

Chlorophyllase in Citrus Leaves. Kinetic Aspects of the Reaction*

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Abstract. The reaction: Chlorophyll + Enzyme $\longrightarrow$ Chlorophyllide + Phytol follows a first order kinetics with regard to the quantity of enzyme, when it is saturated by substrate. K_m and V_m were determined from the average reaction rates for the substrates: Chlorophyll *a* and *b*, pheophytin *a* and *b*, methylechlorophyllide *a* and methylpheophorbide *a*: The lowest K_m corresponded to chlorophyll *a* and the highest V_m to methylpheophorbide *a*. For a substrate of chlorophyll (*a* + *b*), K_m and V_m were determined also using the initial reaction rates. "Enzyme efficiency" was calculated using both methods of determination. The reaction products partially inhibit the hydrolytic process.

Kinetics of enzymatic reactions shows special characteristics (McFEETERS *et al.* 1971, McFEETERS 1975). For that reason, it is necessary to establish the minimum concentrations of substrate for enzyme saturation (NEILANDS and STUMPF 1967); in this way, we get a first order reaction with regard to the enzyme concentration.

The factors directly affecting the enzymatic reaction rate are: enzyme quantity, substrate concentration, and products (SUD'INA *et al.* 1972a, b). The measurement of enzyme activity requires a linear relationship to exist between the reaction rate and the enzyme quantity under controlled conditions of the medium. The dependence of the initial reaction rate on substrate concentration is hyperbolic (NEILANDS and STUMPF 1967). At low substrate concentrations, the rate is directly proportional to substrate concentration, while at high concentrations, the rate is constant (enzyme saturation). The hyperbolic equation is $V = V_m \cdot S / (K_m + S)$, where V = rate for any substrate concentration, V_m = maximum rate of reaction on the substrate, S = substrate concentration, K_m = Michaelis-Menten constant. Substances produced in this type of reactions (SHIMIZU and TAMAKI 1963) can either shift the equilibrium to the opposite side, or block the enzyme active center, when certain concentrations are reached.

The effect of these factors on the hydrolysis of chlorophylls by chlorophyllase (chlorophyll-chlorophyllidohydrolase, E.C. 3.1.1.14) action are studied in this paper.

Received June 19, 1979; accepted October 2, 1979

* Part II.

MATERIAL AND METHODS

Preparation of Chlorophyllase Extract from Acetone Powders

1 g of acetone powders and 25 ml of the buffer 0.041 M tris-0.32 M glycine-0.67 M glucose-0.5% dodecyl sulfate sodium salt (w/v), pH 8.5 (NAVARRO *et al.* 1979) were mixed and the suspension was stirred at room temperature for 8 h and maintained in maceration till 24 h. Afterwards it was filtered through a 0.101 mm mesh in order to eliminate the big particles and then it was centrifuged at $5000 \times g$ for 40 min at 4 °C. The enzymatic extract, with a protein concentration of 21 mg ml⁻¹ (ELLSWORTH *et al.* 1976), is obtained from the supernatant once filtered.

Substrates Preparation

The Citrus leaves were powdered with 4 ml g⁻¹ acetone cooled previously and the homogenates were filtered through a porous plate. The extract is kept at -20 °C during several days and before its use, it is filtered again in order to eliminate the greatest part of carotenoids that will precipitate in these conditions. In the solution, the chlorophylls (*a* + *b*) concentrations are determined according to NAVARRO *et al.* (1979).

a) Chlorophylls *a* and *b*. A part of the acetone extract containing chlorophyll (*a* + *b*) is transferred to diethyl ether and concentrated in vacuo to a determined volume. The concentrate is put on a 0.25 mm silica gel thin layer suitably activated. The separation is made by ascendent chromatography in a solvent mixture petroleum ether/benzene/ethanol (200 : 75 : 18, v/v/v). Chlorophylls *a* and *b* are extracted by centrifugation (VERDU 1972) and are determined according to NAVARRO *et al.* (1979).

b) Pheophytins *a* and *b*. Another part of the chlorophyll (*a* + *b*) extract is dried, redissolved in acetone adding oxalic acid, stirred and left for 1 h in the dark at room temperature. The pigments are then transferred to diethyl ether and washed with distilled water until all the oxalic acid is eliminated (WHITE *et al.* 1963).

c) Methylpheophorbide *a*. Another part of the chlorophylls extract is dried, redissolved in methanol, adding 22% HCl it is then heated under reflux for 1 h. The cooled solution is transferred to diethyl ether in a separatory funnel, washed first with 10% HCl and afterwards with distilled water, until the washing water has a neutral pH (PENNINGTON *et al.* 1964).

d) Methylchlorophyllide *a*. The fresh leaves material is powdered with methanol; the homogenate is maintained for 24 h under stirring in the dark. The suspension is filtered and transferred to diethyl ether.

The extracts of chlorophyll derivatives are concentrated to a determined volume and the pigments are separated by thin layer chromatography and determined similarly as the chlorophylls.

Calculation of the Rate Constant

The general expression of the first order equation for the reaction rate is

$$v = -\frac{d[S]}{dt} = +K[S] \longrightarrow -\frac{d[S]}{[S]} = K dt$$

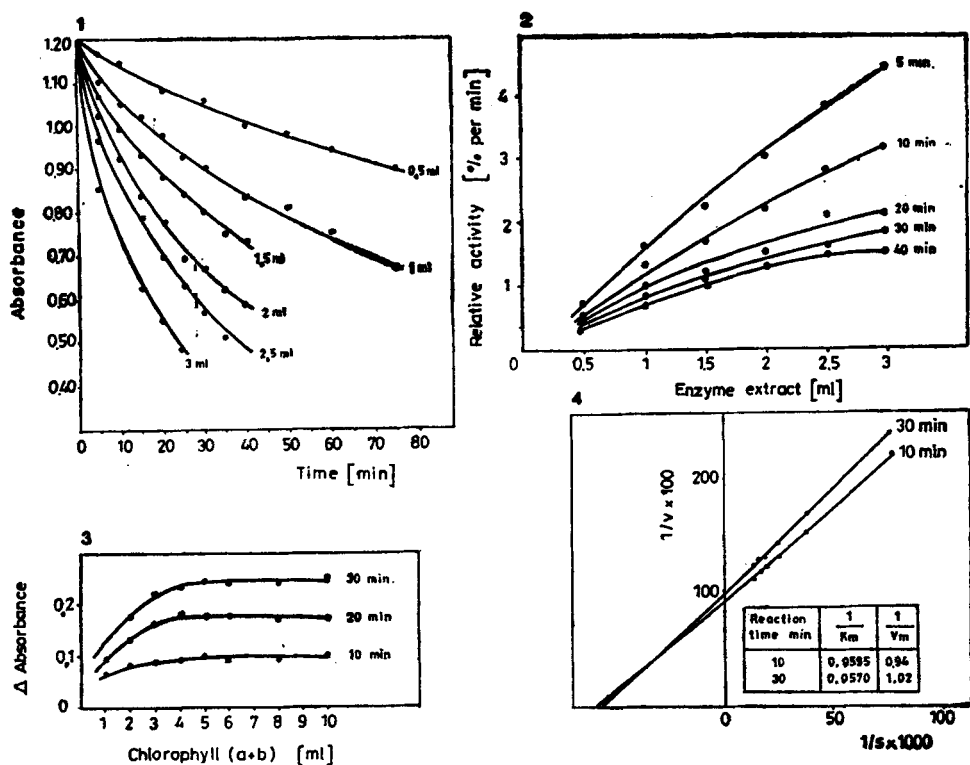


Fig. 1. Effects of different chlorophyllase concentration (figures at curves = amount of enzyme extract) on rate of chlorophyll (*a* + *b*) hydrolysis (expressed in absorbance values).

Fig. 2. Variation of the relative activity with the quantity of chlorophyllase extract, at different reaction times. Chlorophyll (*a* + *b*) as substrate.

Fig. 3. Variation in reaction rate with substrate concentration at different reaction times.

Fig. 4. Plot of the Lineweaver-Burk equation for calculation of V_m and K_m for chlorophylls (*a* + *b*) with average rates for 10 and 30 min reaction time.

and integrating:

$$\begin{aligned}
 -\ln [S] &= Kt + C \longrightarrow \ln \frac{[S_0]}{[S]} = Kt \\
 \lg \frac{[S_0]}{[S]} &= \frac{Kt}{2.30}, \text{ and } [S] = \frac{[S_0]}{2} \longrightarrow t_{1/2} \\
 \lg 2 &= \frac{Kt_{1/2}}{2.30} \longrightarrow K = \frac{0.693}{t_{1/2}} \quad (1)
 \end{aligned}$$

When the hydrolysis rate of the substrate is plotted *vs.* time, graphs like Fig. 1 are obtained. When the absorbance of the hexane phase (in a logarithmic scale) is plotted *vs.* time (in a linear scale), straight lines are obtained (see Fig. 6). From both graphs it is possible to deduce the semi-reaction times ($t_{1/2}$) and when they are substituted in (1) K and from K the initial reaction rates $V_0 = K[S_0]$ are obtained.

TABLE 1

Variation of chlorophyll ($a + b$) concentration (expressed as absorbance) as function of the reaction time and the initial chlorophyll ($a + b$) content

Chlorophyll solution [ml]	Reaction time [min]			
	0	10	20	30
1	0.153	0.092	0.060	0.035
2	0.306	0.225	0.175	0.130
3	0.459	0.360	0.300	0.240
4	0.595	0.500	0.415	0.360
5	0.800	0.700	0.625	0.560
6	0.922	0.835	0.750	0.680
8	1.224	1.130	1.050	0.985
10	1.530	1.420	1.340	1.260

The enzymatic activity is expressed as described by NAVARRO *et al.* (1979), although the "relative activity" concept is introduced, being defined as the per cent difference between the extract absorbance at time zero and the absorbance at a given time.

RESULTS AND DISCUSSION

Effect of Enzyme Quantity

This effect has been studied using reaction mixtures containing 0.5 to 3.0 ml of chlorophyllase extract and the same 97 μ M concentration of chlorophyll ($a + b$) (Fig. 1).

For amounts of the chlorophyllase extract from 0.5 to 1.5 ml, the rate of hydrolysis is proportional to time, but the reaction passes so slowly that a 50% degradation is not obtained even after 80 min. For quantities between 1.5 and 3.0 ml, larger than 50% hydrolysis is obtained in half the time, and its rate is proportional to the time of reaction. In all cases, the kinetic is a first order one.

This conclusion becomes clearer when we relate the different quantities of the enzymatic extract to the relative activities* for the different reaction times (Fig. 2). The "relative activity" decreases quickly with increasing time, the relationship for 5 and 10 min being almost linear for the same substrate. For other times, such activity decreases only slightly with the quantity of the enzymatic extract.

TABLE 2

Variation in maximum rate of chlorophyllase activity [ΔA per 10 min per ml of enzymatic extract] with reaction time

Reaction time [min]	Maximum rate $\times 100$
10	10.0
20	8.7
30	8.3

* "Relative activity" is the difference between the absorbance of the extract at time zero and the absorbance at a given time [% per min].

TABLE 3

1/S and 1/v values obtained from the concentration of chlorophyll (*a* + *b*) as substrate (S) and reaction rates after 10 and 30 min

30 min	S [μM]	78	65	52	39	26	13
	V [$\mu\text{M t}^{-1}$]	23.9	23.0	22.3	20.5	17.5	12.5
	v [$\mu\text{M min}^{-1}$]	0.797	0.767	0.743	0.683	0.583	0.417
	1/S	0.013	0.015	0.019	0.026	0.038	0.077
	1/v	1.255	1.304	1.345	1.463	1.714	2.400
10 min	S [μM]	78	65	52	39	26	13
	V [$\mu\text{M t}^{-1}$]	8.8	8.3	8.0	7.5	6.5	4.5
	v [$\mu\text{M min}^{-1}$]	0.880	0.830	0.800	0.750	0.650	0.450
	1/S	0.013	0.015	0.019	0.026	0.038	0.077
	1/v	1.136	1.205	1.250	1.333	1.538	2.222

Effects of Substrate Concentration; K_m and V_m Determination

The goal of these experiments was to find the concentration of the substrate saturating the enzyme when the reaction follows a first order kinetics. The reaction media contained 1, 2, 3, 4, 5, 8 and 10 ml of a 649 μM chlorophyll (*a* + *b*) solution and always 1 ml of the chlorophyllase extract used in the previous experiments (see Table 1).

When the absolute absorbance increments are plotted *vs.* the amount of substrate (Fig. 3), we can observe that from 4 to 5 ml of chlorophylls in the reaction media (corresponding to *ca.* 50 to 60 μM), the hydrolysis rate is constant and, therefore, the enzyme is saturated by the substrate.

Fig. 3 seems to show that the maximum reaction rate is reached after 30 min nevertheless, when that rate is expressed as ΔA per 10 min per ml enzyme (Table 2), it is proved that the maximum rate decreases as the reaction time increases.

In order to determine the $V_{\max}$ and K_m characteristics of chlorophyllase we used the Lineweaver-Burk linear representation. In it, the absolute value of the ordinate in the origin is the inverse of V_m , and the absolute value of the abscissa in the origin is the inverse of K_m .

$$\frac{1}{v} = \frac{K_m}{V_m} \frac{1}{S} + \frac{1}{V_m}$$

The experimental determination of those constants has been made in two ways: (a) Calculation of the average reaction rate after a certain time, and (b) calculation of the initial rate (V_0) at the semi-reaction time, $t_{1/2}$.

In the first method, we used six reaction media with an increasing concentration of the substrate and 1 ml of the enzymatic extract. The reaction rates were determined after 10 and 30 min (see Table 3).

The plot of $1/S \times 1000$ values *vs.* $1/v \times 100$ gives directly the V_m and K_m values (Fig. 4): for 10 min V_m is 1.06 $\mu\text{M min}^{-1}$, and K_m is 17.54 μM . These values depend upon the pre-established reaction times (SUD'INA *et al.* 1973).

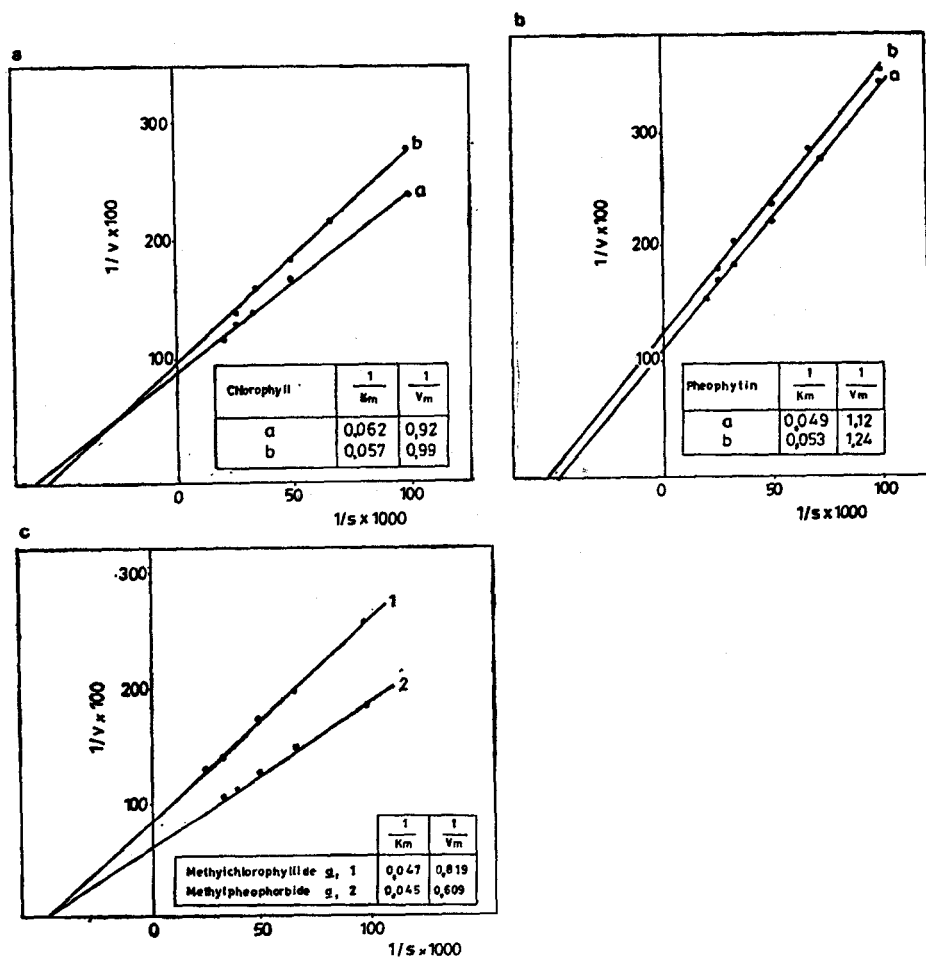


Fig. 5. Plots of the Lineweaver-Burk equation for the: a) chlorophylls *a* and *b*, b) pheophytins *a* and *b*, c) methylchlorophyllide *a* and methylpheophorbide *a*.

Similarly and by the same method, such constants have been determined for the following substrates: chlorophylls *a* and *b*, pheophytins *a* and *b*, methylchlorophyllide *a* and methylpheophorbide *a*, using the 10 min reaction time (Fig. 5a, b, c).

With these data the "enzyme efficiency" is calculated, defined as the $V_m/2 K_m$ relationship by LOZANO (1965), since the Michaelis constant (K_m) is equal to the substrate concentration which is responsible for the reaction rate being half the maximum.

The Michaelis constant values are between 16 and 22 μM (ELLSWORTH 1971, OGURA 1972), and the maximum rates are between 0.81 and 1.64 $\mu M \text{ min}^{-1}$ (Table 4). However, the values obtained for the "efficiency" are more significant: they are maximum for chlorophyll *a* and even more for methylpheophorbide *a*.

TABLE 4

 K_m , V_m and "enzyme efficiency" values for different substrates

Substrates	K_m [μM]	V_m [$\mu M \text{ min}^{-1}$]	$V_m/2 K_m \times 100$
Chlorophyll <i>a</i>	16.13	1.09	3.38
Chlorophyll <i>b</i>	17.54	1.01	2.88
Pheophytin <i>a</i>	20.41	0.89	2.18
Pheophytin <i>b</i>	18.87	0.81	2.15
Methylchlorophyllide <i>a</i>	21.05	1.22	2.90
Methylpheophorbide <i>a</i>	21.74	1.64	3.77

Although from a theoretical point of view, those substrates are considered normal for enzymatic complexes whose K_m is low (chlorophylls in our case), when the term "enzyme efficiency" is introduced, the highest value is that for the methylpheophorbide *a* substrate exerting the maximum K_m .

The second method of calculating V_m and K_m is more accurate and consists in the determination of the initial rates V_0 , taking into account that the reaction in the initial moments follows a first order kinetics. In such conditions, the rate is directly proportional to the substrate concentration and this proportionality constant is calculated using the semi-reaction times ($t_{1/2}$).

To calculate $t_{1/2}$, nine reaction media were prepared with decreasing concentrations of chlorophylls (*a* + *b*) (80 to 5 μM) and 2 ml of enzyme extract. The activity was determined as long as more than 50% of the initial amount of chlorophylls was hydrolysed.

Plotting in a semilogarithmic scale the substrate concentrations as a function of the reaction time (Fig. 6) give straight lines, from which the

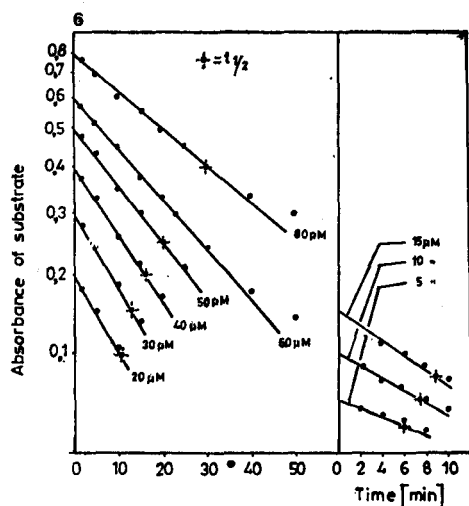


Fig. 6. $t_{1/2}$ values for different concentrations of substrate, chlorophylls (*a* + *b*) (figures at the lines).

semi-reaction times are obtained. The initial rate for each of the substrates, V_0 is calculated by the expressions:

$$K = \frac{0.693}{t_{1/2}} \quad V_0 = K [S_0]$$

The plot of $1/S_0$ and $1/V_0$ is a straight line by which V_m and K_m are calculated (Fig. 7).

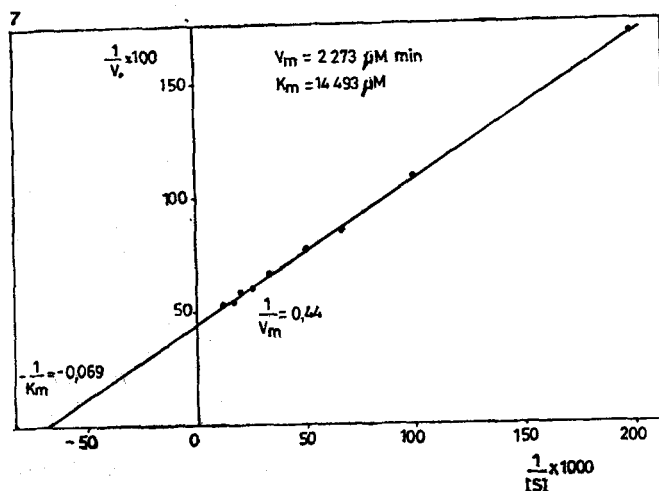


Fig. 7. Lineweaver-Burk equation for the V_m and K_m calculation from initial rates of chlorophyll (a + b) hydrolysis.

K_m and V_m values are $14.93 \mu\text{M}$ and $2.27 \mu\text{M min}^{-1}$, respectively, which shows that this last method is more adequate for calculating the characteristics of the enzyme as it provides lower K_m and higher V_m values than the first method.

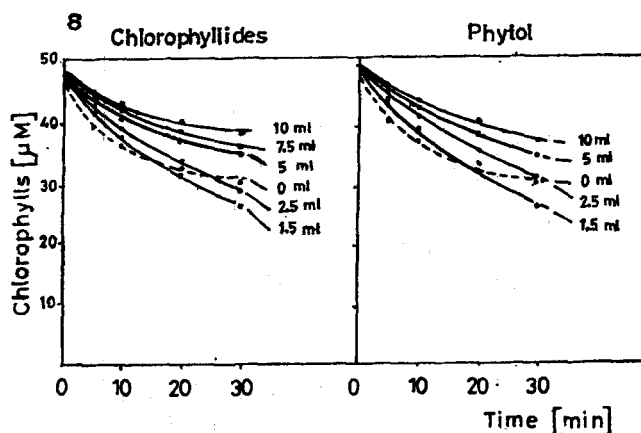


Fig. 8. Inhibition of chlorophyllase activity due to chlorophyllides or phytol (figures at curves are amounts of their 1 mM solutions).

Effects of Reaction Products

The enzymatic reaction may be partially inhibited by the reaction products, as its activity is higher when the media do not initially contain chlorophyllides. To study this effect, we prepared two series of identical samples with a 50 μM initial concentration in chlorophyll ($a + b$); to one of

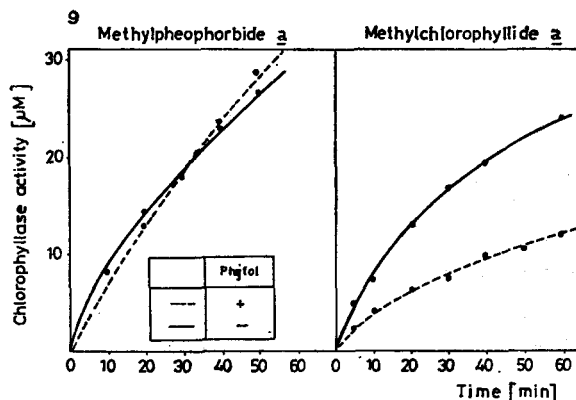


Fig. 9. Inhibition of chlorophyllase activity by phytol using methylpheophorbide *a* or methylchlorophyllide *a* as a substrate.

them we added increasing quantities of 1 mM chlorophyllide solution in acetone, and to the other one, phytol in acetone of the same concentration was added. For all tests, 2 ml of enzyme extract were used.

Low concentrations of chlorophyllides or phytol (1.5 and 2.5 ml of solutions corresponding to 30 and 50 μM , respectively) inhibit only in the first minutes of the reaction. With higher quantities of products, the low enzyme activity is maintained during the reaction time considered.

On the other hand, to study the inhibiting effect of phytol, we used as substrates 32 μM methylpheophorbide *a* and 40 μM methylchlorophyllide *a* (Fig. 9).

In the case of methylpheophorbide *a*, there is practically no inhibitory effect. For methylchlorophyllide *a*, the inhibition produced by phytol is very important. It may be ascribed, rather than to an inhibitory action, to a substitution of the methyl group by phytol (ELLSWORTH 1971, 1972, ICHINOSE and SASA 1973).

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BOOK REVIEW

SENGBUSCH, P. v.: MOLEKULAR- UND ZELLBIOLOGIE. — Springer Verlag, Berlin–Heidelberg–New York 1979. 667 S., 616 Abb., DM 88,—.

Die biochemischen Methoden wie auch die elektronen- und lichtmikroskopischen Analysen führten in den letzten Jahren zu einem tieferen Verständnis der Organisation und Funktion der Zellen. Das Buch von Prof. Sengbusch (Univ. Bielefeld) bietet eine wertvolle Zusammenstellung der für alle lebenden Systeme wichtigsten Zellteile und Komponenten-Makromoleküle um supramolekularen Strukturen. Die einleitenden Kapitel behandeln die wichtigsten Makromolekülklassen: 1. Nukleinsäuren (Struktur, Isolierung, Fraktionierung, Sequenzierung, Replikation, Transkription, Rekombination) und 2. Proteine (Funktion, Isolierung, Röntgenstrukturanalyse, Enzyme, Faserproteine, Antikörper). Einige Kapitel befassen sich mit den Strukturen und der Zusammensetzung von Membranen, die heute im Mittelpunkt des Interesses biochemischer Studien stehen. Es handelt sich nicht nur um Plasmamembranen, aber auch um Kern-, Chloroplast- und Mitochondrienmembranen. Weitere Kapitel beschreiben die Gesamtcharakterisierung unterschiedlicher Zelltypen, z.B. eukaryotischer Zellen in Kultur, Kalluskulturen, Protoplasten, Zellhybride, Heterokarien. Dann folgen Kapitel, die eingehend die supramolekularen Strukturen behandeln (Cytoskelette, Ribosomen, Chromatin, Viren u. a.). Besonders wird die Bedeutung von Zelloberfläche für die Zell-Zell-Kommunikation, sowie für den Informationsaustausch von normalen Zellen und Tumorzellen hervorgehoben. Zum Schluss werden u. a. die Erkenntnisse über die Entstehung, Organisation und Funktion des Immunsystems und einfacher Nervensysteme zusammengefasst. Nach jedem der 70 Kapitel folgen Angaben über weiterführende Literatur.

Das Buch, das in einer umfassenden Form über alle wichtigsten Fragen der Molekular- und Zellbiologie berichtet, kann jedem, der an diesem Gebiet interessiert ist oder darin tätig ist, empfohlen werden.

T. GICHNER (*Praha*)