH 8 monomer binding decreased considerably, most probably due to charge repulsion between the phosphate moiety of the phospholipid analog and the negatively charged NO_2-Tyr-69 residue which has a lower pK than Tyr. The corresponding NH_2-Tyr-69 PLA does not show this difference. In addition to Tyr-69 and Tyr-19 another Tyr residue, most probably Tyr-52 located in the active site cavity close to His-48 and Tyr-69, is also perturbed on monomer binding. So far this residue could not be modified by TNM nor by other Tyr-modifying reagents (*Meyer*, unpublished observations).

Ca^{2+} binding affects the NO_2-Tyr-69 residue as was shown by ultraviolet difference spectroscopy and the lowering of the pK of NO_2-Tyr-69, whereas no such effects were found for NO_2-Tyr-19 and -124.

The introduction of the NO_2 group and in particular of the dansyl-NH_2 group on Tyr-69 and Tyr-19 greatly enhances the penetrating power of these modified enzymes for monomolecular L-di C_{10} PC films. When the pH is increased from 6 to 9 the penetrating power of the NO_2-Tyr-69 porcine and equine PLAs, however, decreased considerably due to the introduction of a negative charge.

The availability of various pure NO_2-Tyr PLAs was of great help for the identification of resonances in the ^1H-NMR spectrum of PLA originating from Tyr residues. By using the Photo CIDNP method, developed by *Kaptein* et al. (1978), it was possible to assign resonances corresponding

to $H_{3,5}$ protons of Tyr-69 and Tyr-124 in porcine PLA. Monomer binding as studied with this technique showed, in agreement with the above mentioned results, that Tyr-69 is perturbed while Tyr-124 is not (*Jansen* et al. 1978).

For various studies, in particular for monolayer experiments (see Sect. 5), the availability of highly radioactively labeled, enzymatically active PLA is required. For this purpose iodination of Tyr residues is very attractive. Reaction of bovine pancreatic (pro)-PLAs with an equimolar amount of iodine resulted for the bovine proteins in the exclusive monoiodination of Tyr-69 while in the porcine proteins in addition to extensive monoiodination of Tyr-69 Tyr-124 also monoiodinated to a small extent (*Slotboom* et al. 1978c). As compared to the native enzyme the iodinated enzyme has a higher specific activity when assayed in the egg yolk assay, while similar V_{max} values were found using micellar diC_8 PC. The introduction of one atom of iodine on Tyr-69 in pancreatic PLA slightly increases the penetration capacity of the enzyme in monolayers of L-di C_{10} PC which is compatible with a better K_m found for monoiodinated PLA activity on micelles of di C_8 PC (*Pattus* et al. 1979a).

Crotalus adamanteus PLA upon reaction with iodine retained 88% of its activity when one mole of di-iodotyrosine per protein molecule was present (*Wells* 1973b).

Bon et al. (1979) also used iodination to radioactively label the subunits of crotoxin. Upon incorporation of one atom of iodine per mole of protein the iodinated component B showed no significant decrease of the PLA activity and retained full neurotoxic potential when tested after complexing with native component A. When the extent of iodination was 0.5–1.0 atom of iodine per mole component A its efficiency in potentiating neurotoxic effects of component B was decreased by about 40%.

Upon reaction of purified bee venom PLA with imidazolide derivatives of long-chain fatty acids, a single acyl residue is covalently coupled, presumably to a Tyr residue (*Drainas* et al. 1978; *Drainas* and *Lawrence* 1978; *Lawrence* and *Moore* 1975; *Lawrence* 1975). Kinetic analysis of the acylated enzyme shows an increase of the enzymatic activity which is almost entirely determined by enhancement of the V_{max} term (53-fold), with a small modification of the K_m value. Addition of free fatty acids has the same effect, though to a lesser extent. Similar phenomena were observed for PLAs from *Vipera ammodytes* and *Naja naja* venoms. Of the possible explanations for this phenomenon given by the authors, the most attractive mechanism is that activation faciliates functional penetration of the lipid interface by the enzyme.

6.11 Miscellaneous

6.11.1 Modification of Phospholipase A_2 with Ethoxyformic Acid Anhydride

Ethoxyformic acid anhydride (EOFA) is a very reactive nonspecific reagent which reacts in proteins with several amino acid side chains such as phenolates, imidazoles, carboxylates, sulfhydryls, α- and ϵ-amines, and guanidino groups (*Larroquère* 1964; *Melchior* and *Fahrney* 1970; *Mühlrád* et al. 1967; *Burstein* et al. 1974). The reagent is unstable and hydrolyzes rapidly in aqueous media ($t_{1/2}$ at pH 7.0 and 20–25°C in between 8 and 25 min; c.f. *Berger* 1975; *Larroquère* 1964; *Melchior* and *Fahrney* 1970). Despite these drawbacks the reagent has been used for chemical modification of a large variety of proteins, including PLA.

Wells (1973b) used this reagent to identify whether a Lys of His residue might be important in the active site of *Crotalus adamanteus* PLA. Because no radioactive EOFA was used, the modification of His was determined by spectral changes at 230 nm. These measurements are not a reliable measure of the involvement of His when Tyr residues are simultaneously ethoxyformylated. Spectral changes during selective de-ethoxyformylation of His by mild hydroxylamine treatment (20 m*M*) not affecting O-ethoxyformyl Tyr residues are much easier to interpret. While *Wells* found almost no ethoxyformylation of His but a complete loss of enzymatic activity, the same modification in the presence of 4-*M* urea showed the modification of 4 His residues (per dimer) with retention of 26% of the enzymatic activity. The fact that four His residues per dimer were modified would also mean that the active site His-48 was modified, which is not compatible with retention of 26% of the activity. Therefore, the result of the spectroscopic determination is probably misleading. During subsequent treatment with 0.1-*M* instead of 0.02-*M* hydroxylamine not only His but also Tyr residues are probably de-ethoxyformylated. These results suggest that other important group(s) were modified as well. The observation that EOFA modification is first order with respect to dimeric enzyme and EOFA led *Wells* to conclude that this modification is an example of "half-site reactivity". This hypothesis was supported by the findings that only one Lys residue/dimer is modified and that there were still detectable cation-induced optical effects and by the recovery of the theoretically predicted specific activities upon dissociation-reassociation of 50% and 100% inactivated PLA at pH 5.0. Based mainly on these observations it was concluded that within the active site of *Crotalus adamanteus* PLA a Lys residue was identified. Besides the observation that until now no Lys residue in any sequenced PLA has been reported on a position which in the tertiary structure of

the bovine pancreatic PLA forms part of the active site (see Sect. 9), there are in the reviewers' opinion several reasons to re-evaluate this modification. It is now known that the *Crotalus adamanteus* PLA has a free α-NH_2 group which could have reacted also with EOFA. Moreover Tyr residue(s) are very likely simultaneously ethoxyformylated. Taking into account the large variety of possible sites for incorporation, a more direct determination of the residue(s) modified as well as of the number of residues modified by radioactive EOFA should be considered. Furthermore, it would have been worthwhile to determine whether enzymatic activity is also lost when assayed using monomeric substrate.

Upon reaction of EOFA with *Naja naja naja* PLA the group of *Dennis* (*Roberts* et al. 1977a) claimed that two amino groups, one Tyr and half a His per enzyme molecule, were modified with retention of 15% of enzymatic activity. Based on this observation and the results obtained after consecutive EOFA-BPB or BPB-EOFA modifications, it was concluded that EOFA also shows half-site reactivity. Most likely the same arguments which led to the withdrawal of the half of the site reactivity of BPB (*Darke* et al. 1980) also hold for EOFA modification.

EOFA and acetic anhydride have been reported to modify only NH_2 groups and no His or Tyr residues in crotoxin (*Jeng* and *Fraenkel-Conrat* 1978; *Bon* et al. 1979). With a 50-fold excess two NH_2 groups reacted in crotoxin with retention of all PLA activity and neurotoxicity, while higher concentrations of EOFA modified progressively more NH_2 groups with increasing losses of PLA activity and neurotoxicity. In this respect the separate crotoxin B-chain (basic PLA) behaves in a way almost identical with that of the complex, whereas in the A-chain (Crotapotin) EOFA acylates only one NH_2 group.

Simlarly, all PLA activity and neurotoxicity are lost upon reaction of EOFA with β-bungarotoxin, although no data were reported in which amino acid residues were modified (*Howard* and *Truog* 1977; *Ng* and *Howard* 1978). Ca^{2+} and di C_6 PC (above the CMC) were found to protect almost all PLA activity against inactivation by EOFA, whereas the neurotoxic properties are still lost. The authors suggest that there are possible two sites on the protein, one responsible for PLA activity which can be protected and another one for neurotoxicity which can not be protected against EOFA modification.

Reaction of *notechis* II-5 with EOFA showed the modification of one Tyr, one Lys, and two His residues (*Eaker* 1978). One of the His residues reacts slowly, the other fast. Although contradictory results were obtained concerning whether PLA activity is lost or not, depending on the use of egg yolk or *purified* egg yolk PC, the authors claimed to have modified His-14 and His-21, which would mean that His-48 was not modified. Most probably His-21 is involved in the binding of the enzyme to lipid-water

interfaces. More extensive treatment with EOFA led to inactivation which could not be reversed with hydroxylamine. It is suggested that a Lys residue has been modified, although no supporting evidence was presented.

6.11.2 Cross-Linking of Phospholipase A_2

In order to demonstrate cross-linking of *Naja naja naja* PLA under conditions in which the enzyme exists in an aggregated state, *Lewis* et al. (1977) used various photoactivatable heterobifunctional arylazides. The reagents used, *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS), ethyl *N*-5-azido-2-nitrobenzoyl-aminoacetimidate HCl (ANB-AI), and ethyl 4-azido benzimidate HCl (AB-I), were found to react with about 1.5–1.8 Lys residues without appreciable loss of enzymatic activity and without polymerization in the absence of irradiation. After irradiation of the modified PLAs at a concentration of 1 mg/ml it was found that except for the PLA-AB-I complex the other two complexes gave rise to ± 80%–90% cross-linking with the formation of roughly similar ratios of di-, tri-, and tetramers. Only about 50% of the PLA-AB-I complex was cross-linked, 31% of which resulted in the formation of dimers. The unpurified, cross-linked PLAs all had retained 20%–80% of the enzymatic activity. Because this activity is significantly higher than can be explained by the presence of monomeric PLA in the mixture, a portion of the cross-linked proteins retains PLA activity.

To test the hypothesis that crotoxin A serves as a "chaperon" to enhance the specificity of crotoxin B *Hendon* and *Tu* (1979) cross-linked both polypeptide chains using the bifunctional cross-linking agent dimethylsuberimidate. An average of three cross-links were introduced as found from the number of Lys residues blocked. Most likely two of these cross-links occur between the subunits A and B, while the third is presumably present as an intrapeptide cross-link on subunit B. No loss of PLA activity of the cross-linked crotoxin was observed indicating that cross-linking does not interfere with the PLA active site present in the B chain. In contrast, neurotoxicity of the cross-linked crotoxin is lost. Since the PLA activity of the cross-linked complex remains unaffected and since this activity is believed to be directly involved in presynaptic action in neurotoxicity, it appears that the loss of neurotoxicity occurs from some form of interference between the cross-linked complex and the target site, thus adding credence to the chaperon concept for crotoxin A.

Roberts et al. (1977c) also used dimethylsuberimidate for cross-linking experiments to determine whether *Naja naja naja* PLA at low concentrations aggregates in the presence of substrate micelles. Moderate amounts

of covalently linked enzymes are formed when cross linking was performed in the absence of micelles at concentrations where the enzyme is a dimer. Only under conditions favoring binding of the enzyme to mixed micelles was it found that not only the dimer-monomer ratio increased but also that the trimer-monomer ratio increased even more as compared to conditions where no binding exists between the enzyme and mixed micelles (c.f. also Sect. 5).

6.11.3 Photoaffinity Labeling

So far, only *Huang* and *Law* (1978, 1981) used photoaffinity labeling to study the interaction of PLA *(Crotalus atrox)* with phospholipids. They synthesized a racemic 1,2-dihexyl ether analog of PE which contains in the polar head group an ethyl diazomalonyl group and which was found to be an effective substrate analog. After photolysis of a mixture of the PLA and the photolabile PE analog (present in a concentration of only four times its CMC), they observed covalent linkage of the enzyme with the PE by the photochemically generated carbene. From the amount of incorporated substrate analog the ratio of bound ligand to 14 000-dalton polypeptide was 1.04 and no half-site reactivity was found. Extensive inhibition was observed, whether or not Ca^{2+} was present, although inhibition was greater in the presence of Ca^{2+} as could be expected for an ordered mechanism of binding with Ca^{2+} adding first. When the diether PE analog without the photolabile group was added along with the photolabile PE analog the enzyme was protected from inactivation, suggesting that the two phospholipids compete for the same site. Based on the assumption that *Crotalus atrox* PLA, like the *Crotalus adamanteus* PLA, is only active in the dimeric form, *Huang* and *Law* (1978) suggest that alkylation of one subunit in the dimeric enzyme leads to rapid dissociation and reassociation of unalkylated monomers to give active dimers.

The radioactivity associated with the PE analog incorporated into the PLA was found to be localized in two fragments, viz. a large peptide comprising residues 43-97 and the N terminal segment residues 1-15. Undoubtedly important information toward a better understanding of the architecture of the enzyme-substrate interaction can be expected upon further exploration of this attractive approach.

6.11.4 Semisynthesis of Pancreatic Phospholipase A_2

The α-helical N terminal region of pancreatic PLAs has been shown to be directly involved in the binding of these enzymes to lipid-water interfaces (*Van Dam-Mieras* et al. 1975). Furthermore, the absence of micellar activity of the zymogen as well as of various α-amino-blocked porcine AMPAs (vide infra) led *Abita* et al. (1972) to conclude that the α-amino

group stabilizes the active geometry of the catalytic site. To further elucidate the important role of the N terminal region on activity and lipid-binding properties of the pancreatic PLAs chemical modification was less attractive or not possible. Therefore semisynthesis was used to substitute various amino acid residues at the N terminal region (*Slotboom* and *de Haas* 1975; *Slotboom* et al. 1978a). Such a semisynthetic approach requires that the ϵ-amino groups of Lys residues must be selectively protected, enabling removal and reintroduction of amino acid residues or peptides to take place exclusively at the free α-amino group. For the pancreatic PLAs this was done by amidination of the zymogens with methylacetimidate followed by tryptic activation. The resulting ϵ-amidinated PLAs (AMPAs) have about 70% of the enzymatic acticity of that of native PLAs when assayed on micelles of L-diC_8 PC; their behavior is almost identical in all respects to that of the unmodified PLAs. It is therefore not necessary to remove afterwards the protecting amidino groups. Using this procedure *Pattus* et al. (1979a) prepared ^{3}H-labeled AMPA for monolayer studies (see Sect. 5). Upon successive removal of N terminal amino acid residues of porcine AMPA by the Edman procedure des-Ala-1-, des-Ala-1.Leu-2-, and des-Ala-1.Leu-2.Trp-3-AMPAs were obtained which are devoid of enzymatic activity on micellar substrate. Although des-Ala-1 AMPA still possesses some activity toward monomeric substrate, removal of more than one amino acid residue further decreases this activity. Various amino acids were covalently coupled to des-Ala-1 AMPA, resulting in AMPA analogs which were always catalytically active on monomeric substrate. Whereas substitution of L-Ala-1 by Gly, β-Ala, L-Asn, L-Asp, or L-NorLeu produced AMPA analogs catalytically active on micellar substrates, this was found not to be the case for AMPA analogs having N terminal D-Ala, L-α-amino isobutyric acid, *N*-methyl-L-Ala, L-Leu, or L-Phe. These latter analogs do not bind to lipid-water interfaces despite the availability of a free α-amino group (*Slotboom* et al. 1978a; *Slotboom* et al., to be published). Most likely this is due to the presence of a rather bulky, branched, or D-amino acid residue, which for steric reasons prevents the proposed interactions shown in Fig. 13 with concomitant distortion of the IRS (*Slotboom* et al. 1977). Similarly various ^{13}C-enriched amino acids have been introduced at the N terminal position of pancreatic AMPAs, enabling the determination of the pK values of the α-amino groups. A pK of 8.4 was found for the α-amino group of porcine AMPA, in good agreement with similar values (8.3 and 8.45, respectively) determined by proton titration (*Janssen* et al. 1972) and by titration of protons released during tryptic activation of the zymogen (*Slotboom* et al. 1978b). Even higher pK values were found for the α-amino group of equine and bovine [(3-^{13}C)-L-Ala-1] AMPA, viz. 8.8 and 8.9, respectively (*Jansen* 1979; *Jansen* et al. 1979). In contrast, [(3-^{13}C)-D-Ala-1] porcine

AMPA was found to have a more normal pK value of 7.8 for its α-amino group (*Slotboom* et al. 1978b). These results, together with the observation that introduction of an octan-2-one moiety on His-48 or addition of specifically Ca^{2+} ions increase the pK of the α-amino group of [(3-^{13}C)-L-Ala-1] AMPA from 8.4 to 9.0 and not that of [(3-^{13}C)-D-Ala-1] AMPA, once more stresses the special environment of L-Ala-1 in pancreatic PLA.

Using the same technique, but now coupling the tripeptide Ala.Leu.Phe to des Ala-1.Leu-2.Trp-3-AMPA, [Phe-3] AMPA was obtained. This analog was found to have about 40% of the enzymatic activity of AMPA, indicating that Trp-3 is not essential (*Slotboom* and *de Haas* 1975). [Phe-3] AMPA enabled the unambiguous conclusion that in addition to Trp perturbation one or more Tyr residues are also perturbed upon interaction with micellar substrate analogs (*van Dam-Mieras* et al. 1975).

Substitutions further on in the N terminal region have been performed by covalent coupling of preassembled peptides to AMPA fragments shortened at the N terminal which were prepared by selective proteolytic cleavage or CNBr splitting of tri-, hexa-, and octapeptides. It has to be mentioned that these splittings caused the loss of all enzymatic activity which could not be restored by noncovalent combining of the peptide and protein fragment as observed for RNASe S'. Similar findings were reported also for PLA for *Naja naja oxiana* (*Magazanik* et al. 1979). Using chymotryptic cleavage at Trp-3 *Jansen* (1979) prepared [Gly-3] and [Glu-4] porcine AMPAs and showed that substitution of Trp-3 by Gly abolishes almost all micellar activity, most likely due to distortion of the α-helical structure. Although Gln-4 is absolutely conserved in all sequenced PLAs, [Glu-4] AMPA possesses about 40% of the activity of AMPA. Interestingly, the penetrating power of [Gly-3] AMPA into monolayers of L-diC_{10} PC was decreased, whereas that of [Glu-4] AMPA was increased as compared to that of unmodified AMPA. Recently *van Scharrenburg* et al. (1981) substituted Asn-6 in the bovine AMPA by Arg which occurs at this position in the porcine enzyme. This substitution was found to increase both the low affinity for lipid-water interfaces and the low penetrating capacity of the bovine AMPA for monolayers to comparable values found for the porcine AMPA. Substitution of the absolutely conserved Phe-5, located in the hydrophobic wall around the active site cleft (see Fig. 13), by a Tyr residue in bovine AMPA causes the loss of almost all catalytic activity, probably due to a distortion of the active site (*van Scharrenburg* et al., to be published). It can thus be concluded that these substitutions may yield valuable information on the role of the N terminal amino acid residues in enzymatic activity and lipid binding properties of pancreatic PLAs, but more work has to be done to properly explain the observed findings.

7 Direct Binding Studies

7.1 Binding of Ca^{2+}

7.1.1 Pancreatic Phospholipases A_2

Equilibrium gel filtration studies demonstrated that both porcine PLA and its zymogen possess only one high-affinity Ca^{2+} binding site per protein molecule (*Pieterson* 1973; *Pieterson* et al. 1974a; *Slotboom* et al. 1978b). Binding of Ca^{2+} to porcine PLA and pro-PLA induces ultraviolet difference spectra which are characterized by a large peak at 242 nm and two small peaks at 282 and 288 nm. It was tentatively concluded that the observed difference spectrum originates from a shift of a Tyr residue to a more polar environment and a charge effect on a His residue. Qualitatively identical difference spectra were obtained for both proteins with Ba^{2+} and Sr^{2+}, while Mg^{2+} did not produce a difference spectrum nor interfered with the appearance of the normal Ca^{2+} difference spectrum. From the appearance of the Ca^{2+}-induced ultraviolet difference spectrum and the observation that Ca^{2+} substantially slows down the rate of tryptic inactivation of PLA, it was concluded that saturation of the Ca^{2+} binding site produces a conformational change in the protein. Both from ^{1}H-NMR and fluorescence titration studies using native and His-48-modified pancreatic PLAs it was demonstrated that Ca^{2+} binding decreases the pK value of His-48 from about 7 to 5.7 (*Aiguiar* et al. 1979; *Verheij* et al. 1980a). This finding is in agreement with the conclusion that catalysis depends on the unprotonated form of a group with a pK of 5.5 (*Volwerk* et al. 1979) which has therefore been assigned to the imidazole side chain of His-48 (*Verheij* et al. 1980a; see Sects. 8 and 9).

The conclusion that Tyr is the only aromatic chromophore perturbed on Ca^{2+} binding is in agreement with the finding that Ca^{2+} does not influence the fluorescence spectra of PLA and pro-PLA. However, addition of Ca^{2+} enhances the ANS fluorescence induced by PLA and its zymogen, enabling the determination of the metal ion dissociation constants (*Pieterson* et al. 1974a). A similar conclusion was reached by *Brittain* et al. (1976) who used Tb^{3+} as a luminescent probe of Ca^{2+} sites in proteins. They showed that irradiation in the tyrosine region produces emission from added Tb^{3+} which was similar for PLA and pro-PLA.

Ca^{2+} dissociation constants were also derived from inactivation of PLA by BPB (*Pieterson* et al. 1974a; *Volwerk* et al. 1974). The dissociation constants for the porcine PLA-Ca^{2+} complex obtained by these different techniques showed good agreement. Rather similar values were found also for the dissociation constants of the zymogen-Ca^{2+} complex as well as for the Ba^{2+} and Sr^{2+} complexes of both proteins. Values were found

ranging from 10^{-1} M at pH 4 to 2.10^{-4} M at pH 10, and the pH dependency suggests that the metal ion binding site contains one or more carboxylates.

Using methods similar to those described above $K_{Ca^{2+}}$ values for bovine, ovine and equine PLAs were determined by ultraviolet difference spectroscopy at pH 6 and found to be 4.5, 4.5, and 1.1 mM, respectively (*Dutilh* et al. 1975; *Fleer* et al. 1981a; *Meyer* 1979). For the bovine PLA the pH dependency of $K_{Ca^{2+}}$ was shown to be controlled by a single carboxylate group with an apparent pK of 5.2 which by chemical modification studies was tentatively assigned to Asp-49 (*Fleer* et al. 1981a). A similar pK value was very recently reported by *Andersson* et al. (1981) for porcine pro-PLA using ^{43}Ca-NMR. With this technique the authors found a dissociation rate constant of 2.5×10^3 s^{-1}. Together with the reported $K_{Ca^{2+}}$ value (0.4 mM at pH 7.5) it was concluded that the Ca^{2+} binding site of porcine pro-PLA is more rigid or generally less accessible to an incoming Ca^{2+} ion as has also been observed for rabbit skeletal muscle troponin C.

It has to be mentioned that the chemically modified pancreatic PLAs like AMPA and [D-Ala-1]-AMPA (*Slotboom* et al. 1978a) and the various nitrated PLAs (*Meyer* et al. 1979b) all possess $K_{Ca^{2+}}$ values similar to their native PLAs. Obviously, no Ca^{2+} binding could be detected for the Asp-49 modified bovine PLA (*Fleer* et al. 1981a), whereas Ca^{2+} binding to BPB-modified pancreatic PLA is greatly impaired, probably due to steric hindrance (*Verheij* et al. 1980a).

So far Gd^{3+} is the only metal ion which can substitute for Ca^{2+} with retention of some of its enzymatic activity on L-diC_8 PC. Dissociation constants for PLA and pro-PLA were evaluated from water proton relaxation (PRR) titrations. At pH 5.8 the $K_{Gd^{3+}}$ for porcine PLA and pro-PLA was found to be 0.5 and 0.18 mM, respectively. The K_{Me} for Ca^{2+}, Eu^{3+}, and Tb^{3+} were evaluated in PRR titrations by competition of these cations with Gd^{3+}. The $K_{Ca^{2+}}$ values determined in this way agreed very well with those obtained directly, whereas K_{Me} for Eu^{3+} and Tb^{3+} for PLA were 0.07 and 0.08 mM at pH 5.3, respectively (*Hershberg* et al. 1976b).

Finally, it has to be mentioned that the affinity of the enzyme for Ca^{2+} is considerably enhanced at neutral pH by micellar substrate analogs (*Pieterson* et al. 1974a,b; *Hershberg* et al. 1976b; *Slotboom* et al. 1978b). This synergistic effect explains the discrepancies observed between Ca^{2+} dissociation constants determined directly and those obtained from kinetic analysis (see also Sect. 7.3).

7.1.2 Venom Phospholipases A_2

Binding of Ca^{2+} to *notexin* (*Halpert* et al. 1976), *notechis* II-1 (*Halpert* and *Eaker* 1976b), and taipoxin (*Fohlman* et al. 1979) induced ultraviolet difference spectra almost identical to those observed for porcine PLA. Somewhat lower $K_{Ca^{2+}}$ values were calculated for these proteins as compared to that of porcine PLA. In addition, it was concluded that one Ca^{2+} was bound per protein molecule, except for taipoxin which binds two Ca^{2+} ions. In this latter protein one Ca^{2+} is bound to the α-subunit and one to the γ-subunit, while the β-subunit has no affinity for Ca^{2+}. Although it appears very likely that indeed one Ca^{2+} is bound per polypeptide chain, this conclusion is based on the assumption that the maximal absorbance is due to the binding of one Ca^{2+} per protein molecule. From ultraviolet difference spectroscopy it was concluded that BPB-modified *notexin* is still able to bind one Ca^{2+} per protein molecule, although its $K_{Ca^{2+}}$ value (25 m*M* at pH 7.4) was 178-fold higher than that found for native notexin.

Abe et al. (1977) demonstrated by equilibrium dialysis that β-bungarotoxin binds one mole of Ca^{2+} per mole of protein and a $K_{Ca^{2+}}$ of 0.15 m*M* was found at pH 8. Similarly as found for porcine PLA this Ca^{2+} binding induces a conformational change as detected by fluorescence measurement in the presence of the dye ANS. Using this method comparable K_{Me} values for Ca^{2+}, Ba^{2+}, and Sr^{2+} were obtained as determined by equilibrium dialysis, whereas Mg^{2+} and Mn^{2+} do not bind. Fluorescence experiments with BPB-modified β-bungarotoxin showed that Ca^{2+} up to 5 m*M* produced only a very small effect on the fluorescence of the dye-toxin complex. These fluorescence studies indicate that BPB-modified β-bungarotoxin has lost its Ca^{2+} binding properties.

Using equilibrium dialysis *Wells* (1973a) showed for the *Crotalus adamanteus* PLA the presence of two cation binding sites per dimer with a dissociation constant of about 5×10^{-5} *M* at pH 8 for the alkaline earth cations. Ultraviolet difference spectroscopy revealed that the binding of Ca^{2+}, Ba^{2+}, or Sr^{2+} to PLA causes a decrease in the absorbance, with peaks near 292 and 286 nm, and an increase in the absorbance, with a broad peak near 260 nm. These spectral perturbations were interpreted as arising primarily from the removal of a charged group from the vicinity of a Trp residue. The pH dependency in the presence of Ca^{2+} of the spectral perturbations is controlled by a group with an estimated pK of 7.6. There is also a pH-dependent spectral perturbation in the absence of Ca^{2+} which has identical characteristics to that seen in the presence of cations and which is controlled by a group with a pK of 8.9. Both groups are not yet assigned. No fluorescence enhancement of ANS by this PLA was observed in the presence or absence of Ca^{2+} (*Wells* 1974b).

Although *Crotalus atrox* PLA like all other PLAs requires Ca^{2+} for activity, no ultraviolet difference spectrum was produced up to 20-m*M* Ca^{2+} at pH 7.4 (*Purdon* et al. 1977). The observed effects of Ca^{2+} on the CD spectrum, the enhancement of fluorescence of ANS-PLA complex by Ca^{2+}, and the heat effect in microcalorimetry suggest that the enzyme binds Ca^{2+}. So far only a kinetically determined $K_{Ca^{2+}}$ value ($1.1 \times 10^{-3} M$ at pH 7.5) was reported. Taking into account the very similar amino acid sequences of the *Crotalus adamanteus* and *Crotalus atrox* PLA in which all aromatic residues are conserved (see Sect. 4), it is remarkable that the metal ion induced difference spectra are so different.

Binding of Ca^{2+} to *Bitis gabonica* PLA produces an ultraviolet difference spectrum rather similar to that observed for *Crotalus adamanteus* PLA (*Viljoen* et al. 1975). The difference spectrum of the *Bitis gabonica* PLA was ascribed to both solvent- and charge-induced perturbations of predominantly Trp, while at low Ca^{2+} concentrations in addition some Tyr perturbation was observed. Moreover, Ca^{2+} binding to *Bitis gabonica* PLA also shows a red shifted peak with a maximum at 240–245 nm, which was not observed for *Crotalus adamanteus* PLA, and which was used to determine the dissociation constant ($K_{Ca^{2+}} = 6.8 \times 10^{-4}$ *M* at pH 7.8) and the number of Ca^{2+} binding sites (vide supra). Similar to the report of *Wells* for *Crotalus adamanteus* PLA, *Viljoen* et al. (1975) also observed pH-dependent spectral perturbations both in the absence and presence of Ca^{2+}. More recently *Viljoen* and *Botes* (1979) found from the pH dependency of spectral changes in the presence of Ca^{2+} three transition zones from which pK values of 5.66, 6.75, and 9.15 (at 25°C) were calculated. Based on the heats of ionization of groups associated with these various pK values, the group with pK 5.66 was assigned to a carboxylate involved in Ca^{2+} binding. The other two groups with pK values of 6.75 and 9.15 were assigned to a His and a Tyr residue, respectively. From kinetic data the group involved in Ca^{2+} binding was found to have a pK value of 6.4. From the observation that Ca^{2+} induces a difference spectrum in BPB-modified PLA *Viljoen* and *Botes* (1979) conclude that Ca^{2+} is still able to bind, but no dissociation constant is reported.

At basic pH Ca^{2+} binding to *Naja naja naja* PLA induces a blue shifted ultraviolet difference spectrum with minima at 292 and 283 nm, due to charge-induced perturbation of Trp. In contrast, at acid pH Ca^{2+} induces a red shifted ultraviolet difference spectrum with maxima at 290.5 and 282 nm due to solvent-induced perturbation of Trp and possibly Tyr (*Roberts* et al. 1977b). Binding constants for Ca^{2+} in the pH range 3.5–8.5 were thus determined and were found to be in good agreement with those obtained from quenching effects of Ca^{2+} on the fluorescence intensity. In calculating binding constants only a single binding site for divalent

metal ions was observed. The binding of Ca^{2+} to the enzyme is pH dependent with a pK of 5.9 and a $K_{Ca^{2+}}$ of 0.15 m*M* for the unprotonated form of the enzyme. The difference spectrum induced by Ca^{2+} at acidic pH is similar to the titration difference spectrum observed in the absence of Ca^{2+} which shows a pH dependency with a pK of about 7. It has been concluded that Ca^{2+} binding to *Naja naja naja* PLA triggers a conformational change lowering the pK of a critical residue, probably the active site His residue. Ca^{2+} binding also affects the monomer-dimer equilibrium. Inhibitory metals include Zn^{2+}, Ba^{2+}, and Sr^{2+}, whereas Mn^{2+}, Mg^{2+}, and Cd^{2+} bind without altering enzymatic activity. The ultraviolet difference spectrum induced by Ca^{2+} with BPB-modified enzyme was consistent with Trp perturbation and perturbation of the newly added chromophore. The binding constant for Ca^{2+} was not changed.

The Ca^{2+}-induced difference spectra of PLAs from *Naja nigricollis* (*Yang* and *King* 1980a) and from *Hemachatus haemachatus* (*Yang* and *King* 1980b) are negative with minima at 290 and 283 nm and are interpreted to be primarily charge-induced perturbations of Trp. In addition a positive peak at 260 nm was also observed which upon titration enabled the authors to determine the dissociation constants ($K_{Ca^{2+}}$ 0.2 m*M* and 0.23 m*M* at pH 8, respectively). Also these authors conclude that only one Ca^{2+} binding site per protein molecule is present. Essentially similar results were obtained for both BPB-modified enzymes, although the Ca^{2+}-induced difference spectra drastically changed. Both PLAs from *Naja nigricollis* and *Hemachatus haemachatus* also markedly enhance the emission intensity of ANS, but in contrast to pancreatic PLA and β-bungarotoxin, Ca^{2+} decreases the fluorescence of the complex. The corresponding BPB-modified proteins, however, did not enhance emission intensity at all, irrespective of the presence of Ca^{2+}.

7.2 Binding of Monomeric Substrate Analogs

A prerequisite for these studies is the availability of suitable phospholipids fulfilling at least the conditions (1) that they are not hydrolyzed by the enzyme, (2) that they must behave as competitive inhibitors, and (3) that they must possess a large enough monomer concentration range together with a good affinity. Similarly, as previously discussed (see Sect. 5) for monomer kinetics direct binding studies are also hampered by the phenomenon that quite often the dissociation constants exceed the CMC values. Because short-chain 1-*sn*-phosphatidylcholines like D-diC_6- or D-diC_7 PCs have been shown to be competitive inhibitors, these lecithins have been used as suitable substrate analogs to study monomer binding. Similarly 1-acyl lyso-PCs also appeared to be useful.

Although it could not strictly be proven that these lysolecithins are indeed competitive inhibitors, similar results were obtained as with D-lecithins. However, the use of either D-lecithins or 1-acyl lyso-PCs has the drawback that in particular in the presence of Ca^{2+} ions a slow aspecific hydrolysis might occur due to the rather high enzyme concentrations used as compared to kinetic studies. It is, however, possible to substitute Ca^{2+} by Ba^{2+} of Sr^{2+} ions which are competitive for Ca^{2+}. Alternatively, one can use nonhydrolyzable substrate analogs. The *n*-alkylphosphocholines having alkyl moieties of 10, 12, or 14 carbon atoms and CMC values of about 10, 1, and 0.1 m*M*, respectively, turned out to be most useful. As is the case for lysolecithins, no conclusive evidence is yet available that these substrate analogs are competitive inhibitors. Nevertheless, their behavior is in all respects similar to that of monomeric short-chain D-lecithins or 1-acyl lysolecithins.

Binding or monomers of short-chain D-lecithins or 1-acyl lyso-PCs to porcine PLA or pro-PLA induces similar red shifted ultraviolet difference spectra with peaks at 282 and 288 nm caused by perturbation of (a) Tyr residue(s) (*Pieterson* 1973; *Pieterson* et al. 1974b). In agreement with this observation hardly any perturbation of the unique Trp residue at position 3 was observed in fluorescence spectroscopy with these or other substrate analogs present as monomers (*Pieterson* et al. 1974b; *Van Dam-Mieras* et al. 1975). Equilibrium gel filtration was also used to study monomer binding of D-lecithins to porcine PLA and pro-PLA. Both techniques enabled the determination of the dissociation constants for binding of monomeric D-diC_7 to porcine PLA, which were found to be 0.9 and 0.4 m*M*, respectively, whereas for the zymogen somewhat higher values were reported. When using D-diC_6 PC the K_d values increased six- to sevenfold. Recently, *Volwerk* et al. (1979) using equilibrium dialysis found one monomer binding site to be present in porcine PLA for the *n*-decylphosphocholine. It was found from ultraviolet difference spectroscopy and from BPB inactivation that the dissociation constant of monomeric 1-acyl lyso-PCs decreases from 43 to 0.06 m*M* when the acyl moiety increases from 7 to 14 carbon atoms, from which it was concluded that monomer binding is mainly due to hydrophobic interactions (*Volwerk* et al. 1974; *Pieterson* et al. 1974b). The affinity of monomers of D-diC_7 PC or *n*-dodecylphosphocholine for porcine PLA remains constant between pH 4 and 7 and is not much affected by Ca^{2+}. In particular in the absence of Ca^{2+} the affinity decreases above pH 7 (*Pieterson* 1973; *van Dam-Mieras* et al., unpublished observations). The dissociation constants for binding of *n*-decylphosphocholine to porcine, equine, and bovine PLA at pH 6 were found to be 1.6, 20, and 5 m*M*, respectively, while somewhat higher values were found for the zymogens (*van Dam-Mieras* et al. 1975; *Verheij* et al. 1980a; *Fleer* 1980).

Methyl-His-48-porcine and -equine PLAs bind monomers of *n*-decylphosphocholine with the same affinities as their respective native enzymes (*Verheij* et al. 1980a). In contrast, no detectable binding was observed for monomers of D-diC_7 to BPB-inhibited porcine PLA using equilibrium gel filtration (*Pieterson* et al. 1974b). This lack of binding is probably due to steric hindrance. Various N-terminal-blocked AMPAs as well as des-Ala-1 AMPA bind monomeric D-diC_7 PC with comparable affinities as AMPA or native PLA, in contrast to des(Ala-1-Arg-6) AMPA which had lost its affinity for monomers (*J.C. Vidal,* unpublished observations). Also the various nitrated porcine PLAs show at pH 6 affinities for monomers of *n*-dodecylphosphocholine similar to those of the unmodified PLA (*Meyer* et al. 1979b). More interestingly, it was found that monomer binding of nitro Tyr-69 porcine PLA was greatly impaired at pH 8 compared to native PLA, most likely due to deprotonation of the nitro Tyr-69 residue. No such effect was observed for amino Tyr-69 PLA nor for NO_2 Tyr-124, suggesting that Tyr-69 is perturbed upon monomer binding. From the observed difference spectrum of the NO_2 Tyr-69 PLA it has been suggested that in addition to Tyr-69 another Tyr residue is also perturbed, which probably could be Tyr-52.

7.3 Binding to Aggregated Lipids

As has been already extensively discussed (vide supra), a number of theories have been developed in the last decade to explain the high catalytic activity of PLA towards substrate present in organized lipid-water interfaces as compared to its low activity on the same substrate present in monomeric form. Irrespective of the particular model, it is therefore obvious that investigations providing detailed information on the protein-lipid interaction are of utmost importance. With the present thorough knowledge of the properties of various PLAs and of different lipid-water interfaces such studies are now feasible, and valuable qualitative and quantitative data about the lipid-protein complex can be obtained. Unfortunately, direct binding studies consume rather large quantitites of enzyme, and this is probably the main reason that up until now most attention has been paid to the pancreatic PLAs. Although most of these studies so far are limited to micellar substrate analogs, there is a growing interest in also extending these investigations to bilayer-type structures.

7.3.1 Pancreatic Phospholipase A_2

Binding of micelles of D-diC_7 PC, lyso-PC, or *n*-alkylphosphocholine to porcine PLA further increases the peaks in the ultraviolet difference

spectrum already produced by monomer phospholipid binding while a concomitant shift of the maximal difference absorption from 288 to 292 nm is observed, indicative of both Tyr and Trp perturbation (*Pieterson* et al. 1974b; *van Dam-Mieras* et al. 1975). Binding of micelles to PLA can also be monitored by fluorescence spectroscopy where a large increase in fluorescence intensity and a blue shift of about 10 nm of the emission maximum is observed. No such effects are observed for pro-PLA (*van Dam-Mieras* et al. 1975). Elution of a mixture of PLA and pro-PLA in the presence of lysolecithin micelles on Sephadex G-75 showed that only PLA elutes at the void volume bound to the lipid micelles, whereas pro-PLA elutes at its normal position according to its molecular weight (*Pieterson* 1973). These observations are in agreement with the presence of a binding site for aggregated lipids on the enzyme in addition to the monomer binding site. A similar conclusion was reached by *Hershberg* et al. (1976a) from PRR studies.

Equilibrium gel filtration studies using either micelles of C_{14} lyso-PC or mixed micelles of D-diC_{10} PC plus C_{14} lyso-PC were performed by *Pieterson* et al. (1974b) to obtain quantitative data on the binding. It was concluded that one molecule of porcine PLA was bound to about 35 lipid monomers in the mixed micelle and to about 15 in the lysolecithin micelle. The affinity of porcine PLA was found to be higher for the mixed micelles ("K_d" = 2.1×10^{-5} *M*) at pH 6 than for the C_{14} lyso-PC micelles ("K_d" = 1.6×10^{-4} *M*) (*J.C. Vidal*, unpublished results). The bovine PLA, although having the same PLA-phospholipid ratio in the complex as the porcine PLA, possesses a lower affinity ("K_d" = 1.0×10^{-4} *M*) for the mixed micelles. BPB-inactivated porcine PLA was found to have a similar capacity to interact with these lipid-water interfaces as the native PLA, and it was concluded that the recognition site for interfaces is not only functionally but also topographically distinct from the monomer binding and catalytic site.

More recently, *Araujo* et al. (1979), *Hille* et al. (1981), and *Donné-Op den Kelder* et al. (1981) used equilibrium gel filtration and light scattering to study the complex formation of porcine PLA with micelles of various *n*-alkylphosphocholines and lysolecithins. From the results obtained it turned out that the binding is not a simple additive process but rather an insertion of two enzyme molecules into the micelle followed by a reorganization of the detergent monomers.

Araujo et al. (1979) found from microcalorimetry that the binding of PLA to micelles of *n*-hexadecylphosphocholine is a rapid, exothermic process. Using nonlinear regression analysis of binding data it is possible from these measurements to determine the enthalpy changes (Δ H), the number of lipid molecules complexed with one PLA molecule (N), and the dissociation constant (K_d). The low Δ H values and the positive Δ S

changes together with the negative value of the heat capacity ΔC_p are in support of the idea that mainly hydrophobic interactions determine the stability of the PLA-lipid complex. A highly schematic drawing of the complex formation in agreement with the stoichiometry found by the various techniques is given in Fig. 8. At least two possible pathways (A and B) can be considered (*Robinson* and *Tanford* 1975) along which the final complex is built up.

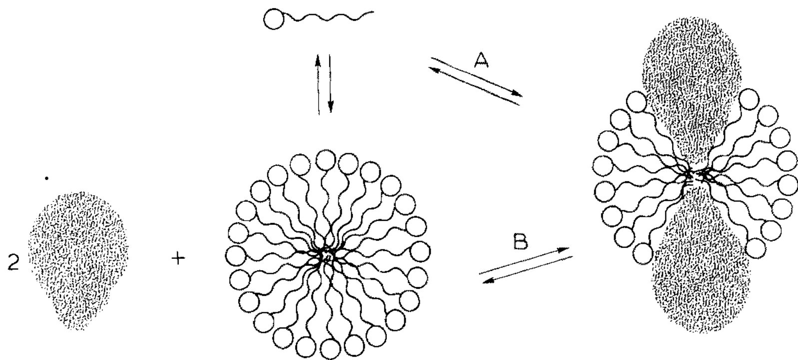

Fig. 8. Schematic view of the pathways for the formation of a complex between PLA and micelles of *n*-hexadecylphosphocholine(*Araujo* et al. 1979)

The comicellization mechanism (pathway A) has been proposed for some water-soluble proteins containing several high-affinity lipid-binding sites (*Makino* et al. 1973; *Haberland* and *Reynolds* 1975; *Rosseneu* et al. 1976). *Araujo* et al. (1979) strongly favored the concept of insertion of pancreatic PLA into the micelle (pathway B). The authors emphasized that the dimeric structure of pancreatic PLA in the complex shown in Fig. 8 should not be interpreted to mean that an enzyme dimer is functionally active in catalysis.

Although these physicochemical techniques provide valuable information, these measurements are rather time consuming and need large quantities of protein. It is therefore more advantageous to use fluorescence or ultraviolet difference spectroscopy. These techniques were used by *van Dam-Mieras* et al. (1975) to study the binding of porcine PLA to *n*-hexadecylphosphocholine micelles. In this study dissociation constants were calculated from *total* lipid concentrations. However, recently this method has been shown to be incorrect, since it leads to apparent K_d values which are too high (Fig. 9) (*Hille* et al. 1981). As shown in Fig. 9 plotting of the ultraviolet absorption difference signals relative to *free* lipid concentration (expressed as monomers) requires nonlinear regres-

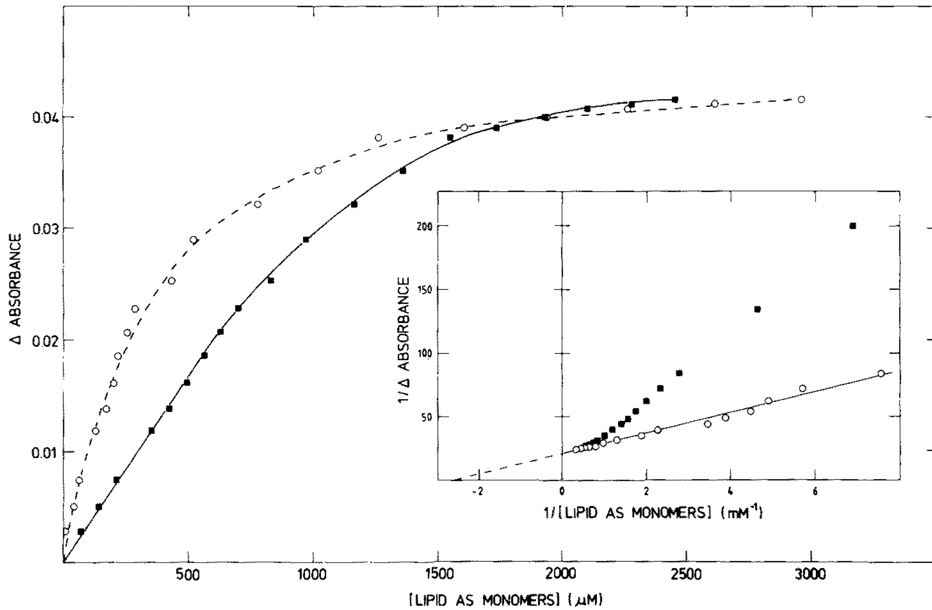

Fig. 9. A direct plot of the ultraviolet absorption difference spectroscopy signal at 292 nm relative to the *n*-octadecylphosphocholine concentration expressed as monomers. The difference signal at 292 nm relative to total lipid concentration (■) is shown. The *solid curve* through these points represents the result of the computer fit. In addition, the observed signal is plotted as a function of free lipid (○). The *broken curve* gives the calculated difference signal relative to free lipid monomers. *Inset:* a double reciprocal plot of the observed difference signal at 292 nm as a function of total lipid (■) and free lipid (○), respectively. The concentration of PLA is 27.4 *μM*. All measurements were done at 25°C and pH 4.0 (*Hille* et al. 1981)

sion analysis to obtain quantitative data. When the signal is plotted versus free lipid concentration the direct plot fits a hyperbola. Consequently, the corresponding double reciprocal plot is a straight line, whereas it is curved when lipid *total* is plotted. *Donné-Op den Kelder* et al. (1981) showed that only when complex formation is measured by titrating enzyme to lipid can K_d and the number of lipid molecules complexed with one PLA molecule (N) be obtained graphically without the use of a computer. However, this latter procedure requires large amounts of enzyme. Using both techniques the authors determined the K_d values as well as the stoichiometry of the porcine PLA complexes formed with a series of saturated and unsaturated *n*-alkylphosphocholines and lysolecithins. In good agreement with the results obtained from microcalorimetry they found that all the PLA-lipid complexes formed with the saturated phospholipid analogs consisted of two PLA molecules and about half the number of monomers present in the original pure micelle.

The PLA-lipid complexes formed with the unsaturated phospholipid analogs were found to contain three PLA molecules and about 70% of the monomers originally present in the pure micelles. The dissociation constants were found to be dependent on the chain length of the phospholipid analog and range from 23 μM for *n*-tetradecylphosphocholine micelles to 6.6 μM for *n*-octadecylphosphocholine micelles at pH 6, wheras the affinity for lyso-PCs was two- to sixfold lower. These observations further support the conclusion of *Araujo* et al. (1979) that the stability of the PLA-lipid complex is predominantly due to hydrophobic interactions. Determination of the molecular weight of the protein part in the enzyme *n*-octadecylphosphocholine complex using the sedimentation equilibrium centrifugation method described by *Reynolds* and *Tanford* (1976) gave a value of 30 000 which was in good agreement with the proposed model (*Hille* et al. 1981).

Studying the pH dependency of the stability of the PLA *n*-octadecylphosphocholine complex *Donné-Op den Kelder* et al. (1981) found that a protonated group with a pK of 6.25 controls this binding, and it has been suggested that the active site residues His-48 and/or Asp-49 are the most likely candidates involved in the lipid binding process. In particular, at basic pH Ca^{2+} is required for binding of PLA to micellar compounds by stabilizing the conformation of the enzyme that has optimum micelle-binding properties. Similar studies, but now using methyl-His-48- and octan-2-one-His-48-modified PLAs showed that the micelle binding of these proteins is now controlled by a group with pK 4.6, while addition of Ca^{2+} at high pH values again restores the micelle-binding properties of these modified PLAs. Therefore, most probably the group having a pK 4.6 should be assigned to Asp-49. Apparently, upon alkylation of the N-1 atom of His-48 the rather high pK value of Asp-49 drops from 6.25 to 4.6, the latter value being normal for a carboxylate group in a protein. In this respect it should be mentioned that it is perhaps not necessary to assume a second binding site for Ca^{2+} (*van Dam-Mieras* et al. 1975; *Slotboom* et al. 1978b), even when different affinities are found for Ca^{2+} in the absence or presence of micelles, respectively.

7.3.2 Snake Venom Phospholipase A_2

Prigent-Dachary et al. (1980) used fluorescence spectroscopy to study binding of various snake venom PLAs to vesicles of long-chain phospholipids. They found that strong inhibitors of blood clotting (PLAs from *Naja nigricollis, Naja mossambica mossambica,* and *Vipera berus orientale*) interact with PC, PC + PS, and PS vesicles, although a higher affinity was found for the PS-containing vesicles than for the pure PC vesicles. Poor inhibitors of blood coagulation (PLAs from *Bitis gabonica, Crotalus*

adamanteus, Crotalus atrox, and *Naja melanoleuca DE II*) do not or only weakly bind to these vesicles. Using the "nonhydrolyzable" diC_{16} ether PC it was demonstrated that Ca^{2+} promotes the complex formation which can occur whenever the lipids are in the crystal or fluid phase. Inactivation of the anticoagulant PLA from *Naja nigricollis* with BPB decreased the affinity of the enzyme for the phospholipids twofold.

Very recently *Jain* et al. (to be published) compared the binding of porcine and *Naja melanoleuca* PLAs to long-chain phospholipid dispersions (vesicles) using various techniques. Qualitatively, gel filtration, differential scanning calorimetry, and freeze-fracture electron microscopy showed binding of *Naja melanoleuca* PLA to vesicles of pure diC_{14} ether PC. Similar experiments with porcine PLA did not reveal any binding to the diC_{14} ether PC vesicles alone. However, only when vesicles of the ternary system PC + lyso-PC + FA were used does the porcine PLA show affinity for the bilayer phospholipids. More quantitative data about the binding of these two PLAs to bilayer structures were obtained from fluorescence and ultraviolet difference spectroscopy. Binding of *Naja melanoleuca* PLA to pure diC_{14} ether PC vesicles causes an increase in fluorescence intensity and in parallel a blue shift of the emission maximum, which for the porcine PLA again occurs exclusively in the ternary bilayer system. Using the curve-fitting procedure for lipid binding as described by *Araujo* et al. (1979) and *Hille* et al. (1981) it was found that the K_d values for *Naja melanoleuca* PLA were lower than for porcine PLA for the same ternary system and that the number of phospholipid molecules contributing to the binding is lower for the *Naja melanoleuca* PLA than for the porcine PLA. The product-facilitated binding of the pig PLA to bilayers is also manifested in the time course and kinetics of hydrolysis of substrate bilayers. Thus pig PLA catalyzed hydrolysis of diC_{10} PC and diC_{14} PC dispersions is accompanied by a lag phase which is reduced or abolished by the externally added products. The results thus suggest that the binding of pig PLA is regulated by the organization of the bilayer and the factors favoring phase separation in bilayers also favor the binding of the pancreatic PLA to bilayers.

Recently, *Verheij* et al. (1980b) using ultraviolet difference spectroscopy determined the dissociation constants and the stoichiometry of the PLA-*n*-hexadecylphosphocholine complexes for a number of snake venom PLAs in the presence of Ca^{2+} (*Vipera berus, Naja melanoleuca,* and *Crotalus atrox*). The dissociation constants were found to be in the range from 1.6 to 8 μM which is comparable to that of the porcine PLA, but the lipid to protein ratio (N) is considerably lower for snake venom PLAs than for the porcine PLA. BPB-inactivated *Vipera berus* also binds to micelles, though with a twofold lower affinity as compared to the native enzyme.

In the absence of Ca^{2+} *Wells* (1973a) did not observe an ultraviolet difference spectrum of *Crotalus adamanteus* PLA with micelles of D-diC_6 PC. Similar observations have been reported by *Tinker* for *Crotalus atrox* PLA (personal communication).

In direct binding studies of *Bitis gabonica* PLA with diC_{16} PC, lyso-PC, or fatty acid, *Viljoen* et al. (1975) found ultraviolet difference spectra originating from perturbation of Trp residues, both in the presence and absence of Ca^{2+}. It was assumed that Ca^{2+} is necessary for producing an active conformation of the enzyme allowing the productive binding of substrate and that in the absence of Ca^{2+} unproductive binding gives rise to the observed difference spectrum.

Roberts et al. (1977c) and *Adamich* et al. (1979) used equilibrium gel filtration to study binding of native and BPB-modified *Naja naja naja* PLAs to mixed micelles of Triton X-100 plus long-chain PCs (and other phospholipids). They found binding only when divalent metal ions were present. In contrast, no metal ions were required for binding of *Naja naja naja* PLA to mixed micelles of Triton X-100 and fatty acid or lyso-PC. The reported K_d values (*Adamich* et al. 1979) have no physical meaning, since it was assumed that the complex formed is additive (vide supra).

8 Immunology

Ouchterlony's double immunodiffusion showed that only cow and sheep pancreatic PLA gave precipitin lines of complete identity to both antisera. Horse PLA only partially cross reacts with pig PLA using anti-horse PLA serum, whereas pig PLA shows a partial cross reaction with horse, cow, and sheep PLA towards anti-pig serum (*Meyer* et al. 1978; *Meyer* 1979). Similar results were obtained from the microcomplement fixation assay. With this technique horse and cow PLA show in particular considerable immunological differences, whereas the pig enzyme takes an intermediate position between these phospholipases. Ouchterlony's immunodiffusion did not discriminate between the enzyme and its zymogen, since a complete cross reaction toward anti-PLA serum was observed. However, the complement fixation assay detects a considerable difference. Using this assay iso-porcine PLA could be clearly distinguished from porcine PLA, although there are only four substitutions in their sequences (*Puijk* et al. 1979). Moreover, with the microcomplement fixation assay it turned out that most likely the N terminal sequence Ala^1-Arg^6 is part of an antigenic determinant of PLA. Radioimmune assay, using monovalent PLA-specific F_{ab} fragments revealed a maximum number of three antigenic sites of PLA that can simultaneously be occupied

by antibody. The F_{ab} fragments were separated into three fractions, using three immunoadsorbent columns in series. These F_{ab} fragments showed different inhibitory properties toward binding of PLA to micellar substrate. One of these F_{ab} fragments turned out to effectively protect PLA against BPB modification.

9 X-Ray Analyses

X-ray analysis of protein crystals is a powerful technique to obtain detailed structural information about the protein. The elucidation of the spatial arrangements of the side chains may contribute to our knowledge of the catalytic mechanism of the enzyme. A prerequisite of a successful X-ray crystallographic analysis is the availability of good protein crystals and heavy atom derivaties. Not all phospholipases crystallize readily to yield crystals suitable for X-ray analysis. The enzyme from porcine pancreas never yielded suitable single crystals, despite numerous attempts, while its precursor produced crystals of poor quality which allowed calculation of an electron density map at a resolution of only 3 Å (*Drenth* et al. 1976). The revised sequence of porcine PLA (*Puijk* et al. 1977) could, however, not be incorporated into this electron density map. This observation and the absence of regular α-helices and β-plated sheets suggest that the crystals contained denatured protein.

In the meantime it was found that both the active enzyme and the precursor of bovine pancreatic PLA crystallized readily as high quality single crystals. Using these crystals and three heavy-atom derivatives, the three-dimensional structure was determined to a resolution of 2.4 Å (*Dijkstra* et al. 1978). Subsequently diffraction data to 1.7 Å resolution was collected and the phospholipase model was crystallographically refined at this resolution to a final R factor of 17.1% (*Dijkstra* et al. 1981b).

Phospholipases from *Crotalus adamanteus* and *Crotalus atrox* also yield crystals suitable for X-ray analysis. In both cases one dimer per asymmetric unit was present (*Pasek* et al. 1975). Interpretation of the electron density map at a resolution of 2.5 Å shows that the main chain folding of *Crotalus atrox* PLA is very similar to that of bovine PLA (*Keith* et al. 1981). Furthermore it was found that the C-terminal appendage is linked via a disulfide bridge to Cys-50 (see also Sect. 4). In the dimer both active sites are shielded from the surrounding water, an observation which raises serious doubts on the catalytic function of the dimer.

Notexin, a neurotoxic basic phospholipase, forms crystals diffracting to a resolution of 1.8 Å. There are six molecules in the unit cell (*Kannan* et al. 1977). No further data obtained with this phospholipase has been published so far.

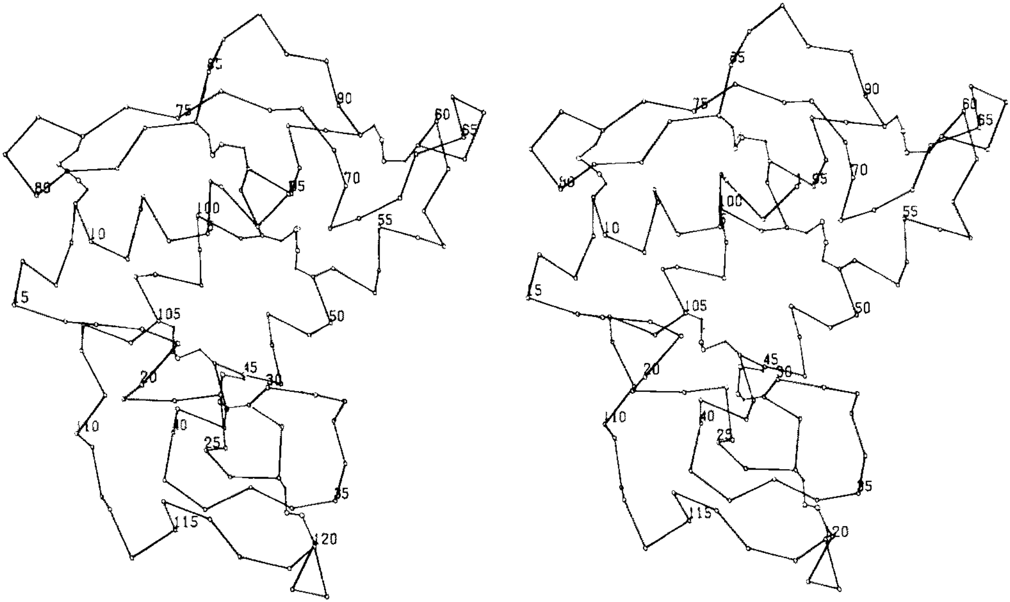

Fig. 10. Stereo diagram showing the conformation and disulfide bridges of the bovine pancreatic phospholipase molecule (*Verheij* et al. 1980a)

In the absence of three-dimensional structures of other phospholipases we assume that the results obtained with the bovine pancreatic and the rattlesnake PLAs can be applied to other (venom) phospholipases as well. For this reason we will give a somewhat detailed description of the structure of bovine PLA.

The molecule is kidney-shaped with dimensions of 22Å × 30 Å × 42 Å; it has a high content of secondary structure with about 50% α-helix and 10% β-structure (Fig. 10). The structure is stabilized by a large number of hydrogen bridges linking (1) backbone to backbone, (2) backbone to side chain atoms, and (3) side chain to side chain atoms. In addition the loops are held together by seven disulfide bridges. For example, the two long antiparallel α-helices corresponding to residues 40 to 58 and 90 to 108 are connected by two disulfide bridges (Cys-44 to Cys-105 and Cys-51 to Cys-98). In these helices the active center residues His-48, Asp-49, Tyr-52, and Asp-99 are brought tightly together.

Figure 11 shows a three-dimensional view of the active center of bovine PLA, including the backbones of residues 28–33, 48–52, and 98–99 and some of the side chains. Note that the amino acids in this part of the sequence are invariant in all phospholipases except for residues 31 and 50 (see Fig. 2).

The main chain of residues 28–33 is part of the calcium-binding loop which runs from residues 25–42 and contains the five glycines conserved

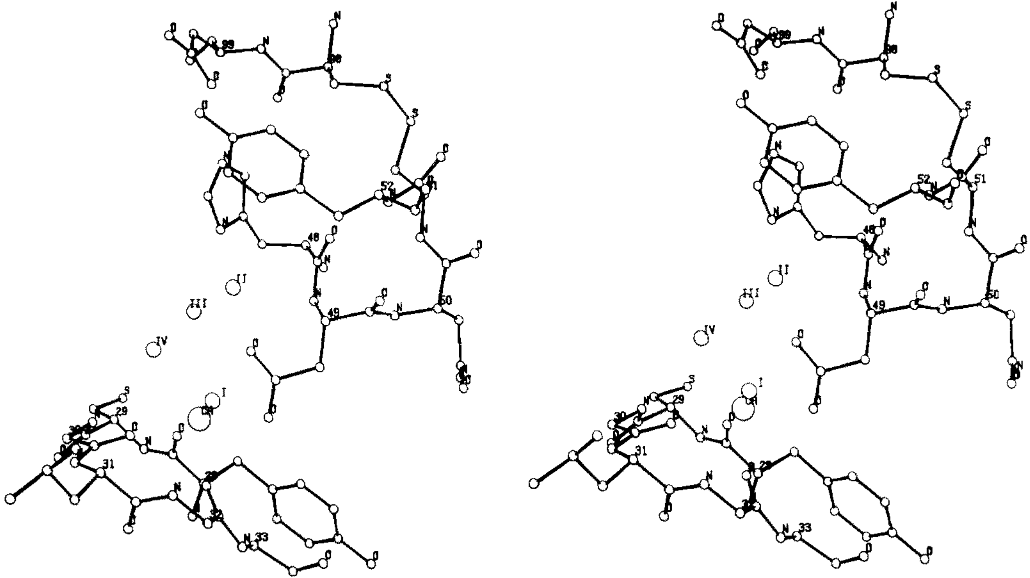

Fig. 11. Stereo picture of the active site of PLA, including the calcium ion and several water molecules (*Dijkstra* 1980)

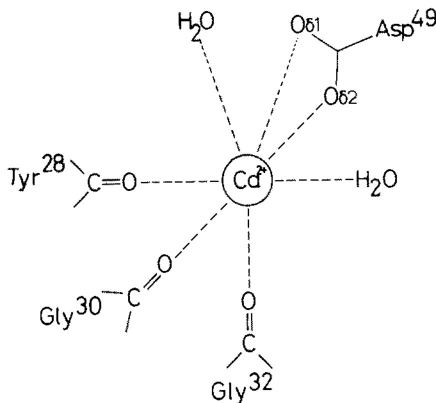

Fig. 12. Schematic representation of the calcium ion and its ligands (*Dijkstra* 1980)

in all phospholipases. When the folding pattern of bovine PLA is summarized in a Ramachandran plot these five glycine residues are found in regions disallowed for other amino acids. Substitution of these glycines for other amino acids, while maintaining the chain folding pattern, would be highly unfavorable in energy terms (*Dijkstra* 1980).

The calcium ion is located in the active site surrounded by seven oxygen ligands (Fig. 12), viz. three carbonyl oxygens, the δ^1 and δ^2 oxygens of Asp-49 (not Asp-99 as reported before; *Dijkstra* et al. 1978), and two water molecules. Six of these ligands are found at the corners of an octahedron. The Ca^{2+} ion can be replaced by a Ba^{2+} ion, although Ba^{2+} does

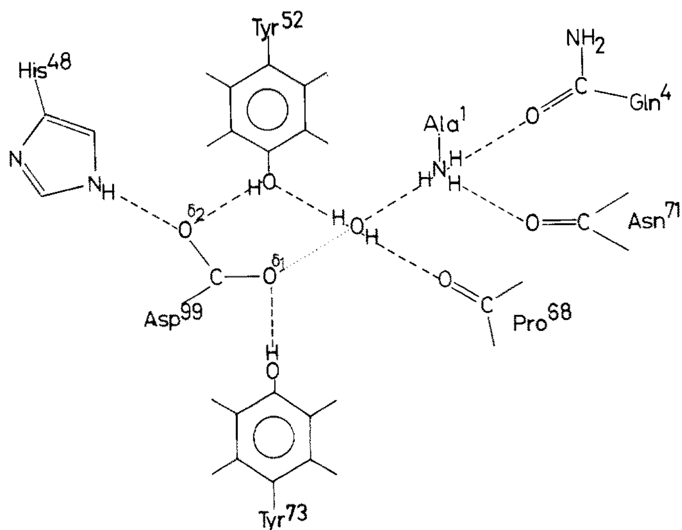

Fig. 13. Proton relay system of phospholipase (*Dijkstra* 1980)

not orient itself exactly into the same position, probably due to its larger size (*B.W. Dijkstra*, personal cummunication).

By chemical modification studies (*Volwerk* et al. 1974; *Verheij* et al. 1980a) it has been shown that His-48 is involved in catalysis. Fig. 13 shows that the imidazole ring is in close proximity to the side chains of Asp-99 and Tyr-52 and a water molecule. The N-3 atom of His-48 is at hydrogen-bonding distance (2.8 Å) of one of the carboxylate oxygens of Asp-99. Close to the N-1 of His-48 (about 3 Å) a water molecule is found (water molecule I in Fig. 11). This water molecule could very well perform the nucleophilic function in the ester hydrolysis in analogy to the active center serine in the serine esterases. The carbonyl oxygens of Asp-99 are also hydrogen bonded to the hydroxyl groups of Tyr-52 (2.55 Å) and Tyr-73 (2.50 Å). Both tyrosine residues are invariant in all phospholipases. Via a water molecule these residues are also hydrogen bonded to the α-amino group, the side chain of Gln-4, and the carbonyl oxygens of Pro-68 and Asn-71. Gln-4 again is invariant in all phospholipases and the interactions with the α-amino group and the main chain carbonyl oxygens do not necessarily depend on the side chains.

Therefore, one might predict that in all phospholipases such an extended proton relay system does exist. This system probably has a structural function rather than a catalytic function, since proteins devoid of the α-amino group (e.g., precursor) effectively hydrolyze monomeric substrates. The system is buried in the interior of the protein and the Asp-99

His-48 couple is shielded from the surrounding solvent by a number of invariant hydrophobic residues: Phe-5, Ile-9, Ala-102, Ala-103, Phe-106, and the disulfide bridge between Cys-29 and Cys-45. In addition Phe-22 (Tyr in most venom enzymes) is part of this hydrophobic active site wall. Whereas the hydrophobic residues forming the active site wall are mostly invariant, the situation at the surface surrounding the active site is quite different. As already amply discussed (see Sect. 4) the entrance of the active site is composed of highly variable, mainly hydrophobic amino acid side chains. The fact that the surface does not put strict spatial requirements on the size of the side chains (as is the case with residues surrounding the Asp-99-His-48 couple) gives rise to a great variety of in general hydrophobic residues.

If we finally try to predict how the primary structures of about 30 venom phospholipases (Fig. 2) would fit the three-dimensional structure of the bovine pancreatic phospholipase we come to the following conclusions: In all phospholipases the residues around the Asp-99-His-48 couple and the potential Ca^{2+} ligands are invariant (or highly conserved). There is no obvious reason why all phospholipases could not form an extended proton relay system as depicted in Fig. 13. The residues around the entrance of the active site are variable, but with few exceptions they are hydrophobic. The large deletion between residues 57 and 68 found in the venom phospholipases shortens two external loops around the disulfide bridge between Cys-61 and Cys-91 without affecting the gross shape of the molecule. Therefore, we tentatively conclude that the phospholipases from the different sources not only show a high degree of sequence homology but also have very similar three-dimensional properties. This conclusion is supported by the results of the X-ray analysis of the *Crotalus atrox* PLA at 2.5 Å resolution (*Keith* et al. 1981).

Another X-ray determination deals with the structure of the precursor of bovine pancreatic PLA. Good crystals of this protein have been obtained and the results show that the structure is nearly identical to that of the active PLA, except for the N terminal region and Tyr-69. In the precursor, these residues show a high mobility, whereas they are fixed in the active PLA. Because the N terminal residues and the side chains around Tyr-69 are part of the IRS, this observation is of utmost interest (*J. Drenth*, personal communication).

10 Mechanism of Catalysis

In this section we will make an attempt to compare data emerging from chemical modifications, direct binding studies, and X-ray crystallography and see how this data fits a proposed catalytic model for bovine pancreatic PLA. Kinetic analyses of the hydrolysis of aggregated substrate require a binding step of the enzyme to the lipid-water interface prior to the Michaelis-Menten complex formation. It has been discussed (see Sect. 5) that such an additional binding step complicates the interpretation of kinetic data in terms of well-defined rate and binding constants. Only by using monomeric short-chain phospholipids can interpretable kinetic data be obtained (*Roholt* and *Schlamowitz* 1961; *Wells* 1972; *Volwerk* et al. 1979). As has been pointed out in the previous sections we know that:

1. Hydrolysis requires an ester bond which is separated by five or six atoms from a negative charge and which must be present in a specific stereochemical orientation.
2. Ca^{2+} ions are required for the reaction while Ba^{2+} and Sr^{2+} ion are competitive inhibitors. They bind in a 1:1 ratio to the enzyme in a pocket formed by three backbone carbonyl groups and the side chain of Asp-49.
3. Monomeric substrates or substrate analogs bind in a 1:1 ratio; in this binding process hydrophobic interactions predominate.
4. His-48 is involved in catalysis with its N-1 group oriented toward the solvent. The pK of this group is about 6.5, a value that drops to about 5.5 in the presence of Ca^{2+} ions.
5. Although the enzyme hydrolyzes esters, it is not a classical serine esterase. It does not react with organophosphates, and no results have been obtained in favor of the existence of an acyl enzyme. Therefore, *Wells* (1973b) proposed that a water molecule must be the nucleophile attacking the ester bond.

The proposed catalytic mechanism depends heavily on the X-ray structure of bovine pancreatic PLA. We assume that this structure does not differ significantly from the structure of any PLA (from pancreas or venom). Such an assumption is not unrealistic, since we have seen that venom and pancreatic phospholipases show a high degree of homology. Crystallographic data of other homologous proteins (e.g., serine proteases, cytochromes C, and acid proteases) show that their three-dimensional folding is fairly independent of variations in the primary structure.

In the X-ray structure His-48 is located in a cleft near the absolutely conserved side chains of Asp-49, Tyr-52, and Asp-99 (see Fig. 2). The wall of the cleft is constituted by residues with highly conserved, hydro-

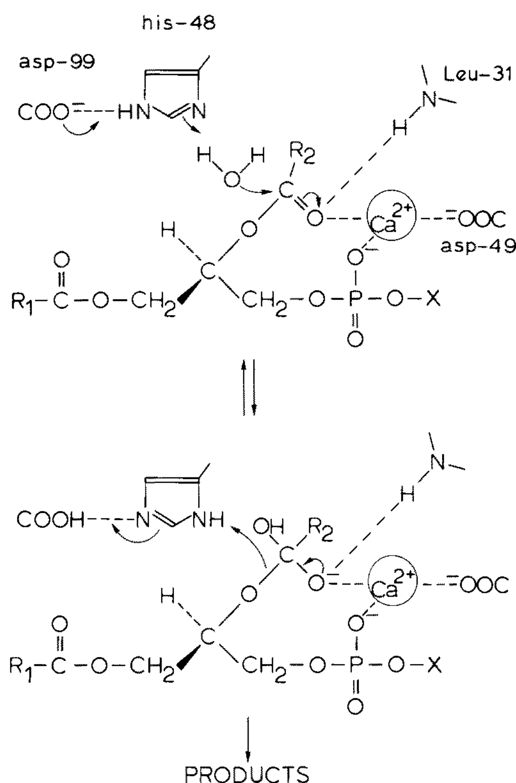

Fig. 14. Proposed catalytic mechanism for PLA (*Verheij* et al. 1980a)

phobic side chains. Based on the chemical evidence (vide supra) and the spatial arrangement of the side chains, a mechanism has been proposed (*Verheij* et al. 1980a) which is described in Figure 14.

The presence of the Asp-99-His-48 couple suggests a comparison with the serine esterases. The serine residue found in the serine esterases is lacking in PLA, but instead a water molecule about 3 Å away from the N-1 nitrogen of His-48 is supposed to perform the nucleophilic function in the ester hydrolysis in analogy to the active center serine in the esterases. When this water molecule attacks the substrate carbonyl carbon atom, the imidazole ring of His-48 picks up a proton from the water molecule, thereby facilitating the reaction. This proton is subsequently donated by the imidazole ring to the alkoxy oxygen, just as in the serine enzymes where the proton from serine is transferred by His to the leaving group (*Kraut* 1977; *Komiyama* and *Bender* 1979).

The function of the Ca^{2+} ion may be to bind the negative phosphate group. If this were the only role of the Ca^{2+} ion it is not clear why in the presence of the slightly larger Ba^{2+} ions (0.99 Å and 1.34 Å) a ternary complex is formed but not hydrolyzed.

A possible explanation is that because Ca^{2+} is a stronger Lewis acid than Ba^{2+} it can more easily polarize the ester carbonyl function and stabilize the tetrahedral intermediate in concert with the backbone NH group of residue 30. A similar role for the metal ion cofactor has been suggested for the Zn^{2+} ion in carboxypeptidase (*Quiocho* and *Lipscomb* 1971) and for Ca^{2+} in staphylococcal nuclease (*Cotton* et al. 1979).

No X-ray crystallographic data of an enzyme-substrate (analog) complex are available. However, it is possible to fit a substrate molecule in the active center with the susceptible ester bond in the required position relative to the attacking water molecule, the phosphate group close to the Ca^{2+} ion, and the remaining part of the polar head group (e.g., choline) pointing towards the solvent. The two acyl chains, while running parallel to each other, can be fitted into a shallow cleft on the enzyme surface in between the apolar side chains of Leu-2, Leu-19, Leu-20, and Leu-31 (Fig. 15).

How does this mechanism fit data of phospholipases other than the bovine pancreatic PLA? The side chains of the calcium ligand Asp-49, the Asp-99-His-48 couple, and Tyr-52 are invariant in all phospholipases and most probably fulfil a similar role. The role of Tyr-52 is not very clear, although it is at hydrogen bridge distance of Asp-99 and may help to stabilize the charge of the Asp-99-His-48 couple.

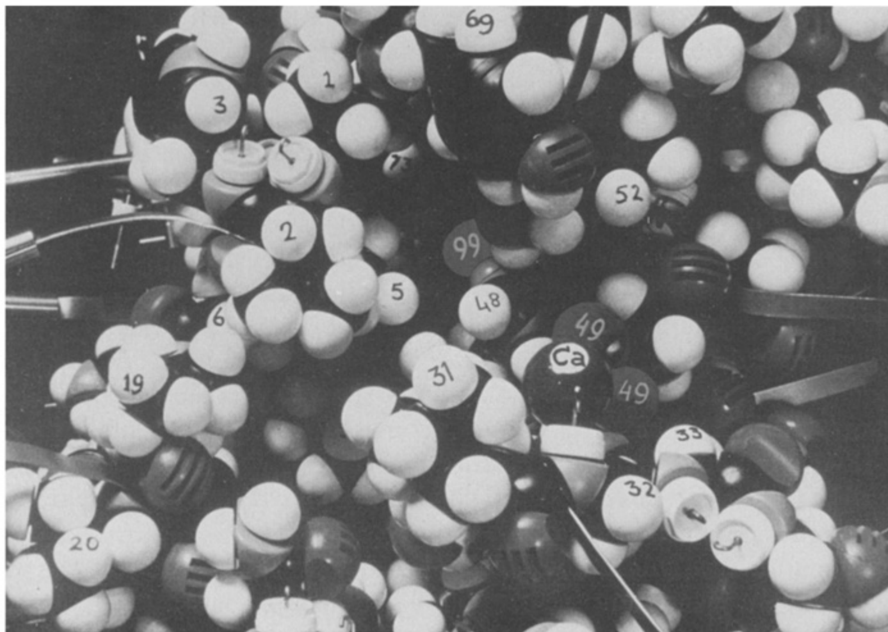

Fig. 15. The space-filling model of bovine pancreatic phospholipase

Although somewhat variable, the residues forming the wall of the active site cavity are very hydrophobic in all phospholipases (see Sect. 9). Consequently, we must assume that in all phospholipases the Asp-99-His-48 couple is accommodated in a hydrophobic microenvironment. Despite this similarity the reported pK values of the group controlling catalysis – and according to Fig. 14 this must be histidine – vary between 5.5 and 7.6 (*Wells* 1972; *Viljoen* and *Botes* 1979; *Volwerk* et al. 1979) and may suggest that subtle changes near the Asp-99-His-48 couple might change its pK drastically. For all pancreatic enzymes it has been shown that the active site histidine shows a "normal" pK value of about 6.5, and this value is lowered to about 5.5 in the presence of Ca^{2+} ions (*Dutilh* 1977; *Aguiar* et al. 1979; *Verheij* et al. 1980a). Also in *Naja naja naja* PLA the pK of the active center histidine is lowered upon addition of Ca^{2+} (*Roberts* et al. 1977b); the same influence of Ca^{2+} on the pK of His-48 has been observed with PLAs from *C. adamanteus* and *N. melanoleuca* fraction DE III (*J. v. Eijk,* unpublished results). A further increase in k_{cat} values above pH 7 observed in PLA from pancreatic as well as venom phospholipases might be ascribed to a conformational change induced by deprotonation of a residue with a pK value around 8. The nature of this group has not yet been elucidated, although it has been suggested to be a lysine (*Wells* 1973b) or the α-amino group (*Volwerk* et al. 1979).

The binding of monomeric substrates or substrate analogs to both pancreatic, *Naja oxiana* and *C. adamanteus* PLA has been shown to be a mainly hydrophobic process resulting in a threefold improvement in binding for each additional methylene group (*Wells* 1972, 1974a; *Zhelkovski* et al. 1978a; *Volwerk* et al. 1974; *Volwerk* 1979). Also, modification of His-48 with alkylating reagents is only successful when the reagents possess an apolar part (*Roberts* et al. 1977a; *Verheij* et al. 1980a). If indeed the side chains of the residues 2, 19, 20, and 31 contribute most to this binding, then we may expect from Fig. 2 that this hydrophobic interaction plays an important role for all phospholipases. These residues are also an integral part of the larger hydrophobic surface (see Sect. 4) that is supposed to interact with lipid-water interfaces. Therefore, one expects a somewhat different orientation of the substrate molecule bound to the active site when the enzyme becomes embedded in a lipid-water interface. Whether this confirmational change alone is responsible for the fact that aggregated substrates are hydrolyzed with high velocity compared to monomeric substrates is not yet clear. Other factors like the conformation and the hydration of the substrate (*Brockerhoff* 1973) and the entropy loss upon binding (*Wells* 1974a) may play an important role as well. Finally it is also conceivable, that in the hydrolysis of monomers the release of products is slow whereas *in* the interface the product is

replaced rapidly by a new substrate molecule by lateral diffusion. This diffusion is rapid enough to allow turnover numbers at least one order of magnitude higher than the observed maximal turnover numbers (about 7000 sec^{-1}).

11 Concluding Remarks

The combined efforts of a number of investigators using protein sequencing, kinetic analysis, nuclear magnetic resonance, and high resolution X-ray have led to a proposed mechanism for the hydrolysis of monomeric phospholipids by PLA. Further studies on the interaction of PLA with aggregated phospholipids on a molecular basis are required to understand how the fine structure of the lipid-water interface determines the specific activity of these enzymes. As yet no general agreement exists about the factors causing the dramatic rate enhancements observed with PLA from various sources. Does the microenvironment of the organized lipid-water interface change the conformation of the inserted enzyme improving the catalytic site or are the aggregated substrate lipid molecules in a conformation and orientation particularly favorable for hydrolysis?

The last decade witnessed intense interest in orientation, conformation, and motion of phospholipid molecules in the various forms of lipid aggregates. These properties have been studied using a wide variety of physical techniques (*Hauser* et al. 1980 and references herein). Deuterium magnetic resonance, neutron diffraction and single crystal X-ray studies have revealed a preferred phospholipid conformation which seems to be general for various phospholipid classes, independent of their aggregation state (monomer, micelle, bilayer). On the average the *sn*-1-chain is extended perpendicular to the bilayer surface at all segments while the *sn*-2-chain begins parallel to the membrane surface and is bent perpendicular to it after the C_2 segment. The more exposed and less hydrophobic microenvironment of the *sn*-2-ester bond as compared to the *sn*-1-ester linkage has even tempted several investigators to "explain" the specific action of PLA and lecithin cholesterol acyltransferase! Unfortunately the detailed information available on the structure of the hydrocarbon region has not been matched with information on the conformation of the polar head groups. Though refuted, the over-all orientation of the phosphorylcholine group in PC and of the phosphorylethanolamine group in PE seems to be parallel to the bilayer surface, and this preferred polar group conformation is determined by intramolecular forces (*Hauser* et al. 1980). The head group is engaged in intermolecular interactions with neighboring phospholipid molecules and the extent of hydration is correlated with

the strength of association of the head groups. The interaction of the charged hydrophilic polar groups with each other and with water and the van der Waals attractions between the hydrophobic fatty acyl chains constitute the physical basis for the arrangement of the lipid molecules in different structures such as micelles, cylindrical rods, or bilayers.

As discussed before, the bilayer aggregates are usually too closely packed to allow interaction with phospholipases and only after the introduction of surface defects in the bilayer does penetration of the enzyme become possible. Such surface defects or cracks can be formed in several ways and in general any change in the environment resulting in lateral phase separation usually allows the enzyme to interact with the bilayer. At the thermotropic phase transition of a single PL species some surface heterogeneity is present, surface defects are formed in small unilamellar vesicles prepared by sonication below the phase transition temperature. The presence in the bilayer of small amounts of reaction products, lyso-PC + FA, or mixtures of different PLs creates phase separation, and especially with negatively charged phospholipids, ion binding or pH-induced charge alteration of the polar group can lead to completely different long-range order of the PLs and isothermal phase separation. Although all these surface defects allow the enzyme to penetrate the bilayer, the interaction process is not always accompanied by a high interfacial activity. Our knowledge on a molecular level of how the enzyme is oriented in the interface is still scanty. Is there an "annulus-type" phospholipid binding to the rough protein surface changing the conformation of certain lipid molecules? Does the lipid binding change the conformation of the protein?

The present state of affairs suggests that PLAs from various sources have a different mode of action. Of course, they will differ in details because of their various tasks in vivo; however, it seems highly improbable that proteins with such similar structures and proportions would follow a fundamentally different mechanism. An apparently simple question whether these enzymes degrade organized lipid-water interfaces as monomeric or dimeric protein could not be answered so far. The analysis of inhibition kinetics both in the monomeric and aggregated substrate region is plagued with experimental difficulties which are not easily solved. It is to be hoped that in the near future more high-resolution X-ray structures will be solved and that single crystals can be obtained from enzyme-interface complexes. High resolution NMR studies of enzyme-lipid complexes and application of photoaffinity labels in PLA and PLs are expected to yield detailed information on the dynamic aspects of lipid-protein interaction. Much work must be done to increase our knowledge of local surface charges and pH effects and of the molecular details of hydration at various lipid-water interfaces. It seems evident that in order to surmount the

present problems which are related to the interaction of two biopolymers, protein and PL aggregates, further collaboration between enzymologists, lipidologists, organic chemists, and physical chemists is required.

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Author Index

Page numbers in *italics* refer to the bibliography

Aarsman AJ, Deenen LLM van Bosch H van den 97, *187*
Aarsman AJ, see Bosch H van den 96, *188*
Abe T, Alemà S, Miledi R 100, 144, 145, 146, 165, *187*
Abita JP, Lazdunski M, Bonsen PPM, Pieterson WA, Haas GH de 95, 110, 160, *187*
Adamas ND, see Slatopolsky E 31, *42*
Adamich M, Dennis EA 121, *187*
Adamich M, Roberts MF, Dennis EA 121, 122, 175, *187*
Adamich M, see Roberts MF 98, 120, 121, 122, *198*
Adams DA, see Kleeman CR 3, *39*
Aguiar A, Haas GH de, Jansen EHJM, Slotboom AJ, Williams RJP 163, 184, *187*
Alagón AC, Molinar RR, Possani LD, Gletcher PL Jr, Cronan JE Jr, Juliá JZ 100, *187*
Alagón AC, see Possani LD 101, *197*
Aleksiev B, Shipolini R 99, 102, *187*
Aleksiev B, Tchorbanov B 102, *187*
Aleksiev B, see Tchorbanov B 102, *200*
Alemà S, see Abe T 100, 144, 145, 146, 165, *187*
Alexander E, see Davidman M 21, *37*
Alexandrov SL, see Mal'tser VG 110, 139, *196*
Alexandrová M, see Hocman G 76, 85, *87*
Allgyer TT, Wells MA 118, *187*
Allison ME, Wilson CB, Gottschalk CW 12, *35*
Allison MEM, Wilson CB, Gottschalk CW 21, *35*
Allison WS, see Lewis RV *195*
Anderson T, Drakenberg T, Forsén S, Wieloch T, Lindström M 164, *187*
Andreasen TJ, Doerge DR, McNamee MG 101, *187*
Andreoli M, Robbins J, Cassano C 48, *85*
Andreoli M, see Salvatore G 48, 66, *88*
Anerbach GD, see Sherwood LM 30, *42*
Antonov VK, see Mal'tsev VG 110, 139, *196*
Antonov VK, see Zhelkovskii AM 113, 138, 139, 150, 184, *203*
Apitz-Castro R, see Jain MK 134, 137, 138, 174, *193*
Apitz-Castro RC, see Jain MK 131, 132, *193*
Apsalon UR, Shamborant OG, Miroshnikov AI 100, 101, *188*
Apsalon UR, see Magazanik LG 144, 145, 162, *195*
Apsalon UR, see Ovchinnikov YuA 105, *197*
Apsalon UR, see Zhelkovskii AM 150, *203*
Araujo PS de, Rosseneu MY, Kremer JMH, Zoelen EJJ van, Haas GH de 110, 139, 170, 171, 173, 174, *188*
Arnaud CD 30, *35*
Arnaud CD, Goldsmith RS, Bordier JP, Sizemore GW 30, *35*
Arnesjö B, Barrowman J, Borgström B *188*
Arrington R, see Epstein M 17, *38*
Arruda JA, Carrasquillo T, Cerbria A, Rademacher DR, Kurzmann NA 33, *35*
Atanasov B, see Tchorbanov B 102, *200*
Augustyn JM, Elliott WB 96, 100, *188*
Aynedjian HS, see Bank N 20, 26, 29, *35*

Baartmans PHM, see Slotboom AJ 120, 131, *199*
Backhouse J, see Mawer EB 31, *40*
Bäcker A, see Kramer HJ 24, *39*
Bahlmann J, McDonald SJ, Dunningham JG, de Wardener HE 21, *35*
Bank N, Aynedjian HS 20, 26, *35*
Bank N, Su WS, Aynedjian HS 29, *35*
Bank N, see Welbourne T 33, *43*
Banks BEC, see Shipolini RA 141, *199*
Barden RE, Darke PL, Deems RA, Dennis EA 101, 138, 146, *188*
Barenholz Y, Pieroni G, Verger R 127, *188*
Barenholz Y, see Schmidt CF 118, *199*
Bariety J, see Papanicolaou N 22, *40*
Bar-Khayim Y, see Garella S *38*
Barque JP, Dervichian DG 123, 124, 125, *188*
Barque JP, see Dervichian DG 123, 124, *190*
Barrowman J, see Arnesjö B *188*
Bartfai T, see Gatt S 135, *191*
Bassett S, see Goldman R 14, 29, *38*
Basti CP, Haylett JP, Binder HJ 27, *35*
Bastomsky CH, Kalloo H, Frenkel-Leith D 80, *85*
Bauer M, see Pickardt CR 82, *88*
Bauer M, see Scriba PC 81, *88*

Bdolah A, see Simon T 99, 102, 138, *199*
Beck LH, Senesky D, Goldberg M 25, *35*
Begin R, Epstein M, Sackner MA, Levinson R, Dougherty R, Duncan D 17, *35*
Beintema A, see Gebhardt DOE 146, *191*
Belen'kii BG, see Mal'tsev VG 110, 139, *196*
Bender ML, see Komiyama M 182, *194*
Bennett C, see Maddox D 12, *40*
Bennett EL, see Hanley MR 100, *192*
Benyajiti S, Goldstein L 33, *35*
Berger A, see Shiloah J 99, 102, 110, *199*
Berger H, see Schultze RG 19, *41*
Berger SL 157, *188*
Bergovitz MA, see Reiss E 29, *41*
Berl T, Katz FH, Henrich WL, de Torrente A, Schreier RW 22, *35*
Berliner RW, Kennedy TJJ 26, *35*
Berliner RW, see Howards SS 21, *39*
Berlyne GM 25, *35*
Berlyne GM, see Danovitch GM 14, *37*
Berman M, see Glinoer D 73, 75, 76, *86*
Bevers EM, Op den Kamp JAF, Deenen LLM van 134, 135, *188*
Bevers EM, Singal SA, Op den Kamp JAF 134, 135, *188*
Bieber AL, see Cate RL 99, 101, *189*
Biltonen RL, see Menashe M 135, *196*
Binder HJ, see Basti CP 27, *35*
Binder HJ, see Fisher K 27, *38*
Blumenfeld O, see Bourgoignie JJ *36*
Bock E, see Ramlau J 145, *198*
Boffa GA, Boffa MC, Winchenne JJ 102, *188*
Boffa GA, Boffa MC, Zakin MM, Burstein M 102, *188*
Boffa MC, Rothen C, Verheij HM, Verger R, Haas GH de *188*
Boffa MC, see Boffa GA 102, *188*
Boffa MC, see Prigent-Dachary J 136, 173, *197*
Boffa MC, see Verheij HM 109, 128, 136, 144, 146, 174, 177, *201*
Bohn E, see Kunze H 138, *195*
Bois M Du, see Clarkson EM 23, *37*
Boisseau MR, see Prigent-Dachary J 136, 173, *197*
Bon C, Changeux JP, Jeng TW, Fraenkel-Conrat H 156, 158, *188*
Bonjour JP, Preston C, Fleisch H 31, *36*
Bonnefis MJ, see Fauvel J 93, *191*
Bonsen PPM, Haas GH de, Pieterson WA, Deenen LLM van 131, 138, 142, *188*
Bonsen PPM, Pieterson WA, Volwerk JJ, Haas GH de 142, *188*
Bonsen PPM, see Abita JP 95, 110, 160, *187*
Bonsen PPM, see Haas GH de 103, 114, 115, 122, 142, *192*
Boonjarern S, see Stein JH 21, 24, *42*
Bordier JP, see Arnaud CD 30, *35*
Borgström B, see Arnesjö B *188*
Bosch H van den 93, *188*
Bosch H van den, Aarsman AJ 96, *188*
Bosch H van den, see Aarsman AJ 97, *187*
Botes DP, Viljoen CC 100, 105, *188*
Botes CP, see Viljoen CC 113, 129, 132, 144, 145, 146, 147, 150, 166, 175, 184, *201*
Bother-By AA, see Roberts MF 119, *198*
Bourgoignie JJ, Hwang KH, Espinel C, Klahr S, Bricker NS 23, *36*
Bourgoignie JJ, Hwang KH, Ipakchi E, Bricker NS 21, 23, 24, *36*
Bourgoignie JJ, Kaplan M, Eun Ch, Favre H, Hwang KH, Blumenfeld O, Bricker NS *36*
Bourgoignie JJ, Klahr S, Bricker NS 23, *36*
Bourgoignie JJ, see Bricker NS 15, 28, 34, *36*
Bourgoignie JJ, see Epstein M 17, 24, *38*
Bourgoignie JJ, see Favre H 22, 23, 24, *38*
Bourgoignie JJ, see Fine LG 15, 23, 25, *38*
Bourgoignie JJ, see Kaplan MA 23, 28, 30, *39*
Bourgoignie JJ, see Schmidt RW 5, 24, *41*
Bouvier P, Op den Kamp JAF, Deenen LLM van 134, 135, *189*
Bowles ME, see Brown JH 141, *189*
Boyd JE, see Hayslett JP 22, *39*
Bradwell AR, Burnett D, Ramsden DB, Burr WA, Prince HP, Hoffberger R 78, *85*
Braganca BM, Sambray YM, Ghadially RC 96, *189*
Braganca BM, Sambray YM, Sambray RY 138, *189*
Brasure EB, Henderson TO, Glonek T, Pattnaik NM, Scanu AM 98, *189*
Bratusch-Marrain P, Haydl H, Waldhäusl W, Dudczak R, Graninger W 81, *85*
Breithaupt H 138, *189*
Breithaupt H, Omori-Satoh T, Lang J 101, 105, *189*
Breithaupt H, Rübsamen K, Habermann E 101, 105, *189*
Breithaupt H, see Habermann E 99, 100, *192*
Brenner BM, see Deen WM 20, *37*
Brenner BM, see Maddox D 12, *40*
Brenner MA, see Hamada S 50, 56, 66, *86*
Brenner MA, see Sterling K 50, 57, 58, 66, 84, *89*
Bricker NS 7, 9, 14, 19, 22, *36*
Bricker NS, Bourgoignie JJ, Weber H, Schmidt RW, Slatopolsky E 28, 34, *36*
Bricker NS, Dewey RR, Lubowitz H, Stokes JN, Krikensgaard T 3, *36*
Bricker NS, Doorhout Mees EJ, Klahr S, Orlowski ZB, Rieselbach RE, Todd LE 3, *36*
Bricker NS, Fine LG, Kaplan MA, Epstein M, Bourgoignie JJ, Licht A 15, *36*
Bricker NS, Kime SW Jr, Morrin PAF 4, 9, 11, *36*

Bricker NS, Klahr S, Lubowitz H, Rieselbach RE 3, 7, *36*
Bricker NS, Orlowski T, Kime SW Jr, Morrin PAF 4, 10, 11, *36*
Bricker NS, Slatopolsky E, Lubowitz H, Schultze R 8, 9, *36*
Bricker NS, see Bourgoignie JJ 21, 23, 24, *36*
Bricker NS, see Dorhout Mees EJ 4, *37*
Bricker NS, see Epstein M 17, 24, *38*
Bricker NS, see Favre H 22, 23, 24, *38*
Bricker NS, see Fine LG 15, 23, 25, *38*
Bricker NS, see Kaplan MA 23, 28, 30, *39*
Bricker NS, see Lubowitz H 4, 12, 20, 23, *40*
Bricker NS, see Morrin PA 3, 9, 33, 34, *40*
Bricker NS, see Purkenson ML 33, *41*
Bricker NS, see Reiss E 7, *41*
Bricker NS, see Rieselbach RE 7, 11, *41*
Bricker NS, see Robson AM 11, *41*
Bricker NS, see Schmidt RW 5, 17, 24, 33, *41*
Bricker NS, see Schultze RG 7, 14, 16, 19, 20, 21, 22, 26, 27, *41*
Bricker NS, see Shankel SW 3, 11, *42*
Bricker NS, see Slatopolsky E 14, 15, 18, 19, 29, 30, 31, 33, *42*
Bricker NS, see Weber H 20, 21, *43*
Brickman AS, see Colodro IH 31, *37*
Brittain HG, Richardson FS, Martin RB 163, *189*
Brochner-Mortenson K 14, *36*
Brockerhoff H 117, 118, 132, 184, *189*
Brockerhoff H, Jensen RG 112, *189*
Brown JH, Bowles ME 141, *189*
Brown PR, Koutsaimanis KG, de Wardener HE 22, 23, *36*
Brown WV, see Rosseneu MY 171, *198*
Bruin SH de, see Janssen LHM 110, 161, *194*
Brychaert MC, see Verheij HM 109, 128, 136, 144, 146, 174, 177, *201*
Buckalew VM 23, *36*
Buckalew VM, Lancaster CD 22, *37*
Buckalew VM Jr, Gruber KA 24, *37*
Buckalew VM Jr, see Gruber KA 23, *39*
Buckalew VM Jr, Lancaster CD Jr 23, *37*
Buklolova-Orlova T, see Tchorbanov B 102, *200*
Burke CW, Shakespear RA 76, *85*
Burnett D, see Bradwell AR 78, *85*
Burns RA, Roberts MF 119, *189*
Burr WA, see Bradwell AR 78, *85*
Burstein E, see Tchorbanov B 102, *200*
Burstein M, see Boffa GA 102, *188*
Burstein Y, Walsh KA, Neurath H 157, *189*

Caglar S, see Schultze RG 7, 14, 16, 26, 27, *41*
Caglar S, see Slatopolsky E 30, *42*
Cahnmann HJ, see Robbins J 46, 65, 81, 85, *88*
Callewaert GL, see Shipolini RA 103, 141, *199*
Canterburry JM, see Kaplan MA 28, 30, *39*
Canterburry JM, see Reiss E 29, 30, *41*
Canterburry JM, see Slatopolsky E 30, *42*
Carlson DM, see Marshall JS 75, *88*
Carlson DM, see Zinn AB 53, 62, *89*
Carlsson FHH, see Louw AI 100, *195*
Caro Ade, see Gratoli R *192*
Carrasquillo T, see Arruda JA 33, *35*
Carriere S, Wong NLM, Dirks JH 20, *37*
Cassano C, see Andreoli M 48, *85*
Castle JN, see Cavalieri RR 60, 75, *86*
Castle JN, see Gavin LA 77, *86*
Cate RL, Bieber AL 99, 101, *189*
Catravas GN, see Tobias GS 100, *200*
Cattaneo P, see Vidal JC 141, *201*
Cavalieri RR, MacMahon FA, Castle JN 60, 75, *86*
Cavalieri RR, see Gavin LA 77, *86*
Cavalieri RR, see Snyder SM 69, *88*
Cerbria A, see Arruda JA 33, *35*
Chang WC, Hsu HP, Lo TB 101, *189*
Changeux JP, see Bon C 156, 158, *188*
Chap H, see Fauvel J 93, *191*
Charbonnier-Augeire M, see Lairon D 128, *195*
Chasis H, Smith HW 13, *37*
Chazan JA, see Garella S *38*
Cheng SY, see Gershengorn MC 57, 60, 61, 65, *86*
Cheng SY, see Robbins J 46, 65, 81, 85, *88*
Chignard M, see Vargaftig BB 146, *201*
Ching-Hsien Han S, see Rosenthal AF 138, *198*
Cid-Dresdner H, see Kannan KK 176, *194*
Clarkson EM, Koutsaimanis KG, Davidman M, Du Bois M, Penn WP, de Wardener HE 23, *37*
Clarkson EM, Raw SM, de Wardener HE 22, 23, *37*
Clemente F, see Durand S 93, *190*
Clemente F, see Figarella C 97, 98, *191*
Coburn JW, Popovtzer M, Massy SG, Kleeman CR 15, *37*
Coburn JW, see Colodro IH 31, *37*
Coburn JW, see Kopple JD 14, *39*
Coburn JW, see Massry SG 14, 29, *40*
Cogan JP, see Glinoer D 75, 76, *86*
Cohen H, see Tsao FHC *200*
Cohen JJ, see Garella S *38*
Cohen JJ, see Harrington JT 3, *39*
Cohen LH, see Jong WW de 111, *194*
Colodro IH, Brickman AS, Coburn JW, Osborn TW, Norman AW 31, *37*

Conrades MB, see Martin KJ 30, *40*
Cooper AG, see Erlanger BF 141, *190*
Cope CL, Person J 22, *37*
Cordes EH, see Jain MK 130, *193*
Cotton FA, Hazen EE Jr, Legg MJ 183, *189*
Cottrell RC, see Shipolini RA 103, 141, *199*
Cox JW, see Stein JH 21, *42*
Cronan JE Jr, see Alagón AC 100, *187*
Crosson JT, see Lubowitz H 20, *40*
Crosson JT, see Mazumdar DC 12, *40*
Crouzat-Reynes G, see Lecureuil M 46, *87*
Crumb CK, Martinez-Maldonado M, Eknoyan G, Suki W 33, *37*

Dam-Mieras MCE van, Slotboom AJ, Pieterson WA, Haas GH de 154, 160, 162, 168, 170, 171, 173, *189*
Dam-Mieras MCE van, see Slotboom AJ 161, *199*
Dam-Mieras MCE van, see Stewart-Hendrickson H 138, *200*
Dam-Mieras MCE van, see Verger R 124, *201*
Danovitch GM, Weinberg J, Berlyne GM 14, *37*
Danovitch GM, see Schmidt RW 11, *41*
Darke PL, Jarvis AA, Deems RA, Dennis EA 121, 139, 145, 148, 150, 158, *189*
Darke PL, see Barden RE 101, 138, 146, *188*
Davidman M, Alexander E, Lalone R, Levinsky N 21, *37*
Davidman M, see Clarkson EM 23, *37*
Davies HEF, see Wrong O *43*
Davis BB, see Howards SS 21, *39*
Davis LG, see Henderson TO 98, *193*
Dedieu AGR, see Volwerk JJ 114, 115, 117, 139, 143, 163, 168, 181, 184, *202*
Dedieu AGR, see Slotboom AJ 156, *200*
Deems RA, Dennis EA 101, 119, 139, *189*
Deems RA, Eaton BR, Dennis EA 120, 132, *189*
Deems RA, see Barden RE 101, 138, 146, *188*
Deems RA, see Darke PL 121, 139, 145, 148, 150, 158, *189*
Deems RA, see Roberts MF 110, 117, 120, 121, 122, 139, 143, 144, 145, 146, 148, 150, 158, 159, 166, 175, 184, *198*
Deen WM, Maddox DA, Robertson CR, Brenner BM 20, *37*
Deen W, see Maddox D 12, *40*
Deenen LLM van, Haas GH de 122, *189*
Deenen LLM van, Haas GH de, Heemskerk CHTh 129, *189*
Deenen LLM van, see Aarsman AJ 97, *187*
Deenen LLM van, see Bevers EM 134, 135, *188*
Deenen LLM van, see Bonsen PPM 131, 138, 142, *188*
Deenen LLM van, see Bouvier P 134, 135, *189*
Deenen LLM van, see Haas GH de 98, 103, 114, 115, 122, 129, 140, 141, 142, *192*
Deenen LLM van, see Jain MK 134, *193*
Deenen LLM van, see Op den Kamp JAF 129, *197*
Deenen LLM van, see Slotboom AJ 120, 131, *199*
Delori PJ 102, *190*
Delori PJ, Tessier M *190*
Dennis EA 119, 120, 132, *190*
Dennis EA, see Adamich M 121, 122, 175, *187*
Dennis EA, see Barden RE 101, 138, 146, *188*
Dennis EA, see Darke PL 121, 139, 145, 148, 150, 158, *189*
Dennis EA, see Deems RA 101, 119, 120, 132, 139, *189*
Dennis EA, see Kensil CR 115, 132, 133, 135, *194*
Dennis EA, see Lewis RV *195*
Dennis EA, see Roberts MF 98, 110, 117, 119, 120, 121, 122, 139, 143, 144, 145, 146, 148, 150, 158, 159, 166, 175, 184, *198*
Dennis EA, see Robson RJ 119, *198*
Denunzio AG, see Epstein M 17, *38*
Dervichian DG, Barque JP 123, 124, *190*
Dervichian DG, see Barque JP 123, 124, 125, *188*
Desnuelle P, see Haas GH de 103, *192*
Desnuelle P, see Rietsch J 123, *198*
Desnuelle P, see Scow RO 128, *199*
Desnuelle P, see Sémériva M 112, *199*
Dewey RR, see Bricker NS 3, *36*
Dijkman R, see Donné-Op den Kelder GM 154, 170, 172, 173, *190*
Dijkman R, see Jain MK 134, 137, 138, 174, *193*
Dijkman R, see Meijer H 154, 164, 169, 175, *196*
Dijkman R, see Volwerk JJ 114, 115, 117, 139, 143, 163, 168, 181, 184, *202*
Dijkstra BW 103, 149, 153, 176, 177, 178, *190*
Dijkstra BW, Drenth J, Kalk KH 109, 141, 151, *190*
Dijkstra BW, Drenth J, Kalk KH, Vandermaelen PJ 103, 149, 176, 178, *190*
Dijkstra K, see Egmond MR 153, *190*
Dijkstra K, see Kaptein R 155, *194*
Dijkstra BW, see Verheij HM 109, 136, 142, 143, 144, 163, 164, 168, 169, 177, 179, 182, 184, *201*
Dirks JH, see Carriere S 20, *37*
Dixon HBF, Fields R 152, 153, *190*
Dlouha V, see Haas GH de 103, *192*
Doe RP, see Seal US 48, 56, 57, 66, *88*
Doerge DR, see Andreasen TJ 101, *187*
Donlon MA, see Tobias GS 100, *200*
Donné-Op den Kelder GM, see Hille JDR 139, 170, 171, 172, 173, 174, *193*
Donné-Op den Kelder GM, Hille JDR, Dijkman R, Haas GH de, Egmond MR 154, 170, 172, 173, *190*
Doonan S, see Shipolini RA 103, 141, *199*
Doren PJ van, see Dutilh CE 98, 110, 164, *190*

Dorhout Mees EJ, Machado M, Slatopolsky E, Klahr S, Bricker NS 4, *37*
Doorhout Mees EJ, see Bricker NS 3, *36*
Dougherty R, see Begin R 17, *35*
Douste-Blazy L, see Durand S 93, *190*
Dousty-Blazy L, see Fauvel J 93, *191*
Dowdall MJ, see Fohlman J 144, 145, 165, *191*
Dowdy AS, see Wilkinson R 19, *43*
Dowling JT, see Freinkel N 47, *86*
Dowling JT, see Ingbar SH 47, *87*
Drainas D, Lawrence AJ 138, 156, *190*
Drainas D, Moores GR, Lawrence AJ 156, *190*
Drakenberg T, see Anderson T 164, *187*
Drenth J 180
Drenth J, Enzing CM, Kalk KH, Vessies JCA 103, 176, *190*
Drenth J, see Dijkstra BW 103, 109, 141, 149, 151, 176, 178, *190*
Drenth J, see Verheij HM 109, 136, 142, 143, 144, 163, 164, 168, 169, 177, 179, 182, 184, *201*
Dua A, see Massry SG 30, *40*
Dubach UC, see Schmidt U 27, *41*
Dubois A, see Glinoer D 73, 75, 76, *86*
Dudczak R, see Bratusch-Marrain P 81, *85*
Dufourq J, see Prigent-Dachary J 136, 173, *197*
Duncan D, see Begin R 17, *35*
Duncan DC, see Epstein M 17, 19, *37*
Dunn MJ 22, *37*
Dunn MJ, see Tannen RL 3, *43*
Dunningham JG, see Bahlmann J 21, *35*
Durand S, Clemente F, Thouvenot JP, Fauvel-Marmouyet J, Douste-Blazy L 93, *190*
Dutilh CE 142, 184, *190*
Dutilh CE, Doren PJ van, Verheul FEAM, Haas GH de 98, 110, 164, *190*
D'ykov VL, see Mal'tsev VG 110, 139, *196*
D'yakov VL, see Zhelkovskii AM 113, 138, 139, 150, 184, *203*

Eaker D 144, 158, *190*
Eaker D, see Fohlman J 99, 101, 102, 105, 144, 145, 165, *191*
Eaker D, see Halpert J 102, 105, 144, 145, 165, *192*
Eaker D, see Kannan KK 176, *194*
Eaker D, see Lind P 105, 109, *195*
Earley LE, see Martino JA 21, *40*
Eaton BR, see Deems RA 120, 132, *189*
Echtheld CJA van, see Jain MK 134, *193*
Edelhoch H, see Gershengorn MC 57, 60, 61, 65, *86*
Edelhoch H, see Johnson ML 61, 62, *87*
Edelhoch H, see Robbins J 45, 65, 81, 85, *88*
Efremov ES, see Gritsuk VI 100, *192*
Egan RW, see Gale PH 96, *191*
Egan TJ, see Holliday NA 3, *39*
Egmond MR, Slotboom AJ, Haas GH de, Dijstra K, Kaptein R 153, *190*
Egmond MR, see Donné-Op den Kelder GM 154, 170, 172, 173, *190*
Egmond MR, see Hille JDR 139, 170, 171, 172, 173, 174, *193*
Egmond MR, see Jain MK 134, 137, 138, 174, *193*
Egmond MR, see Scharrenberg GJM van 162, *199*
Egmond MR, see Verheij HM 118, 154, 174, *201*
Eibl H 94, 96, *190*
Eijk Jr 184
Eknoyan G, see Crumb CK 33, *37*
Eknoyan G, see Herbert CS 33, *39*
Elkan I, see Slatopolsky E 14, 29, *42*
Elkan IO, see Slatopolsky E 15, 18, 19, *42*
Elkington JR *37*
Elliott WB, see Augustyn JM 96, 100, *188*
Elliott WB, see Munjal D 141, *196*
Elliott WB, see Nair BC 101, *196*
Emmert D, see Scriba PC 81, *88*
Engstrom R, see Epstein M 17, *38*
Enzing CM, see Drenth J 103, 176, *190*
Epstein FH 26, *37*
Epstein FH, see Hayslett JP 22, *39*
Epstein FH, see Silva P 26, *42*
Epstein M, Bricker NS, Bourgoignie JJ 17, 24, *38*
Epstein M, Duncan DC, Fishman LM 17, 19, *37*
Epstein M, Pins DS, Arrington R, Denunzio AG, Engstrom R 17, *38*
Epstein M, see Begin R 17, *35*
Epstein M, see Bricker NS 15, *36*
Erlanger BF, Vratsamos SM, Wassermann N, Cooper AG 141, *190*
Espinel CH 27, 33, *38*
Espinel C, see Bourgoignie JJ 23, *36*
Eterovic VA, see Hanley MR 100, *192*
Eun Ch, see Bourgoignie JJ *36*
Evans HJ, Franson R, Qureshi GD, Moo-Penn WF 102, *191*
Evenberg A, Meyer H, Gaastra W, Verheij HM, Haas GH de 105, *191*
Evenberg A, Meyer H, Verheij HM, Haas GH de 97, 98, 105, 110, *191*
Evenson MA, see Steele TH 3, *42*

Fahrney D, see Melchior WB Jr 157, *196*
Fang VS, see Refetoff S 59, *88*
Fateh-Moghadam A, see Scriba PC 81, *88*
Fauvel J, Bonnefis MJ, Sarda L, Chap H, Thouvenot JP, Douste-Blazy L 93, *191*
Fauvel-Marmouyet J, see Durand S 93, *190*
Favre H 22, 24, *38*
Favre H, Hwang KH, Schmidt RW, Bricker NS, Bourgoignie JJ 22, 23, 24, *38*
Favre H, Louis F, Gourjon M 22, 23, *38*
Favre H, see Bourgoignie JJ *36*
Favre H, see Louis F 23, 24, *40*

Feldman D, see Pasek M 176, *197*
Ferlan I, Gubenšek F 100, *191*
Ferrato F, see Lairon D 128, *195*
Ferris TF, see Stein JH 21, 24, *42*
Fields R, see Dixon HBF 152, 153, *190*
Figarella C, Clemente F, Guy O 97, 98, *191*
Figarella C, see Gratoli R *192*
Fine LG, Bourgoignie JJ, Kuo HK, Bricker NS 23, *38*
Fine LG, Bourgoignie JJ, Weber H, Bricker NS 15, 25, *38*
Fine LG, Yanagawa N, Schultze RG 26, *38*
Fine LG, see Bricker NS 15, *36*
Finkelstein FO, Hayslett JP 26, *38*
Fisher K, Binder HJ, Hayslett JP 27, *38*
Fishman LM, see Epstein M 17, 19, *37*
Fleer EAM 152
Fleer EAM, Puijk WC, Slotboom AJ, Haas GH de 150, 152, 164, 168, *191*
Fleer EAM, Verheij HM, Haas GH de 105, *191*
Fleisch H, see Bonjour JP 31, *36*
Fletcher PL Jr, see Possani LD 101, *197*
Foda-van der Hoorn MMEL, see Meijer H 154, 164, 169, 175, *196*
Fohlman J 99, 102, *191*
Fohlman J, Eaker D 99, 101, 144, *191*
Fohlman J, Eaker D, Dowdall MJ, Lüllmann-Rauch R, Sjödin T, Leander S 144, 145, 165, *191*
Fohlman J, Eaker D, Karlsson E, Thesleff S 99, 102, *191*
Fohlman J, Lind P, Eaker D 105, *191*
Fohlman J, see Ramlau J 145, *198*
Fontaliran F, see Papanicolaou N 22, *40*
Forsén S, see Anderson T 164, *187*
Fotino S 30, *38*
Fouque F, see Vargaftig BB 146, *201*
Fox SH, see Gershengorn MC 74, *86*
Fraenkel-Conrat H, Jeng TW, Hsiang M 105, 191
Fraenkel-Conrat H, see Bon C 156, 158, *188*
Fraenkel-Conrat H, see Jeng TW 140, 144, 145, 146, 158, *194*
Fraenkel-Conrat HL, see Slotta KH 100, *200*
Franson R, see Evans HJ 102, *191*
Freitag JJ, see Martin KJ 30, *40*
Freinkel N, Dowling JT, Ingbar SH 47, *86*
Freinkel N, see Ingbar SH 47, *87*
Frenkel-Leith D, see Bastomsky CH 80, *85*
Friedler RM, see Massry SG 14, 29, *40*
Fullerton WW 58, *86*

Gaastra W, see Evenberg A 105, *191*
Gärtner R, Henze R, Horn K, Pickardt CR, Scriba PC 63, *86*
Gärnter R, see Henze R 63, *87*
Gärtner R, see Horn K 53, 54, 61, 83, *87*
Gale PH, Egan RW 96, *191*
Gallai-Hatchard J, see Magee WL 97, *195*
Garella S, Chazan JA, Bar-Khayim Y, Cohen JJ 38
Gatt S, Bartfai T 135, *191*
Gatz J, see Hesch RD 78, 79, 80, *87*
Gavellas G, see Kaplan MA 28, 30, *39*
Gavellas G, see Schmidt RW 33, *41*
Gavin LA, MacMahon FA, Castle JN, Cavalieri RR 77, *86*
Gebhardt DOE, Beintema A, Reman FC, Gent CM van 146, *191*
Gedney WB, see Morrin PA 3, 33, 34, *40*
Gent CM van, see Gebhardt DOE 146, *191*
Gershengorn MC, Cheng SY, Lippoldt RE, Lord RS, Robbins J 57, 60, 61, 65, *86*
Gershengorn MC, Glinoer D, Fox SH, Robbins J 74, *86*
Gershengorn MC, Larsen D, Robbins J 73, *86*
Gershengorn MC, Lippodt RE, Edelhoch H, Robbins J 57, 60, 61, 65, *86*
Gershengorn MC, see Glinoer D 72, 73, 75, *86*
Gershengorn MC, see Johnson ML 61, 62, *87*
Gershengorn MC, see Robbins J 46, 65, 81, 85, *88*
Ghadially RC, see Braganca BM 96, *189*
Giebisch G 27, *38*
Gier J de, see Jain MK 134, *193*
Gier J de, see Op den Kamp JAF 129, *197*
Gilmore JP 17, *38*
Ginodman LM, see Zhelkovskii AM 113, 138, 139, 150, 184, *203*
Giorgio NA, Tabachnik M 49, 51, 56, 57, 66, 67, *86*
Glassock P, see Maddox D 12, *40*
Gletcher PL Jr, see Alagón AC 100, *187*
Glinoer D, Gershengorn MC, Dubois A, Robbins J 73, *86*
Glinoer D, Gershengorn MC, Robbins J 72, 75, *86*
Glinoer D, McGuire RA, Dubois A, Cogan JP, Robbins J, Berman M 75, 76, *86*
Glinoer D, McGuire RA, Gershengorn MC, Robbins J, Berman M 73, 75, *86*
Glinoer D, see Gershengorn MC 74, *86*
Glinoer D, see Robbins J 45, 65, 81, 85, *88*
Glonek T, see Brasure EB 98, *189*
Glonek T, see Henderson TO 98, *193*
Godon JP 20, 21, 23, *38*
Goldberg M, see Beck LH 25, *35*
Goldberg M, see Puchett JB 3, *41*
Goldfine ID, see Snyder SM 69, *88*
Goldman R, Bassett S 14, 29, *38*
Goldsmith RS, see Arnaud CD 30, *35*
Goldstein DA, see Massry SG 30, *40*
Goldstein L, see Benyajiti S 33, *35*
Gonick CH, Saldanha LF 22, 23, *38*

Gonick CH, see Hillgard SD 23, *39*
Gonzales C, see Wilkinson R 19, *43*
Gopalakrishnakone P, Hawgood BJ, Theakston RDG, Reid AH 101, *191*
Gordon AH, Gross J, O'Connor D, Pitt-Rivers R 46, *86*
Gospodinow B, see Kramer HJ 23, *39*
Gotgilf IM, see Magazanik LG 144, 145, 162, *195*
Gottschalk CW 3, *38*
Gottschalk CW, see Allison ME 12, *35*
Gourjon M, see Favre H 22, 23, *38*
Gradowska L, see Slatopolsky E 30, *42*
Graninger W, see Bratusch-Marrain P 81, *85*
Gratoli R, Caro A de, Guy O, Figarella C *192*
Greco F del, Simon NM, Roguska J, Walker C 20, *37*
Green AM, Marshall JS, Pensky J, Stanbury JB 67, *86*
Green AM, see Marshall JS 67, 68, 75, *87*
Griffith OH, Jost P 93
Gritsuk VI, Meshcheryakova EA, Okhanov VV, Efremov ES, Miroshnikov AI 100, *192*
Gross J, see Gordon AH 46, *86*
Gross JB, see Hauley MJ 27, *39*
Gruber KA, Buckalew VW Jr 23, *39*
Gruber KA, see Buckalew VM Jr 24, *37*
Gry R, see Slatopolsky E 31, *42*
Gubenšek F, Žunič D 100, *192*
Gubenšek F, see Ferlan I 100, *191*
Gubenšek F, see Šket D 102, *199*
Guglielmucci E, see Vidal JC 117, *201*
Gul S, see Smith AD 135, *200*
Gundersen CB Jr, see Howard BD 95, *193*
Gutmann FD, see Wagnild JP 4, *43*
Guttierrez-Merino Ć, see Menashe M 135, *196*
Guy O, see Figarella C 97, 98, *191*
Guy O, see Gratoli R *192*
Guyer W, see Hauser H 185, *193*

Haas GH de 93
Haas GH de, Bonsen PPM, Deenen LLM van 122, *192*
Haas GH de, Bonsen PPM, Pieterson WA, Deenen LLM van 114, 115, 142, *192*
Haas GH de, Postema NM, Nieuwenhuizen W, Deenen LLM van 98, 129, 140, 141, *192*
Haas GH de, Slotboom AJ, Bonsen PPM, Deenen LLM van, Maroux S, Puigserver A, Desnuelle P 103, *192*
Haas GH de, Slotboom AJ, Bonsen PPM, Nieuwenhuizen W, Deenen LLM van, Maroux S, Dlouha V, Desnuelle P 103, *192*
Haas GH de, see Abita JP 95, 110, 160, *187*
Haas GH de, see Aguiar A 163, 184, *187*
Haas GH de, Araujo PS 110, 139, 170, 171, 173, 174, *188*
Haas GH de, see Boffa MC *188*
Haas GH de, see Bonsen PPM 131, 138, 142, *188*
Haas GH de, see Dam-Mieras MCE van 154, 160, 162, 168, 170, 171, 173, *189*
Haas GH de, see Deenen LLM van 122, 129, *189*
Haas GH de, see Donné-Op den Kelder GM 154, 170, 172, 173, *190*
Haas GH de, see Dutilh CE 98, 110, 164, *190*
Haas GH de, see Egmond MR 153, *190*
Haas GH de, see Evenberg A 97, 98, 105, 110, *191*
Haas GH de, see Fleer EAM 105, 150, 152, 164, 168, *191*
Haas GH de, see Hershberg RD 117, 118, 164, 170, *193*
Haas GH de, see Hille JDR 139, 170, 171, 172, 173, 174, *193*
Haas GH de, see Jain MK 134, 137, 138, 174, *193*
Haas GH de, see Jansen EHJM 138, 156, 161, *193*
Haas GH de, see Janssen LHM 110, 161, *194*
Haas GH de, see Meijer H 154, 164, 169, 175, *196*
Haas GH de, see Nieuwenhuizen W 97, 98, 110, *196*
Haas GH de, see Pattus F 123, 124, 128, 131, 156, 161, *197*
Haas GH de, see Pieterson WA 94, 95, 114, 115, 116, 117, 142, 143, 144, 154, 163, 164, 168, 169, 170, *197*
Haas GH de, see Puijk WC 103, 105, 175, 176, *197*
Haas GH de, see Scharrenburg GJM van 106, 107, 162, *199*
Haas GH de, see Slotboom AJ 94, 95, 96, 120, 131, 147, 156, 161, 162, 163, 164, 173, *199*
Haas GH de, see Verger R 112, 117, 118, 120, 123, 124, 126, 128, 131, 132, 136, 138, *201*
Haas GH de, see Verheij HM 109, 118, 136, 142, 143, 144, 146, 154, 163, 164, 168, 169, 174, 177, 179, 182, 184, *201*
Haas GH de, see Volwerk JJ 114, 115, 117, 118, 139, 142, 143, 146, 163, 168, 179, 181, 184, *201*
Haas GH de, see Wezel FM van 98, 148, *202*
Haas GH de, see Zografi G 123, *203*
Haberland ME, Peynolds JA 171, *192*
Habermann E, Breithaupt H 99, 100, *192*
Habermann E, Hardt KL 96, *192*
Habermann E, Neumann WP 96, *192*
Habermann E, see Breithaupt H 101, 105, *189*
Hachimori Y, Wells MA, Hanahan DJ 100, 110, *192*
Hall AD, see Luetscher JA Jr 21, *40*
Hall PW, see Schwartz WB 34, *42*
Halpert J, Eaker D 102, 105, 144, 145, 165, *192*
Halpert J, Eaker D, Karlsoon E 144, 145, 165, *192*
Hamada S, Inada M, Takemura Y, Newman ES, Brenner MA, Sterlin K 50, 56, 66, *86*
Hamada S, see Sterling K 50, 57, 58, 66, *89*
Hanahan DJ, Joseph M, Morales R 100, *192*

Hanahan DJ, see Hachimori Y 100, 110, *192*
Hanahan DJ, see Saito K 140, 141, *198*
Hanahan DJ, see Wells MA 100, 110, 140, *202*
Hanley MR, Eterovic VA, Hawkes SP, Hebert AJ, Bennett EL 100, *192*
Hao YL, Tabachnik M 66, 70, *86*
Hao YL, see Tabachnik M 66, 71, *89*
Hardt KL, see Habermann E 96, *192*
Harrah JL, see Holliday NA 3, *39*
Harrington JT, Cohen JJ 3, *39*
Hauber J, see Salach JI 97, 101, 141, *198*
Hauley MJ, Kokko JP, Gross JB, Jacobson HR 27, *39*
Hauser H, Guyer W, Pascher I, Skrabal P, Sundell S 185, *193*
Hauton JC, see Lairon D 128, *195*
Hawgood BJ, see Gopalakrishnakone P 101, *191*
Hawkes SP, see Hanley MR 100, *192*
Hawthorne JN, see White DA 93, *202*
Haydl H, see Bratusch-Marrain P 81, *85*
Hays RM, see Schwartz WB 34, *42*
Hayslett JP, Boyd JE, Epstein FH 22, *39*
Hayslett JP, see Basti CP 27, *35*
Hayslett JP, see Finkelstein FO 26, *38*
Hayslett JP, see Fisher K 27, *38*
Hayslett JP, see Kaufman JM 22, *39*
Hayslett JP, see MacLean AJ 3, 34, *40*
Hayslett JP, see Schon DA 26, *41*
Hayslett JP, see Silva P 26, *42*
Hazen EE Jr, see Cotton FA 183, *189*
Hebert AJ, see Hanley MR 100, *192*
Heemskerk CHTh, see Deenen LLM van 129, *189*
Hegyi G, see Mühlrád A 157, *196*
Hehrmann R, see Hesch RD 78, *87*
Heinrikson RL, Krueger ET, Keim PS 99, 103, 105, 147, *193*
Heinrikson RL, see Randolph A 105, *198*
Heller M, see Ulitzur S 96, *200*
Heller RJ, see Kleeman CR 14, 27, *39*
Henderson TO, Kruski AW, Davis LG, Glonek T, Scanu AM 98, *193*
Henderson TO, see Brasure EB 98, *189*
Hendon RA, Tu AT 159, *193*
Hendriks FFA, see Meijer H 154, 175, *196*
Henrich WL, see Berl T 22, *35*
Henze R, Gärtner R, Horn K 63, *87*
Henze R, see Gärtner R 63, *86*
Herbert CS, Martinez-Maldonado M, Eknoyan G, Suki WN 33, *39*
Hershberg RD, Reed GH, Slotboom AJ, Haas GH de 117, 118, 164, 170, *193*
Hesch RD, Gatz J, Jüppner H, Stubbe D 79, 80, *87*
Hesch RD, Gatz J, McIntosh CHS, Janzen J, Hehrmann R 78, *87*
Hesch RD, Gatz J, Pape J, Schmidt E, Mühlen A von zur 79, *87*
Hill LF, see Mawer EB 31, *40*
Hille JDR, Donné-Op den Kelder GM, Sauve P, Haas GH de, Egmond MR 139, 170, 171, 172, 173, 174, *193*
Hille JDR, see Donné-Op den Kelder GM 154, 170, 172, 173, *190*
Hillgard SD, Lu E, Gonick CH 23, *39*
Hocman G 46, *87*
Hocman G, Michajlovskij N, Alexandrová M 76, 85, *87*
Hocman G, see Takemura Y 58, 61, *89*
Hoffberger R, see Bradwell 78, *85*
Hoffman PM, see Schoolwerth AC 33, *41*
Hofman GC, see Scriba PC 81, *88*
Hoffsten P, see Slatopolsky E 33, *42*
Holick MF, see Popovtzer MM 31, *41*
Holliday NA, Egan TJ, Morris CR, Jarrah A, Harrah JL 3, *39*
Horn K, Gärtner R 53, 54, 61, 83, *87*
Horn K, Kubiczek T, Pickardt CR, Scriba PC 60, 70, 82, *87*
Horn K, Pickardt CR, Scriba PC 53, 80, 82, *87*
Horn K, see Gärtner R 63, *86*
Horn K, see Henze R 63, *87*
Horn K, see Pickardt CR 82, *88*
Horn K, see Scriba PC 81, *88*
Hornych A, see Papanicolaou N 22, *40*
Horton R, see Kaye Z 22, *39*
Howard NL 100, *193*
Howard BD, Gundersen CB Jr 95, *193*
Howard BD, Truog R 141, 148, 158, *193*
Howard BD, see Ng RH 158, *196*
Howard BD, see Wernicke JF 100, *202*
Howards SS, Davis BB, Knox GF, Wright FS, Berliner RW 21, *39*
Howden MEH, see Leonardi TM 102, *195*
Hruska K, see Martin KJ 30, *40*
Hruska K, see Slatopolsky E 30, 31, *42*
Hsiang M, see Fraenkel-Conrat H 105, *191*
Hsu HP, see Chang WC 101, *189*
Huang KS, Law JH 160, *193*
Huang C, see Schmidt CF 118, *199*
Hwang KH, see Bourgoignie JJ 21, 23, 24, *36*
Hwang KH, see Favre H 22, 23, 24, *38*

Inada M, see Hamada S 50, 56, 66, *86*
Inada M, see Sterling K 50, 57, 58, 66, *89*
Ingbar SH, Dowling JT, Freinkel N 47, *87*
Ingbar SH, see Freinkel N 47, *86*
Ingbar SH, see Snyder SM 69, *88*
Ipakchi E, see Bourgoignie JJ 21, 23, 24, *36*

Ishimaru K, Kihara H, Ohno M 102, *193*
Iwanaga S, see Kawauchi S 100, *194*
Iwanaga S, see Samejima Y 105, *199*

Jacobson HR, see Hauley MJ 27, *39*
Jaffe D, see Kaplan MA 28, 30, *39*
Jain MK 127
Jain MK, Apitz-Castro RC 131, 132, *193*
Jain MK, Cordes EH 130, *193*
Jain MK, Echteld GJA van, Ramirez F, Gier J de, Haas GH de, Deenen LLM van 134, *193*
Jain MK, Egmond MR, Verheij HM, Apitz-Castro R, Dijkman R, Haas GH de 134, 137, 138, 174, *193*
Jain MK, Haas GH de 134, *193*
Jain MK, see Upreti GC 130, 131, 132, 133, 134, *200*
Jansen EHJM 110, 161, 162, *193*
Jansen EHJM, Meyer H, Haas GH de, Kaptein R 138, 156, *193*
Jansen EHJM, Scharrenburg GJM van, Slotboom AJ, Haas GH de, Kaptein R 161, *193*
Jansen EHJM, see Aguiar A 163, 184, *187*
Jansen EHJM, see Slotboom AJ 161, 162, 163, 164, 173, *199*
Jansen EHJM, see Verheij HM 109, 136, 142, 143, 144, 163, 164, 168, 169, 177, 179, 182, 184, *201*
Janssen LHM, Bruin SH de, Haas GH de 110, 161, *194*
Janzen J, see Hesch RD 78, *87*
Jarrah A, see Holliday NA 3, *39*
Jarvis AA, see Darke PL 121, 139, 145, 148, 150, 158, *189*
Jeng TW, Fraenkel-Conrat H 140, 144, 145, 146, 158, *194*
Jeng TW, see Bon C 156, 158, *188*
Jeng TW, see Fraenkel-Conrat H 105, *191*
Jensen RG, see Brockerhoff H 112, *189*
Johnson ML, Lippold RE, Gershengorn MC, Robbins J, Edelhoch H 61, 62, *87*
Johnson ML, see Robbins J 83, *88*
Jong WW de, Zweers A, Cohen LH 111, *194*
Jorgensen EC, see Snyder SM 69, *88*
Joseph M, see Hanahan DJ 100, *192*
Jost P, see Griffith OH 93
Joubert FJ 101, 105, *194*
Joubert FJ, Taljaard N 101, 105, *194*
Joubert FJ, Walt SJ van der 101, 110, *194*
Jüppner H, see Hesch RD 79, 80, *87*
Juliá JZ, see Alagón AC 100, *187*
Juliá JZ, see Possani LD 101, *197*

Kagedal B, Kallberg M 52, *87*
Kainagi R, Koizumi K 130, *194*
Kalk KH, see Dijkstra BW 103, 109, 141, 149, 151, 176, 178, *190*
Kalk KH, see Drenth J 103, 176, *190*
Kallberg M, see Kagedal B 52, *87*
Kalloo H, see Bastomsky CH 80, *85*
Kannan KK, Lövgren S, Cid-Dresdner H, Petef M, Eaker D 176, *194*
Kanter A, see Reiss E 30, *41*
Kantrowitz EA, see Vensel LA 151, 152, *201*
Kaplan EL, see Reiss E 29, *41*
Kaplan MA, Bourgoignie JJ, Rosecan J, Bricker NS 23, *39*
Kaplan MA, Canterburry JM, Bourgoignie JJ, Veliz G, Gavellas E, Reis G, Bricker NS 30, *39*
Kaplan MA, Canterburry JM, Gavellas G, Jaffe D, Bourgoignie JJ, Reiss E, Bricker NS 28, 30, *39*
Kaplan M, see Bourgoignie JJ *36*
Kaplan MA, see Bricker NS 15, *36*
Kaptein R, Dijkstra K, Nicolay K 155, *194*
Kaptein R, see Egmond MR 153, *190*
Kaptein R, see Jansen EHJM 138, 156, 161, *193*
Karlsson E 95, 99, *194*
Karlsson E, Ponsawasdi P 101, *194*
Karlsson E, see Fohlman J 99, 102, *191*
Karlsson E, see Halpert J 144, 145, 165, *192*
Katz FH, see Berl T 22, *35*
Kauerz MTh, see Op den Kamp JAF 129, *197*
Kaufman JM, Siegel NJ, Hayslett JP 22, *39*
Kaverzneva ED, see Litvinko NM *195*
Kawamura J, Mazumbar DC, Lubowitz H 11, 12, *39*
Kawamura J, see Lubowitz H 20, *40*
Kawauchi S, Iwanaga S, Samejima Y, Suzuki T 100, *194*
Kaye M 30, *39*
Kaye Z, Mayeda S, Zipser R, Zia P, Horton R 22, *39*
Keim PS, see Heinrikson RL 99, 103, 105, 147, *193*
Keith C, see Pasek M 176, *197*
Kelly RB, see Strong PN 130, *200*
Kennedy TJJ, see Berliner RW 26, *35*
Kensil CR, Dennis EA 115, 132, 133, 135, *194*
Kensil CA, see Roberts MF 119, *198*
Kézdy FJ, see Shen BW 139, *199*
Kézdy FJ, see Tsao FHC *200*
Khurgin YuI, see Litvinko NM *195*
Kihara H, see Ishimaru K 102, *193*
Kime SW, see Morrin PA 9, 33, *40*
Kime SE Jr, see Bricker NS 4, 9, 10, 11, *36*
Kime SW Jr, see Reiss E 7, *41*
King K, see Yang CC 101, 102, 110, 144, 145, 146, 167, *202*
Kirshman JD, see Sealey JE 23, *42*
Klahr S, see Bourgoignie JJ 23, *36*
Klahr S, see Bricker NS 3, 7, 8, 9, *36*
Klahr S, see Dorhout Mees EJ 4, *37*

Klahr S, see Martin KJ 30, *40*
Klahr S, see Schoolwerth AC 33, *41*
Klahr S, see Slatopolsky E 30, 31, *42*
Kleeman CR, Adams DA, Maxwell MH 3, *39*
Kleeman CR, Okun R, Heller RJ 14, 27, *39*
Kleeman CR, see Coburn JW 15, *37*
Klein C, see Morrin PA 9, 33, *40*
Kliabansky C, see Shiloah J 99, 102, 110, *199*
Knox FG 21, *39*
Knox GF, see Howards SS 21, *39*
Knutson D, see Maddox D 12, *40*
Kocholaty W 141, *194*
Kochva E, see Simon T 99, 102, 138, *199*
Koizumi K, see Kainagi R 130, *194*
Kokko JP, see Hauley MJ 27, *39*
Komiyama M, Bender ML 182, *194*
Kondo K, Narita K, Lee CY 100, 105, 106, *194*
Kondo K, Toda H, Narita K 144, 145, 146, *195*
Kopple JD, Coburn JW 14, *39*
Korcek L, Tabachnik M 51, 54, 55, 56, 57, 58, 60, 63, 68, 69, *87*
Korcek L, see Siegel JS 71, *88*
Korcek L, see Tabachnik M 66, 70, 71, *89*
Koutsaimanis KG, see Brown PR 22, 23, *36*
Koutsaimanis KG, see Clarkson EM 23, *37*
Kramer HJ, Bäcker A, Krück F 24, *39*
Kramer HJ, Gospodinow B, Krück F 23, *39*
Kraut J 182, *195*
Kremer JMH, see Araujo PS 110, 139, 170, 171, 173, 174, *188*
Kremer VL, see Luetscher JA Jr 21, *40*
Krikensgaard T, see Bricker NS 3, *36*
Kronberg DS, see Sherwood LM 30, *42*
Krück F, see Kramer HJ 23, 24, *39*
Krueger ET, see Heinrikson RL 99, 103, 105, 147, *193*
Kruski AW, see Henderson TO 98, *193*
Kubiczek T, see Horn K 60, 70, 82, *87*
Kubiczek T, see Pickardt CR 82, *88*
Kudo T, see Yoshida H 101, 148, *203*
Kunze H, Bohn E, Vogt W 138, *195*
Kunze H, Nahas N, Traynor JR, Wurl M 138, *195*
Kunze H, see Nieuwenhuizen W 97, 98, *197*
Kuo HK, see Fine LG 23, *28*
Kurenbin OI, see Mal'tsev VG 110, 139, *196*
Kurtzman NA, Pillay VKG 10, *39*
Kurup PA 140, *195*
Kurzmann NA, see Arruda JA 33, *35*

Lairon D, Charbonnier-Augeire M, Nalbone G, Leonardi J, Hauton JC, Pieroni G, Ferrato F, Verger R 128, *195*
Lalone R, see Davidman M 21, *37*
Lancaster CD, see Buckalew VM 22, *37*
Lancaster CD Jr, see Buckalew VM Jr 23, *37*
Lang J, see Breithaupt H 101, 105, *189*
Laragh JH, see Sealey JE 23, *42*
Larroquère J 157, *195*
Larsen D, see Gershengorn MC 73, *86*
Law JH, see Huang KS 160, *193*
Law JH, see Shen BW 139, *199*
Law JH, see Tsao FHC *200*
Law R, see Tinker DO 133, *200*
Lawrence AJ 156, *195*
Lawrence AJ, Moores GR 156, *195*
Lawrence AJ, see Drainas D 138, 156, *190*
Lawrence AJ, see Moores GR 96, *196*
Lazarus JH, see Sterling K 84, *89*
Lazdunski M, see Abita JP 95, 110, 160, *187*
Leander S, see Fohlman J 144, 145, 165, *191*
Lebez D, see Šket D 102, *199*
Lecureuil M, Lecureuil N, Crouzat-Reynes G 46, *87*
Lecureuil N, see Lecureuil M 46, *87*
Lee CY, see Kondo K 100, 105, 106, *194*
Legg MJ, see Cotton FA 183, *189*
Lemann J, see Slatopolsky E 31, *42*
Leonardi J, see Lairon D 128, *195*
Leonardi TM, Howden MEH, Spence I 102, *195*
Levinsky N, see Davidman M 21, *37*
Levinson R, see Begin R 17, *35*
Lewis J, see Slatopolsky E 31, *42*
Lewis RV, Roberts MF, Dennis EA, Allison WS *195*
Licht A 23, 24, *40*
Licht A, see Bricker NS 15, *36*
Lichtenberg D, see Menashe M 135, *196*
Lin KY, see Weber H 20, 21, *43*
Lind P, Eaker D 105, 109, *195*
Lind P, see Fohlman J 105, *191*
Lindström M, see Anderson T 164, *187*
Lippoldt RE, see Gershengorn MC 57, 60, 61, 65, *86*
Lippoldt RE, see Johnson ML 61, 62, *87*
Lipscomb WN, see Quiocho FA 183, *197*
Litvinko NM, Khurgin YuI, Kaverzneva ED *195*
Lo TB, see Chang WC 101, *189*
Lo TB, see Tsai TH 95, 105, *200*
Lövgren S, see Kannan KK 176, *194*
Long C, Penny IF 140, *195*
Lord RS, see Gershengorn MC 57, 60, 61, 65, *86*
Louis F, Favre H 23, 24, *40*
Louis F, see Favre H 22, 23, *38*
Louw AI, Carlsson FHH 100, *195*
Lu E, see Hillgard SD 23, *39*
Lubowitz H, Mazumdar DC, Kwamura J, Crosson JT, Weisser F, Rolf D, Bricker NS 20, *40*
Lubowitz H, Purkerson ML, Bricker NS 12, *40*

Lubowitz H, Purkerson ML, Rolf D, Weisser F, Bricker NS 33, *40*
Lubowitz H, Purkerson ML, Sugita M, Bricker NS 4, *40*
Lubowitz H, see Bricker NS 3, 7, 8, 9, *36*
Lubowitz H, see Kawamura J 11, 12, *39*
Lubowitz H, see Mazumdar DC 12, *40*
Lubowitz H, see Purkenson ML 33, *41*
Lubowitz H, see Rieselbach RE 11, *41*
Luca HF de, see Popovtzer MM 31, *41*
Luca H de, see Slatopolsky E 31, *42*
Lucassen M, see Tinker DO 133, *200*
Lüllmann-Rauch R, see Fohlman J 144, 145, 165, *191*
Luetscher JA, see Wilkinson R 19, *43*
Luetscher JA Jr, Hall AD, Kremer VL 21, *40*
Lumb GA, see Mawer EB 31, *40*

MacDermot J, Westgaard RH, Thompson EJ 150, *195*
Machado M, see Dorhout Mees EJ 4, *37*
MacLean AJ, Hayslett JP 3, 34, *40*
MacMahon FA, see Cavalieri RR 60, 75, *86*
MacMahon FA, see Gavin LA 77, *86*
Maddox D, Bennett C, Deen W, Glassock P, Knutson D, Brenner BM 12, *40*
Maddox DA, see Deen WM 20, *37*
Magazanik LG, Gotgilf IM, Slavnova TI, Miroshnikov AI, Apsalon UR 144, 145, 162, *195*
Magee WL, Gallai-Hatchard J, Sanders H, Thompson RHS 97, *195*
Magee WL, see Uthe JF 97, *200*
Makino S, Reynolds JA, Tanford C 171, *195*
Malnic G, see Rocha A 20, *41*
Mal'tsev VG, Zimina TM, Kurenbin OI, Belen'kii BG, Alexandrov SL, Pavlova NP, D'yakov VL, Antonov VK 110, 139, *196*
Marcondes M, see Rocha A 20, *41*
Marinetti GV 96, *196*
Maroux S, see Haas GH de 103, *192*
Marshall JS, Green AM, Pensky J, Williams S, Zinn A, Carlson DM 75, *88*
Marshall JS, Pensky J 49, 56, 67, *87*
Marshall JS, Pensky J, Green AM 67, 68, *87*
Marshall JS, Pensky J, Williams S 63, 67, 72, 73, *87*
Marshall JS, see Green AM 67, *86*
Marshall JS, see Pensky J 50, 51, 55, 56, 60, 61, 63, 65, 67, 68, 69, *88*
Marshall JS, see Refetoff S 59, *88*
Marshall JS, see Zinn AB 53, 62, *89*
Martin K, see Slatopolsky E 30, 31, *42*
Martin KJ, Freitag JJ, Conrades MB, Hruska K, Klahr S, Slatopolsky E 30, *40*
Martin RB, see Brittain HG 163, *189*
Martin-Moutot N, Rochat H 101, 129, 132, 144, *196*
Martinez-Maldonado M, see Crumb CK 33, *37*
Martinez-Maldonado M, see Herbert CS 33, *39*
Martino JA, Earley LE 21, *40*
Mason E, see Tinker DO 132, 133, 134, 135, 136, *200*
Massry SG, Friedler RM, Coburn JW 14, 29, *40*
Massry SG, Tuma S, Dua A, Goldstein DA 30, *40*
Massry SG, see Coburn JW 15, *37*
Mawer EB, Backhouse J, Hill LF, Lumb GA, da Silva P, Taylor DM, Stanbury SW 31, *40*
Maxwell MH, see Kleeman CR 3, *39*
Mayeda S, see Kaye Z 22, *39*
Mayer GP, see Sherwood LM 30, *42*
Mazumdar DC, Crosson JT, Lubowitz H 12, *40*
Mazumbar DC, see Kawamura J 11, 12, *39*
Mazumdar DC, see Lubowitz H 20, *40*
McDonald SJ, see Bahlman J 21, *35*
McDowell DR 82, *88*
McGuire RA, see Glinoer D 73, 75, 76, *86*
McIntosh CHS, see Hesch RD 78, *87*
McNamee MG, see Andreasen TJ 101, *187*
Mebs D 96, *196*
Mebs D, Samejima Y 102, *196*
Meddens MJM, see Meijer H 175, *196*
Meijer H 148, 149, 155, 164, *196*
Meijer H, Meddens MJM, Dijkman R, Slotboom AJ, Haas GH de 175, *196*
Meijer H, Puijk WC, Dijkman R, Foda-vander Hoorn MMEL, Pattus F, Slotboom AJ, Haas GH de 154, 164, 169, 175, *196*
Meijer H, Verhoef H, Hendriks FFA, Slotboom AJ, Haas GH de 154, 175, *196*
Melchior WB Jr, Fahrney D 157, *196*
Menashe M, Lichtenberg D, Guttierrez-Merino C, Biltonen RL 135, *196*
Meshcheryakova EA, see Gritsuk VI 100, *192*
Meyer H, see Evenberg A 97, 98, 105, 110, *191*
Meyer H, see Jansen EHJM 138, 156, *193*
Michajlovskij N, see Hocman G 76, 85, *87*
Middelhoff G, see Rosseneu MY 171, *198*
Mieras MCE, see Verger R 117, 118, 124, 131, 132, 136, *201*
Milch PO, see Sterling K 84, *89*
Miledi R, see Abe T 100, 144, 145, 146, 165, *187*
Millieux P, see Papanicolaou N 22, *40*
Mincey TC, see Roberts MF 110, 117, 121, 139, 143, 144, 145, 146, 148, 150, 158, 184, *198*
Miroshnikov AI, see Apsalon UR 100, 101, *188*
Miroshnikov AI, see Gritsuk VI 100, *192*
Miroshnikov AI, see Magazanik LG 144, 145, 162, *195*

Miroshnikov AI, see Ovchinnikov YuA 105, *197*
Mirsohnikov AI, see Zhelkovskii AM 150, *203*
Misiorowski RL, Wells MA 118, *196*
Molinar RR, see Alagón AC 100, *187*
Moody TW, Raftery MA 100, *196*
Moo-Penn WF, see Evans HJ 102, *191*
Moores GR, Lawrence AJ 96, *196*
Moores GR, see Drainas D 156, *190*
Moores GR, see Lawrence AJ 156, *195*
Morales R, see Hanahan DJ 100, *192*
Morrin PA, Bricker NS, Kime SW, Klein C 9, 33, *40*
Morrin PA, Gedney WB, Newmark LN, Bricker NS 3, 33, 34, *40*
Morrin PAF, see Bricker NS 4, 9, 10, 11, *36*
Morrin PA, see Reiss E 7, *41*
Morris CR, see Holliday NA 3, *39*
Mühlen A von zur, see Hesch RD 79, *87*
Mühlrád A, Hegyi G, Tóth G 157, *196*
Munjal D, Elliott WB 141, *196*

Nagy D, see Wosilait WD 71, *89*
Nahas N, see Kunze H 138, *195*
Nair BC, Nair C, Elliott WB 101, *196*
Nair C, see Nair BC 101, *196*
Nalbone G, see Lairon D 128, *195*
Narita K, see Kondo K 100, 105, 106, 144, 145, 146, *194*
Nazimov IV, see Ovchinnikov YuA 105, *197*
Neumann WP, see Habermann E 96, *192*
Neurath H, see Burstein Y 157, *189*
Newman ES, see Hamada S 50, 56, 66, *86*
Newman ES, see Sterling K 50, 57, 58, 66, *89*
Newmark LN, see Morrin PA 3, 33, 34, *40*
Ng RH, Howard BD 158, *196*
Nicolay K, see Kaptein R 155, *194*
Nieuwenhuizen W, Kunze H, Haas GH de 97, 98, *197*
Nieuwenhuizen W, Oomens A, Haas GH de *196*
Nieuwenhuizen W, Steenbergh P, Haas GH de 98, 110, *196*
Nieuwenhuizen W, see Haas GH de 98, 103, 129, 140, 141, *192*
Nilsson SF, Peterson PA 52, 56, 57, 64, 68, *88*
Nishida S, et al. 105, *197*
Nokes GW, see Wilkinson R 19, *43*
Norman AW, see Colodro IH 31, *37*

Oberjat T, see Wernicke JF 100, *202*
Obidairo 105
O'Connor D, see Gordon AH 46, *86*
O'Donnell M, see Tobian L 22, *43*
Ohno M, see Ishimaru K 102, *193*
Okawa Y, see Yamaguchi T *200*
Okhanov VV, see Gritsuk VI 100, *192*
Okun R, see Kleeman CR 14, 27, *39*
Oliver J 3, *40*
Omori-Satoh T, see Breithaupt H 101, 105, *189*
Oomens A, see Nieuwenhuizen W *196*
Op den Kamp JAF 96, *197*
Op den Kamp JAF, Gier J de, Deenen LLM van 129, *197*
Op den Kamp JAF, Kauerz MTh, Deenen LLM van 129, *197*
Op den Kamp JAF, see Bevers EM 134, 135, *188*
Op den Kamp JAF, see Bouvier P 134, 135, *189*
Orlowski T, see Bricker NS 4, 10, 11, *36*
Orlowski ZB, see Bricker NS 3, *36*
Osborn TW, see Colodro IH 31, *37*
Osgood RW, see Stein JH 21, 24, *42*
Otnaess AB, see Roberts MF 119, *198*
Ovchinnikov YuA, Miroshnikov AI, Nazimov IV, Apsalon UR, Soldatova LN 105, *197*
Papahadjopoulos D, see Szoka F Jr 132, *200*
Papanicolaou N, Safar M, Hornych A, Fontaliran F, Weiss Y, Bariety J, Milliez P 22, *40*
Pape J, see Hesch RD 79, *87*
Pascher I, see Hauser H 185, *193*
Pasek M, Keith C, Feldman D, Sigler PB 176, *197*
Pattnaik NM, see Brasure EB 98, *189*
Pattus F, Slotboom AJ, Haas GH de 123, 124, 128, 131, 156, 161, *197*
Pattus F, see Meijer H 154, 164, 169, 175, *196*
Pattus F, see Rietsch J 123, *198*
Pattus F, see Slotboom AJ 161, 162, 163, 164, 173, *199*
Pavkin R. see Sket D 102, *199*
Pavlova NP, see Mal'tsev VG 110, 139, *196*
Peeters H, see Rosseneu MY 171, *198*
Penn WP, see Clarkson EM 23, *37*
Pennell JP, see Schultze RG 7, 14, 16, 26, 27, *41*
Pennell JP, see Slatopolsky E 30, *42*
Penny IF, see Long C 140, *195*
Pensky J, Marshall JS 50, 51, 55, 56, 60, 61, 63, 65, 67, 68, 69, *88*
Pensky J, see Green AM 67, *86*
Pensky J, see Marshall JS 49, 56, 63, 67, 68, 72, 73, 75, *87*
Person J, see Cope CL 22, *37*
Petef M, see Kannan KK 176, *194*
Peterson PA, see Nilsson SF 52, 56, 57, 64, 68, *88*
Pickardt CR, Bauer M, Horn K, Kubiczek T, Scriba PC 82, *88*
Pickardt CR, see Gärtner R 63, *86*
Pickardt CR, see Horn K 52, 60, 70, 80, 82, *87*
Pickardt CR, see Scriba PC 81, *88*
Pieroni G, Verger R 123, 126, *197*
Pieroni G, see Barenholz Y 127, *188*

Pieroni G, see Lairon D 128, *195*
Pieterson WA 142, 163, 168, 170, *197*
Pieterson WA, Vidal JC, Volwerk JJ, Haas GH de 94, 95, 114, 116, 117, 143, 144, 154, 164, 168, 169, 170, *197*
Pieterson WA, Volwerk JJ, Haas GH de 95, 114, 115, 142, 143, 154, 163, 164, *197*
Pieterson WA, see Abita JP 95, 110, 160, *187*
Pieterson WA, see Bonsen PPM 131, 138, 142, *188*
Pieterson WA, see Dam-Mieras MCE van 154, 160, 162, 168, 170, 171, 173, *189*
Pieterson WA, see Haas GH de 114, 115, 142, *192*
Pieterson WA, see Volwerk JJ 114, 117, 118, 142, 143, 146, 163, 168, 179, 184, *201*
Pillay VKG, see Kurtzman NA 10, *39*
Pins DS, see Epstein M 17, *38*
Pitts-Rivers R, see Gordon AH 46, *86*
Pitts RF 8, *40*
Platt R 3, 14, *40*
Polak A 3, *41*
Pongsawasdi P, see Karlsson E 101, *194*
Poon PH, Wells MA 118, *197*
Popovtzer MM, Robinette JB, de Luca HF, Holick MF 31, *41*
Popovtzer M, see Coburn JW 15, *37*
Possani LD, Alagón AC, Fletcher PL Jr, Varela MJ, Juliá JZ 101, *197*
Possani LD, see Alagón AC 100, *187*
Postema NM 141, *197*
Postema NM, see Haas GH de 98, 129, 140, 141, *192*
Potts JF, see Sherwood LM 30, *42*
Pounder DJ, see White DA 93, *202*
Preston C, see Bonjour JP 31, *36*
Prigent-Dachary J, Boffa MC, Boisseau MR, Dufourq J 136, 173, *197*
Prince HP, see Bradwell AR 78, *85*
Puchett JB, Goldberg M 3, *41*
Puigserver A, see Haas GH de 103, *192*
Puijk WC, Verheij HM, Haas GH de 103, 105, 176, *197*
Puijk WC, Verheij HM, Wietzes P, Haas GH de 105, 175, *197*
Puijk WC, see Fleer EAM 150, 152, 164, 168, *191*
Puijk WC, see Meijer H 154, 164, 169, 175, *196*
Puijk WC, see Scharrenburg GJM van 162, *199*
Puijk WC, see Slotboom AJ 156, *200*
Puijk WC, see Verheij HM 109, 136, 142, 143, 144, 163, 164, 168, 169, 177, 179, 182, 184, *201*
Purdon AD, Tinker DO, Spero L 166, *197*
Purdon AD, see Tinker DO 132, 133, 134, 135, 136, *200*
Purkenson ML, Lubowitz H, White RM, Bricker NS 33, *41*
Purkerson ML, see Lubowitz H 4, 12, 33, *40*
Purkerson M, see Slatopolsky E 33, *42*

Quiocho FA, Lipscomb WN 183, *197*
Qureshi GD, see Evans HJ 102, *191*

Rademacher DR, see Arruda JA 33, *35*
Raftery MA, see Moody TW 100, *196*
Rainier S, see Upreti GC 131, *200*
Ramberg CF, see Sherwood LM 30, *42*
Ramirez F, see Jain MK 134, *193*
Ramlau J, Bock E, Fohlman J 145, *198*
Ramsden DB, see Bradwell AR 78, *85*
Randolph A, Sakmar TP, Heinrikson RL 105, *198*
Raw SM, see Clarkson EM 22, 23, *37*
Reaven GM, see Swenson MS 30, *43*
Reed GH, see Hershberg RD 117, 118, 164, 170, *193*
Refetoff S, Fang VS, Marshall JS 59, *88*
Refetoff S, Fang VS, Marshall JS, Robin NI 59, *88*
Regal EM, see Schrier RW 22, *41*
Regal EM, see Tannen RL 3, *43*
Regts J, see Wilshut JC 130, *202*
Reid AH, see Gopalakrishnakone P 101, *191*
Reis G, see Kaplan MA 30, *39*
Reiss E, Bricker NS, Kime SW Jr, Morrin PA 7, *41*
Reiss E, Canterburry JM, Bergovitz MA, Kaplan EL 29, *41*
Reiss E, Canterburry JM, Kanter A 30, *41*
Reiss E, see Kaplan MA 28, 30, *39*
Reiss E, see Slatopolsky E 30, *42*
Relman AS *41*
Relman AS, see Schwartz WB 34, *42*
Reman FC, see Gebhardt DOE 146, *191*
Reynolds JA, Tanford C 173, *198*
Reynolds JA, see Haberland ME 171, *192*
Reynolds JA, see Makino S 171, *195*
Richardson FS, see Brittain HG 163, *189*
Rieselbach RE, Shankel SW, Slatopolsky E, Lubowitz H, Bricker NS 11, *41*
Rieselbach RE, Todd L, Rosenthal M, Bricker NS 7, *41*
Rieselbach RE, see Bricker NS 3, 7, *36*
Rieselbach RE, see Steele TH 3, *42*
Rieselbach RE, see Wagnild JP 4, *43*
Rietsch J, Pattus F, Desnuelle P, Verger R 123, *198*
Rietsch J, see Verger R 124, *201*
Robbins J 53, 83, *88*
Robbins J, Cheng SY, Gershengorn MC, Glinoer D, Cahnmann HJ, Edelhoch H 46, 65, 81, 85, *88*
Robbins J, Johnson ML 83, *88*
Robbins J, see Andreoli M 48, *85*
Robbins J, see Gershengorn MC 60, 61, 65, 73, 74, *86*
Robbins J, see Glinoer D 72, 73, 75, 76, *86*

Robbins J, see Johnson ML 61, 62, *87*
Roberts MF, Adamich M, Robson RJ, Dennis EA 98, 120, 121, 122, *198*
Roberts MF, Bothner-By AA, Dennis EA 119, *198*
Roberts MF, Deems RA, Dennis EA 110, 117, 120, 121, 122, 150, 159, 166, 175, 184, *198*
Roberts MF, Deems RA, Mincey TC, Dennis EA 110, 117, 121, 139, 143, 144, 145, 146, 148, 150, 158, 184, *198*
Roberts MF, Otnaess AB, Kensil CA, Dennis EA 119, *198*
Roberts MF, see Adamich M 121, 122, 175, *187*
Roberts MF, see Burns RA 119, *189*
Roberts MF, see Lewis RV *195*
Robertson CR, see Deen WM 20, *37*
Robin NI, see Refetoff S 59, *88*
Robinette JB, see Popovtzer MM 31, *41*
Robinson NC, Tanford C 171, *198*
Robson AM, Srivastava PL, Bricker NS 11, *41*
Robson AM, see Shakel SW 3, 11, *42*
Robson AM, see Slatopolsky E 14, 29, *42*
Robson RJ, Dennis EA 119, *198*
Robson RJ, see Roberts MF 98, 120, 121, 122, *198*
Rocha A, Marcondes M, Malnic G 20, *41*
Rochat H, see Martin-Moutot N 101, 129, 132, 144, *196*
Roche J, see Salvatore G 48, 66, *88*
Rock CO, Snyder F 99, *198*
Roelofsen B, Zwaal RFA 96, *198*
Roguska J, see Greco F del 20, *37*
Roholt OA, Schlamowitz M 97, 112, 113, 132, 138, 181, *198*
Rolf D, see Lubowitz H 20, 33, *40*
Rosecan J, see Kaplan MA 23, *39*
Rosenthal AF, Ching-Hsien Han S 138, *198*
Rosenthal M, see Rieselbach RE 7, *41*
Rosseneu MY, Soetewij F, Middelhoff G, Peeters H, Brown WV 171, *198*
Rosseneu MY, see Araujo PS 110, 139, 170, 171, 173, 174, *188*
Rothen C, see Boffa MC *188*
Rothen Ch, see Verheij HM 109, 128, 136, 144, 146, 174, 177, *201*
Rübsamen K, see Breithaupt H 101, 105, *189*
Ruggeri JL, see Swenson MS 30, *43*
Rutherford WE, see Slatopolsky E 30, 31, *42*

Sackner MA, see Begin R 17, *35*
Safar M, see Papanicolaou N 22, *40*
Saito K, Hanahan DJ 140, 141, *198*
Sakaguchi K, see Yamaguchi T *202*
Sakmar TP, see Randolph A 105, *198*
Salach JI, Turini P, Hauber J, Seng R, Tisdale H, Singer TP 97, *198*
Salach JI, Turini P, Seng R, Hauber J, Singer P 97, 101, 141, *198*
Saldanha LF, see Gonick CH 22, 23, *38*
Salvatore G, Andreoli M, Roche J 48, 66, *88*
Sambray RY, see Braganca BM 138, *189*
Sambray YM, see Braganca BM 96, 138, *189*
Samejima Y, Iwanaga S, Suzuki T 105, *199*
Samejima Y, see Kawauchi S 100, *194*
Samejima Y, see Mebs D 102, *196*
Sanders H, see Magee WL 97, *195*
Sandler RS, see Schoolwerth AC 33, *41*
Sarda L, see Fauvel J 93, *191*
Sauve P, see Hille JDR 139, 170, 171, 172, 173, 174, *193*
Scanu AM, see Brasure EB 98, *189*
Scanu AM, see Henderson TO 98, *193*
Scarpa A, see Scherphof GL 138, *199*
Schabort JC, see Viljoen CC 129, 132, 166, 175, *201*
Scharrenburg GJM van, Haas GH de, Slotboom AJ 106, 107, *199*
Scharrenburg GJM van, Puijk WC, Egmond MR, Haas GH de, Slotboom AJ 162, *199*
Scharrenburg GJM van, see Jansen EHJM 161, *193*
Scherphof GL, Scarpa A, Toorenberg A van 138, *199*
Scherphof G, see Wilschut JC 130, *202*
Schlamowitz M, see Roholt OA 97, 112, 113, 132, 138, 181, *198*
Schmid J, see Schmidt U 27, *41*
Schmidt CF, Barenholz Y, Huang C, Thompson FE 118, *199*
Schmidt E, see Hesch RD 79, *87*
Schmidt H, see Schmidt U 27, *41*
Schmidt H, see Wittich KA 98, *202*
Schmidt RW, Bourgoignie JJ, Bricker NS 5, 24, *41*
Schmidt RW, Bricker NS 17, *41*
Schmidt RW, Bricker NS, Gavellas G 33, *41*
Schmidt RW, Danovitch GM 11, *41*
Schmidt RW, see Bricker NS 28, 34, *36*
Schmidt RW, see Favre H 22, 23, 24, *38*
Schmidt U, Schmid J, Schmidt H, Dubach UC 27, *41*
Schon DA, Silva P, Hayslett JP 26, *41*
Schoolwerth AC, Sandler RS, Hoffman PM, Klahr S 33, *41*
Schrier RW, Regal EM 22, *41*
Schrier RW, see Berl T 22, *35*
Schrier RW, see Tannen RL 3, *43*
Schultze RG, Berger H 19, *41*
Schultze RG, Shapiro HS, Bricker NS 19, 20, 21, 22, *41*
Schultze RG, Taggart DD, Shapiro H, Pennell JP, Caglar S, Bricker NS 7, 14, 16, 26, 27, *41*
Schultze R, see Bricker NS 8, 9, *36*
Schultze RG, see Fine LG 26, *38*

Schussler GC 68, *88*
Schwartz WB, Hall PW, Hays RM, Relman AS 34, *42*
Scow RO, Desnuelle P, Verger R 128, *199*
Scriba PC, Bauer M, Emmert D, Fateh-Moghadam A, Hofman GC, Horn K, Pickardt CR 81, *88*
Scriba PC, see Gärtner R 63, *86*
Scriba PC, see Horn K 53, 60, 70, 80, 82, *87*
Scriba PC, see Pickardt CR 82, *88*
Seal US, Doe RP 48, 56, 57, 66, *88*
Sealey JE, Kirshman JD, Laragh JH 23, *42*
Sémériva M, Desnuelle P 112, *199*
Senesky D, see Beck LH 25, *35*
Seng R, see Salach JI 97, 101, 141, *198*
Shain W, see Tobias GS 100, *200*
Shakespear RA, see Burke CW 76, *85*
Shamborant OG, see Apsalon UR 100, 101, *188*
Shankel SW, Robson AM, Bricker NS 3, 11, *42*
Shankel SW, see Rieselbach RE 11, *41*
Shannon JA 13, *42*
Shannon JA, Smith HW 13, *42*
Shapiro H, see Schultze RG 7, 14, 16, 26, 27, *41*
Shapiro HS, see Schultze RG 19, 20, 21, 22, *41*
Shen BW, Law JH *199*
Shen BW, Tsao FHC, Law JH, Kézdy FJ 139, *199*
Sherwood LM, Mayer GP, Ramberg CF, Kronberg DS, Anerbach GD, Potts JF 30, *42*
Shier WT, Trotter JT 96, *199*
Shiloah J, Klibansky C, Vries A de, Berger A 99, 102, 110, *199*
Shinkai W, see Yoshida H 101, 148, *203*
Shipley GG, see Untracht SH 128, *200*
Shipolini RA, Callewaert GL, Cottrell RC, Doonan S, Vernon CA, Banks BEC 141, *199*
Shipolini RA, Callewaert GL, Cottrell RC, Vernon CA 103, *199*
Shipolini RA, Doonan S, Vernon CA 103, *199*
Shipolini R, see Aleksiev B 99, 102, *187*
Siegel JS, Korcek L, Tabachnik M 71, *88*
Siegel NJ, see Kaufman JM 22, *39*
Sigler PB 176
Sigler PB, see Pasek M 176, *197*
Silva P, Hayslett JP, Epstein FH 26, *42*
Silva P da, see Mawer EB 31, *40*
Silva P, see Schon DA 26, *41*
Simon NM, see Greco F del 20, *37*
Simon T, Bdolah A 102, *199*
Simon T, Bdolah A, Kochva E 99, 102, 138, *199*
Singal SA, see Bevers EM 134, 135, *188*
Singer P, see Salach JI 97, 101, 141, *198*
Singer TP, see Salach JI 97, 101, *198*
Sisson P, see Waite M 128, *202*
Sizemore GW, see Arnaud CD 30, *35*
Sjödin T, see Fohlman J 144, 145, 165, *191*
Šket D, Gubenšek F, Pavkin R, Lebez D 102, *199*
Skrabal P, see Hauser H 185, *193*
Slatopolsky E, Bricker NS 30, *42*
Slatopolsky E, Caglar S, Gradowska L, Canterburry JM, Reiss E, Bricker NS 30, *42*
Slatopolsky E, Caglar S, Pennel JP, Taggart DD, Canterburry JM, Reiss E, Bricker NS 30, *42*
Slatopolsky E, Elkan IO, Weerts C, Bricker NS 15, 18, 19, *42*
Slatopolsky E, Gry R, Adamas ND, Lewis J, Hruska K, Martin K, Klahr S, de Luca H, Lemann J 31, *42*
Slatopolsky E, Hoffsten P, Purkerson M, Bricker NS 33, *42*
Slatopolsky E, Hruska K, Rutherford WE 31, *42*
Slatopolsky E, Robson AM, Elkan I, Bricker NS 14, 29, *42*
Slatopolsky E, Rutherford WE, Hruska K, Martin K, Klahr S 30, *42*
Slatopolsky E, see Bricker NS 3, 8, 9, 28, 34, *36*
Slatopolsky E, see Dorhout Mees EJ 4, *37*
Slatopolsky E, see Martin KJ 30, *40*
Slatopolsky E, see Rieselbach RE 11, *41*
Slavnova TI, see Magazanik LG 144, 145, 162, *195*
Slotboom AJ, Dam-Mieras MCE van, Haas GH de 161, *199*
Slotboom AJ, Haas GH de 95, 147, 161, 162, *199*
Slotboom AJ, Jansen EHJM, Pattus F, Haas GH de 161, 164, *199*
Slotboom AJ, Jansen EHJM, Vlijm H, Pattus F, Soares de Araujo P, Haas GH de 161, 162, 163, 164, 173, *199*
Slotboom AJ, Verheij HM, Haas GH de 94, 96, *199*
Slotboom AJ, Verheij HM, Puijk WC, Dedieu AGR, Haas GH de 156, *200*
Slotboom AJ, Verger R, Verheij HM, Baartmans PHM, Deenen LLM van, Haas GH de 120, 131, *199*
Slotboom AJ, see Aguiar A 163, 184, *187*
Slotboom AJ, see Dam-Mieras MCE van 154, 160, 162, 168, 170, 171, 173, *189*
Slotboom AJ, see Egmond MR 153, *190*
Slotboom AJ, see Fleer EAM 150, 152, 164, 168, *191*
Slotboom AJ, see Haas GH de 103, *192*
Slotboom AJ, see Hershberg RD 117, 118, 164, 170, *193*
Slotboom AJ, see Jansen EHJM 161, *193*
Slotboom AJ, see Meijer H 154, 164, 169, 175, *196*
Slotboom AJ, see Pattus F 123, 124, 128, 131, 156, 161, *197*
Slotboom AJ, see Scharrenburg GJM van 106, 107, 162, *199*
Slotboom AJ, see Wezel FM van 148, *202*
Slotta KH, Fraenkel-Conrat HL 100, *200*

Smith AD, Gul S, Thompson RHS 135, *200*
Smith CM, Wells MA 113, 139, *200*
Smith HW 13, *42*
Smith HW, see Chasis H 13, *37*
Smith HW, see Shannon JA 13, *42*
Snyder F, see Rock CO 99, *198*
Snyder SM, Cavalieri RR, Goldfine ID, Ingbar SH, Jorgensen EC 69, *88*
Snyder WR, see Tsao FHC *200*
Soares de Araujo P, see Slotboom AJ 161, 162, 163, 164, 173, *199*
Soetewij F, see Rosseneu MY 171, *198*
Soldatova LN, see Ovchinnikov YuA 105, *197*
Sonnenberg H 21, *42*
Spence I, see Leonardi TM 102, *195*
Spero L, see Purdon AD 166, *197*
Srivastava PL, see Robson AM 11, *41*
Stanbury JB, see Green AM 67, *86*
Stanbury SW, see Mawer EB 31, *40*
Steele TH, Wen SF, Evenson MA, Rieselbach RE 3, *42*
Steenbergh P, see Nieuwenhuizen W 98, 110, *196*
Stein JH, Osgood RW, Boonjarern S, Cox JW, Ferris TF 21, *42*
Stein JH, Osgood RW, Boonjarern S, Ferris TF 21, 24, *43*
Sterling K 81, *89*
Sterling K, Hamada S, Takemura Y, Brenner MA, Newman ES, Inada M 50, 57, 58, 66, *89*
Sterling K, Milch PO, Brenner MA, Lazarus JH 84, *89*
Sterling K, see Hamada S 50, 56, 66, *86*
Sterling K, see Takemura Y 58, 61, *89*
Stewart-Hendrickson H, Dam-Mieras MCE van 138, *200*
Stewart-Hendrickson H, see Willman C 125, *202*
Stokes JN, see Bricker NS 3, *36*
Stoppani AOM, see Vidal JC 99, 100, 117, 138, 141, *201*
Strong PN, Kelly RB 130, *200*
Stubbe D, see Hesch RD 79, 80, *87*
Su WS, see Bank N 29, *35*
Sugita M, see Lubowitz H 4, *40*
Suki W, see Crumb CK 33, *37*
Suki WN, see Herbert CS 33, *39*
Sundell S, see Hauser H 185, *193*
Suzuki T, see Kawauchi S 100, *194*
Suzuki T, see Samejima Y 105, *199*
Swenson MS, Weisinger JR, Ruggeri JL, Reaven GM 30, *43*
Szoka F Jr, Papahadjopoulos D 132, *200*

Tabachnik M, Korcek L 70, *89*
Tabachnik M, Hao YL, Korcek L 66, 71, *89*
Tabachnik M, see Giorgio NA 49, 51, 56, 57, 66, 67, *86*
Tabachnik M, see Hao YL 66, 70, *86*
Tabachnik M, see Korcek L 51, 54, 55, 56, 57, 58, 60, 63, 68, 69, *87*
Tabachnik M, see Siegel JS 71, *88*
Taggart DD, see Schultze RG 7, 14, 16, 26, 27, *41*
Taggart DD, see Slatopolsky E 30, *42*
Takahashi K 151, 153, *200*
Takemura Y, Hocman G, Sterling K 58, 61, *89*
Takemura Y, see Hamada S 50, 56, 66, *86*
Takemura Y, see Sterling K 50, 57, 58, 66, *89*
Taljaard N, see Joubert FJ 101, 105, *194*
Tamiya N, see Yoshida H 101, 148, *203*
Tanford C 129, *200*
Tanford C, see Makino S 171, *195*
Tanford C, see Reynolds JA 173, *198*
Tanford C, see Robinson NC 171, *198*
Tannen RL, Regal EM, Dunn MJ, Schrier RW 3, *43*
Tata JR 47, 55, 58, 65, *89*
Taylor CM, see Mawer EB 31, *40*
Tchorbanov B, Aleksiev B, Bukolova-Orlova T, Burstein E, Atanasov B 102, *200*
Tchorbanov B, see Aleksiev B 102, *187*
Tessier M, see Delori PJ *190*
Theakston RDG, see Gopalakrishnakone P 101, *191*
Thesleff S, see Fohlman J 99, 102, *191*
Thompson EJ, see MacDermot J 150, *195*
Thompson FE, see Schmidt CF 118, *199*
Thompson RHS, see Magee WL 97, *195*
Thompson RHS, see Smith AD 135, *200*
Thouvenot JP, see Durand S 93, *190*
Thouvenot JP, see Fauvel J 93, *191*
Tinker DO, Law R, Lucassen M 133, *200*
Tinker DO, Purdon AD, Wei J, Mason E 132, 133, 134, 135, 136, *200*
Tinker DO, Wei J 132, 133, *200*
Tinker DO, see Purdon AD 166, *197*
Tinker DO, see Wu TW 100, 141, *202*
Tisdale H, see Salach JI 97, *198*
Tobian L, O'Donnell M 22, *43*
Tobias GS, Donlon MA, Catravas GN, Shain W 100, *200*
Toda H, see Kondo K 144, 145, 146, *195*
Todd LE, see Bricker NS 3, *36*
Todd L, see Rieselbach RE 7, *41*
Toorenbergen A van, see Scherphof GL 138, *199*
Torrente A de, see Berl T 22, *35*
Tóth G, see Mühlrád A 157, *196*
Traynor JR, see Kunze H 138, *195*
Trotter JT, see Shier WT 96, *199*
Truog R, see Howard BD 141, 148, 158, *193*
Tsai TH, Wu SH, Lo TB 98, 105, *200*
Tsao FHC, Cohen H, Snyder WR, Kézdy FJ, Law JH *200*
Tsao FHC, see Shen BW 139, *199*

Tu 95
Tu AT, see Hendon RA 159, *193*
Tuma S, see Massry SG 30, *40*
Turini P, see Slach JI 97, 101, 141, *198*

Ulitzur S, Heller M 96, *200*
Untracht SH, Shipley GG 128, *200*
Upreti GC, Jain MK 130, 132, 133, 134, *200*
Upreti GC, Rainier S, Jain MK 131, *200*
Uthe JF, Magee WL 97, *200*

Vandermaelen PJ, see Dijkstra BW 103, 149, 176, 178, *190*
Varela MJ, see Possani LD 101, *197*
Vargaftig BB, Fouque F, Chignard M 146, *201*
Veliz G, see Kaplan MA 30, *39*
Vensel LA, Kantrowitz EA 151, 152, *201*
Verger R 112, 123, *201*
Verger R, Haas GH de 112, 120, 123, 126, 138, *201*
Verger R, Mieras MCE, Haas GH de 117, 118, 124, 131, 132, 136, *201*
Verger R, Rietsch J, Dam-Mieras MCE van, Haas GH de 124, *201*
Verger R, see Barenholz Y 127, *188*
Verger R, see Boffa MC *188*
Verger R, see Lairon D 128, *195*
Verger R, see Pieroni G 123, 126, *197*
Verger R, see Rietsch J 123, *198*
Verger R, see Scow RO 128, *199*
Verger R, see Slotboom AJ 120, 131, *199*
Verger R, see Verheij HM 109, 128, 136, 144, 146, 174, 177, *201*
Verger R, see Zografi G 123, *203*
Verheij HM, Boffa MC, Rothen Ch, Bryckaert MC, Verger R, Haas GH de 109, 128, 136, 144, 146, 174, 177, *201*
Verheij HM, Egmond MR, Haas GH de 118, 154, 174, *201*
Verheij HM, Volwerk JJ, Jansen EHJM, Puijk WC, Dijkstra BW, Drenth J, Haas GH de 109, 136, 142, 143, 144, 163, 164, 168, 169, 177, 179, 182, 184, *201*
Verheij HM, see Boffa MC *188*
Verheij HM, see Evenberg A 97, 98, 105, 110, *191*
Verheij HM, see Fleer EAM 105, *191*
Verheij HM, see Jain MK 134, 137, 138, 174, *193*
Verheij HM, see Puijk WC 103, 105, 175, 176, *197*
Verheij HM, see Slotboom AJ 94, 96, 120, 131, 156, *199*
Verheij HM, see Volwerk JJ 114, 115, 117, 139, 143, 163, 168, 181, 184, *202*
Verheul FEAM, see Dutilh CE 98, 110, 164, *190*
Verhoef H, see Meijer H 154, 175, *196*
Vernon CA, see Shipolini RA 103, 141, *199*
Vessies JCA, see Drenth J 103, 176, *190*
Vidal JC 169, 170
Vidal JC, Cattaneo P, Stoppani AOM 141, *201*
Vidal JC, Guglielmucci E, Stoppani AOM 117, *201*
Vidal JC, Stoppani AOM 99, 100, 138, *201*
Vidal JC, see Pieterson WA 94, 95, 114, 116, 117, 143, 144, 154, 164, 168, 169, 170, *197*
Viljoen CC 105, *201*
Viljoen CC, Botes DP 113, 166, 184, *201*
Viljoen CC, Schabort JC, Botes DP 129, 132, 166, 175, *201*
Viljoen CC, Visser L, Botes DP 144, 145, 146, 147, 150, *199*
Viljoen CC, see Botes DP 100, 105, *188*
Visser L, see Viljoen CC 144, 145, 146, 147, 150, *201*
Vlijm H, see Slotboom AJ 161, 162, 163, 164, 173, *201*
Vogt W, see Kunze H 138, *195*
Volwerk JJ 93, 141, 142, 143, 144, *201*
Volwerk JJ, Dedieu AGR, Verheij HM, Dijkman R, Haas GH de 114, 115, 117, 139, 143, 163, 168, 181, 184, *202*
Volwerk JJ, Pieterson WA, Haas GH de 114, 117, 118, 142, 143, 146, 163, 168, 179, 184, *201*
Volwerk JJ, see Bonsen PPM 142, *188*
Volwerk JJ, see Pieterson WA 94, 95, 114, 115, 116, 117, 142, 143, 144, 154, 163, 164, 168, 169, 170, *197*
Volwerk JJ, see Verheij HM 109, 136, 142, 143, 144, 163, 164, 168, 169, 177, 179, 182, 184, *201*
Vratsamos SM, see Erlanger BF 141, *190*
Vries A de, see Shiloah J 99, 102, 110, *199*

Wagnild JP, Gutmann FD, Rieselbach RE 4, *43*
Waite M, Sisson P 138, *202*
Waldhäusl W, see Bratusch-Marrain P 81, *85*
Walker C, see Greco F del 20, *37*
Walsh KA, see Burstein Y 157, *189*
Walt SJ van der, see Joubert FJ 101, 110, *194*
Wardener HE de, see Bahlmann J 21, *35*
Wardener HE de, see Brown PR 22, 23, *36*
Wardener HE de, see Clarkson EM 22, 23, *37*
Wassermann N, see Erlanger BF 141, *190*
Weber H, Lin KY, Bricker NS 20, 21, *43*
Weber H, see Bricker NS 28, 34, *36*
Weber H, see Fine LG 15, 25, *38*
Weber M, see Welbourne T 33, *43*
Weerts C, see Slatopolsky E 15, 18, 19, *42*
Wei J, see Tinker DO 132, 133, 134, 135, 136, *200*
Weinberg J, see Danovitch GM 14, *37*
Weisinger JR, see Swenson MS 30, *43*
Weiss Y, see Papanicolaou N 22, *40*
Weisser F, see Lubowitz H 20, 33, *40*
Welbourne T, Weber M, Bank N 33, *43*

Wells MA 94, 99, 100, 112, 113, 114, 115, 116, 118, 119, 129, 137, 139, 141, 147, 148, 156, 157, 165, 175, 181, 184, *202*
Wells MA, Hanahan DJ 100, 110, 140, *202*
Wells MA, see Allgyer TT 118, *187*
Wells MA, see Hachimori Y 100, 110, *192*
Wells MA, see Misiorowski RL 118, *196*
Wells MA, see Poon PH 118, *197*
Wells MA, see Smith CM 113, 139, *200*
Wen SF, see Steele TH 3, *42*
Wernicke JF, Oberjat T, Howard BD 100, *202*
Wesson LG 6, *43*
Westenberg H, see Wilschut JC 130, *202*
Westgaard RH, see MacDermot J 150, *195*
Wezel FM van, Haas GH de 98, *202*
Wezel FM van, Slotboom AJ, Haas GH de 148, *202*
White DA, Pounder DJ, Hawthorne JN 93, *202*
White RM, see Purkenson ML 33, *41*
Wieloch T, see Anderson T 164, *187*
Wietzes P, see Puijk WC 105, 175, *197*
Wilkinson R, Luetcher JA, Dowdy AS, Gonzales C, Nokes GW 19, *43*
Williams RJP, see Aguiar A 163, 184, *187*
Williams S, see Marshall JS 63, 67, 72, 73, 75, *87*
Willman C, Stewart-Hendrickson H 125, *202*
Wilschut JC, Regts J, Westenberg H, Scherphof G 130, *202*
Wilson CB, see Allison ME 12, 21, *35*
Winchenne JJ, see Boffa GA 102, *188*
Wittich KA, Schmidt H 98, *202*
Wong NLM, see Carriere S 20, *37*
Wosilait WD 71, 72, *89*
Wosilait WD, Nagy D 71, *89*
Wright FS 25, *43*
Wright FS, see Howards SS 21, *39*
Wrong O, Davies HEF *43*
Wu SH, see Tsai TH 98, 105, *200*
Wu TW, Tinker DO 100, 141, *202*
Wurl M, see Kunze H 138, *195*

Yamaguchi T, Okawa Y, Sakaguchi K *202*
Yanagawa N, see Fine LG 26, *38*
Yang CC, King K 101, 102, 110, 144, 145, 146, 167, *202*
Yoshida H, Kudo T, Shinkai W, Tamiya N 101, 148, *203*

Zakin MM, see Boffa GA 102, *188*
Zhelkovskii AM, Apsalon UR, D'yakov VL, Ginodman LM, Miroshnikov AI, Antonov VK 150, *203*
Zhelkovskii AM, D'yakov VL, Antonov VK 150, *203*
Zhelkovskii AM, D'yakov VD, Ginodman LM, Antonov VK 113, 138, 139, 150, 184, *203*
Zia P, see Kaye Z 22, *39*
Zimina TM, see Mal'tsev VG 110, 139, *196*
Zinn AB, Marshall JS, Carlson DM 53, 62, *89*
Zinn A, see Marshall JS 75, *88*
Zipser R, see Kaye Z 22, *39*
Zoelen EJJ van, see Araujo PS 110, 139, 170, 171, 173, 174, *188*
Zografi G, Verger R, Haas GH de 123, *203*
Zunic D, see Gubensek F 100, *192*
Zwaal RFA, see Roelofsen B 96, *198*
Zweers A, see Jong WW de 111, *194*

Subject Index

acid base homeostasis in chronic renal disease 31–34
–, tritable 34
adaptations, solute-specific 2
adsorption equilibrium 124
affinity chromatography 51, 53, 54, 60, 68, 99, 100
albumin 128
aldosterone activity in chronic renal disease 22
n-alkylphosphocholines 152
allopurinol 14
ammonia, excretion of 33
– to inulin ratio 8
– secretion 8, 9
– synthesis 8
ammoniagenesis per nephron 34
ammonium sulfate precipitation 49, 52
1-anilino naphthalene-8-sulfonic acid 146
antibodies to carboxy terminals 30
arginine 151, 152
arginine-phenylglyoxal complex 153
association constant 68, 69, 70

Ba^{2+} 142, 163, 167, 178, 181
bee venom phospholipase, see phospholipase
bicarbonate reabsorption 3, 32, 33
bilayers, osmotically shocked 131
–, phospholipids in 129 ff.
bile salt micelles 128
binding studies with PLA 163 ff.
bioselective adsorption chromatography, see affinity chromatography
blood volume, central 19
body temperature and T4 concentration 83
bromoketones 143
β-bungarotoxin 97, 145, 146, 148, 150
–, B-chain of 102, 103, 106, 109
–, Ca^{2+} binding 165
– and ethoxyformic acid 158

cabbage lecithin 130
calcium ions 110, 121, 125, 129, 144, 145, 155, 174, 178, 181, 183
–, binding of, to PLA and pro-PLA 163 ff.
– as cofactor of PLA 142
– dissociation constants 163
–, relation to phosphate 28, 29
catalytic model for PLA 181 ff.

capillary pressure, peritubular 21
carbohydrates in TBG 62
chaperon concept 159
chronic renal disease, acid-base regulation in 31–34
– – –, adaptation of ammoniagenesis 34
– – –, adaptation of phosphate excretion in 28–30, 34
– – –, clearance ratios in stage II 6–9
– – –, functional integrity of the surviving nephron 3, 4
– – –, glucose titration studies in 11
– – –, homogeneity of G-T balance in 6, 7
– – –, ion balance in 3
– – –, level of functional organization in 6–12
– – –, metabolic acidosis in 32, 33, 34
– – –, potassium balance in 26, 27
– – –, potassium transport in 25 ff.
– – –, regulation of excretion in 13–15
– – –, regulation of specific solutes in 18–31
– – –, single nephron glomerular filtration rate in 20
Cibracron blue 138, 146
cirrhosis and TBG 81
clearance ratios, equality of 7–9
– –, mathematical analysis 8
– – in renal diseases 7, 8
cleavage, selective proteolytic 162
Cohn fractions 46, 47
column chromatography 50
comicellization mechanism 171
complement fixation assay 175
concanavalin-Sepharose 4B 100
corticosteroid-binding protein, isolation of 48
cracks 127, 128, 130, 134, 135
CRD, see chronic renal disease
creatinine, regulation of excretion 13
critical micelle concentration 113, 114, 115
crotapotin 158
crotoxin 144, 145, 156, 158, 159
– complex 100
–, iodinated 156
1,2-cyclohexanedione 150, 151
cytochromes C 181

desialylation of TBG 63, 68
detector element, location of 17, 19
dibutyryl lecithin 122
dihexanoyl lecithin 112, 113, 114
1,25 dihydroxy D_3 30
dioctanoyllecithin, as substrate 97
dissociation constants 163
disulfide bridges, mismatched 107
– connections 103, 106

egg lysolecithin 113
– yolk assay 96, 97, 148, 149
electrophoresis 51, 53
enzyme adsorption 136
–, amount of adsorbed 124
– desorption 136
– penetration 136
–, specific activity of 124
– theory 112
equilibrium gel filtration 122
– surface concentration 125
estradiol and TBG production 73
ethoxyformic acid anhydride 157
extracellular fluid, isosmotic expansion 17
– – volume, expansion of 11, 33
– – –, intrathoracic 19

fasting and TBG 81
fatty acids 58, 142, 143, 156
– –, determination of liberation 96
– – and phospholipase 94
– –, release of 133, 129
fever and T4 concentration 83
fluid reabsorption, fractional 12
fluorescence polarization 61, 62
– quenching 66, 67
– spectroscopy 170, 171, 173

gel chromatography 46, 47
– electrophoresis 51, 53, 54
– filtration 52, 53, 60
glomerular filtration rate in renal disease 6, 20
– – –, relation to 1,25-dihydroxy D_3 31
– plasma flow in CRD, redistribution 20 ff.
glomerulonephritis, immunologic 4
–, SNGFR in 20
glomerulo-tubular balance, homogeneity of 6, 7
– –, inhomogeneity of 10, 11
– – micropuncture studies 12
– – resetting of level 13
glucose reabsorption 12
– titration technique 9–11
glycolipids 134
glycoprotein synthesis 63, 64
–, TBG as 53, 54
glycosuria 11
– threshold 10
glyoxylic acid 153
goiter and TBG 83
guanidinium chloride 61, 62

H^+, buffering of 32
– excretion 33 f.
–, properties of 31
– transport 8
half-site reactivity 139, 145, 150, 158, 160
hematocrit 21
hepatocarcinoma cells 74
hepatocytes, isolation of TBG from 72
–, synthesis of TBG in 74
histidine 141–146
hopping model 133, 136, 137
hydrophobic chromatography 100
– interaction and PLA 108, 109, 113, 114, 115
hyperaldosteronism 27
hyperparathyroidism, secondary 30, 31
hyperthyroidism 79
– and TBG 81, 82, 83
hypoalbuminemia 21
hypothyroidism and TBG 79, 81, 82, 83

immunodiffusion, Ouchterlony's 175
immunology of PLA 175
interface recognition site 117, 143, 144, 161
ion exchange chromatography 47, 52, 53

kaliuresis, adaptive 26, 27
kaliuretic hormone 28
kidney, see also renal 6

D-lecithin 142
lecithin concentration, two-dimensional 120
lecithins 119, 120, 167
–, micellar short-chain 115
–, saturated long-chain 129
lipase, enzyme hydrolysis 124
–, pancreatic, kinetics of 123
–, penetration capacity of 125
lipolysis, kinetic model of 132, 133
–, mechanisms of 112–119
–, models of 117, 120, 124, 125, 136
–, rate increase in 126
–, study of 126
–, surface inhibitors and kinetics of 126
lipolytic enzymes, investigation of kinetics 123 ff.
– –, monolayer technique 125
– – and pH 125
local anesthetics 138
low molecular weight binder for T3 76
lysine 150, 157, 161
lysolecithin 113, 114, 121, 142, 146, 150, 168
– micelles 154
lysophosphatidylcholine 98
lysophospholipids appearance, determination of 96

magnesium excretion 15
magnification phenomenon 15, 16, 20, 21, 26, 29

methionine 148, 149
micelles of phospholipids with detergents 119–122
microcalorimetry 170
microheterogeneity of TBG 63
monolayer technique 128

Na/K-ATPase activity 23, 26
natriuresis 19
natriuretic forces, deactivation of 17
– hormone 22, 23
– –, isolation of 23
– –, mechanism of action 25
N-bromosuccinimide 147, 148
nephrotic uremia 21
neurotoxicity 144, 156, 159
neurotoxins, presynaptic 144, 145, 156
newborns, TBG in 82
NH_3 trapping 8
p-nitrobenzenesulfonate 144
notexin, Ca^{2+} binding of 165
–, X-ray analysis of 176

oral contraceptives and TBG 81, 82, 83
ovalbumin 69

pancreatin, commercial 98
parathormone 33
parathyroid hormones 28, 29, 30
–, antibodies 30
–, and vitamin D_3 metabolites 31
phase transition temperature 129, 132, 134
phenylglyoxal 150, 151
phosphate excretion, normal 28
– – in chronic renal disease 14, 29, 30, 34
–, postprandial increments 28
–, relation to Ca^{++} 28, 29
phosphatidyl-choline 98, 126, 127, 129, 132, 133
1-sn-phosphatidylcholines 167
phosphaturia, adaptive 34
phosphoglycerol 134, 135
phospholipase A_2, activators of 138
–, active site of 170, 184
– activity and organic phosphorus compounds 141
–, acylated, kinetics of 156
–, adsorption of 137
–, affinity constant 120
–, ε-amidinated 161, 162
–, amino acid sequences of 102 ff.
–, α-amino group of 95, 110, 152–154, 160, 161
–, anticoagulant activity 95, 146
–, arginine residue of 151
–, aspartate residue 150, 151
–, assays for 96–98
–, basic properties of 93–96, 109
–, from bee venom 103
–, binding of, to aggregated lipids 169 ff.
–, – to bilayer structures 174
–, – of Ca^{2+} 163–167
– and Ca^{2+} 97, 108, 111
–, Ca^{2+} interaction 113, 115, 116
–, carboxylate groups in 150
– catalysis, mechanism of 181 ff.
–, charge properties of 99, 109
–, chemical modification studies 140 ff.
–, complexes with 99
–, conformational change in 119, 122, 136 154
– and cracked interfaces 134
–, cross-linking of 159
–, desorption of 136, 137
–, digestive function of 95
–, dimeric structure 139, 160, 171
–, dissociation constants 168, 171, 174
– in DPPC unilamellar vesicles 135
–, dual-phospholipid model of 121
–, elapid 106, 108, 109
–, enzyme-CA^{2+} dissociation constant 113, 114
– equilibrium constant 111
–, evolution of 108
–, half-site reactivity 139, 145, 150, 158, 160
–, hemolytic action 95
–, histidine residue of 141–146, 157
–, hydrolysis by 181
–, – kinetics 125, 127, 131
–, – of mixed micelles 119–122
–, hydrophobic side chains 108, 109, 113, 114, 117
–, immunology of 175, 176
–, inhibition of 120, 125
–, – kinetics 137, 138
– inhibitors 138, 168
–, interaction with medium-chain phospholipids 123 ff.
– interface recognition site 117
–, iodinated 156
– isoenzymes 101
–, isolation of 98–102
–, kinetic analysis 111 ff.
–, kinetics of alcohol-modified PLA 131
– -lipid complex 169, 171, 173
–, lipid-water interaction 109
–, – interfaces 154
– and long-chain substrate 132
–, lysine residue of 150, 157, 161
–, methionine residues of 148, 149
–, methylated 144
– and micellar substrate binding 170 ff.
–, – –, interactions with 115–122
–, microheterogeneity of 100
–, molecular weight 111
–, monomer binding 167–169
–, monomer or dimer 139, 160
–, monomeric substrate interaction 112–115 154
–, multiple forms of 98
–, myonecrotic effects 95

phospholipase A_2, neurotoxic action of 95
–, neurotoxicity of 144, 145
–, N-terminal region 161
–, occurrence of 93
–, pancreatic 93, 94, 95
–, –, molecular structure of 177 ff., 183
–, –, proteolysis of 98
–, –, semisynthesis of 160–162
–, penetration of 118, 128, 130, 131
–, – capacity 156
–, pH dependence 113
– and phosphocholine bilayers, intact 129 ff., 134
–, photoaffinity labeling 160
– precursor 94, 95
–, production of 95
–, proton relay system 179
–, purification methods 99, 100
–, rate enhancement of 116, 118
–, reaction with ethoxyformic acid anhydride 157, 158
–, serine residues 141
–, space-filling model of 183
–, specificity reversal 121
–, structural differences between various types of 108
–, – and molecular properties of 102–111
–, substrate interaction, kinetics of 111 ff.
–, substrates for 94, 95, 97
– and sulfhydryl groups 140
– and temperature 133, 134
–, titrimetric assay for 114
– and β-toxins 100
– and transamination 150, 151
–, tryptic inactivation of 163
–, tryptophan residues 147, 148, 168, 175
–, tyrosine residues 154–156, 157, 158, 169, 179
–, ultraviolet difference spectra 163, 164, 165, 166, 170, 171
– variants, electrophoretic 96
– from venoms 98, 100, 101, 103
– X-ray analysis 176–180
–, – structure 181
phospholipid binding 155
phospholipids 134
– in bilayer structures 129 ff.
– in biomembranes, studies of 96
–, dissociation constants of 114
–, micellar 146
–, negatively charged 122
–, short-chained 97
phosphoryl choline 122
PLA, see phospholipase A_2
polyacrylamide gel electrophoresis 51, 52, 53, 54, 60
potassium, excretion of, in chronic renal disease 14, 16, 26
– ingestion 25
– reabsorption 25
pregnancy 63
– and TBG 81, 82
pressure dialysis 50
product theory 112
proflavine 138
prophospholipase 95
(pro)phospholipases, purification of 98
prostaglandins, modulating sodium transport 22
–, release of 146
pyelonephritis, unilateral 4
pyridoxal-5′-phosphate 150

radioimmunoassay of TGB 78, 79
rate enhancement for PLA 116, 118, 126
receptor proteins for thyroid hormones 84
remnant kidney 4
renal disease, chronic 2
– lesions, chronic, techniques for 4

Scatchard equation 71
scooting 133, 136
semisynthetic processes for PLA 162
sequence difference matrix 107, 108
serine 141
– esterase 181
single encounter mechanism 116
– nephron glomerular filtration rate 6, 9, 10, 11, 12
slow TGB 55, 59, 68
snake venom neurotoxins 144
sodium excretion, factors in its regulation 205 20 ff.
– –, fractional 15, 18, 19, 20
– –, regulation of 17, 23
– –, – in CRD 14, 15, 18–25
– reabsorption 12
– –, influence of physical factors on 21
– transport inhibitor 22, 23
sonication 130, 132
spectral changes, pH dependency of 166
sphingomyelin 121, 122, 127, 128
splay zone of glucose titration curve 10, 11
Sr^{2+} 163, 167, 181
stage I–III studies, definition of 5
standard free energy, changes of 70
steroid hormones, solubility of 81
substrate analogs, monomeric binding of 167–169
–, monomeric versus micellar 118
–, surface dilution of 120
– theory 112
sulfhydryl reagents 141
surface defects 127
– pressure, changes in 124, 127
– –, constant 123

T3 clearance 76
T3/TBG ratio 81
T3-uptake values 82
T4, see thyroxine
taipoxin 144, 145
–, Ca^{2+} binding 165

taipoxin, γ-chain of 102, 103, 109, 110
TBG, see thyroxine binding globulin
thermotropic phase transition 130
thioester substrates 97
thyroid disorders, TBG in 82
– function, tests for 82
– hormones during aging 79, 80
– –, association constant to 84
– –, binding to receptor proteins 84
– –, solubility of 81
thyroidectomy 75
thyrotoxicosis 59, 79
thyroxine 54
– analogues, association constants 70
–, association constant 69
– binding globulin, abnormalities of 59
– – –, absence of, inherited 81
– – –, amino acid composition 57, 60, 61, 64, 65
– – – antiserum 80
– – – assay 82
– – – binding of thyroid hormones 46, 65, 66, 69
– – –, biodegradation of 76, 77
– – –, biosynthesis of 72 ff.
– – –, biosynthesis in liver 73
– – –, carbohydrate composition 57, 61, 62, 64
– – –, concentration of 59, 79, 80, 81
– – –, conformational transition 62, 68
– – –, contemporary research on 46
– – –, crystallization of 58
– – – denaturation 58, 59, 61, 69
– – – desialylation of 63
– – –, determination of 78–80
– – – distribution space 59
– – –, electrophoretic mobility of 58
– – – excretion, renal 76, 77
– – –, genetic disorders 83
– – –, half-life 59, 60, 75, 76
– – –, immunoassay 78, 79
– – –, interaction with iodothyronine 70
– – –, isolation of, present methods 53
– – –, isolation techniques 47 ff.
– – –, metabolism of, in monkeys 75, 76
– – –, microheterogeneity of 63, 64
– – –, mobilities of 55
– – –, molecular structure 61, 62
– – –, molecular transitions 61, 62
– – –, molecular weight 55, 60, 64
– – –, physical and chemical properties 55–58
– – –, physiological role of 81–83
– – –, pipsylilated 71
– – – in pregnancy serum 52
– – –, preparations of 60
– – –, properties of 55
– – – purification, principle of 46
– – – in receptor tissues 84
– – – relaxation time 61
– – –, subunit structure of 64, 65
– – – synthesis, gene control of 59, 75, 76
– – –, T3 binding by 67, 68, 69
– – –, T4-binding ability of 47, 60, 61, 67, 80
– – –, T4-interaction, theoretical analysis 71
– – –, thermal denaturation of 58
– – –, ultracentrifugal analysis of 55
– – pre-albumin 46, 48, 54, 55
– calculation, mathematical model for 83
– clearance 76
– /TBG ratio 81, 82, 83
– and synthesis of TBG 74
toxin, postsynaptic and presynaptic 100
trapping experiments 130
Triton X-100 97, 119, 146, 175
– micelles 121
tryptophan 147, 148, 168
TSH 82
tubular reabsorption, changes of, in CRD 14
– secretion, changes of, in CRD 14
– transport, regulation of, in CRD 16
tyrosine 154, 163, 179

ultraviolet difference spectrum and Ca^{2+} 166
urate levels in chronic renal disease 14
urea, excretion in chronic renal disease 13
uremia 21, 23, 33
– and kindney function 3, 5
urine concentration in chronic renal disease 3
–, pH of, in chronic renal disease 3

vitamin D_3 30, 31

water excretion, fractional 13

X-ray crystallographic analysis 176

zinc modulation of excretion 15
zymogen 116, 117, 128, 143, 153, 161, 163, 168
– -like proteins 140